The Role of the Cytoplasmic Domain of the Macrophage Scavenger Receptor MARCO in Adhesion and Uptake

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ABSTRACT

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The macrophage receptor with collagenous structure (MARCO) is a class A scavenger receptor. Class A scavenger receptors are multifunctional type II transmembrane glycoproteins that have roles in modified lipoprotein uptake, innate immunity, and macrophage adhesion. It has been shown that the extracellular cysteine-rich domain of MARCO is important for ligand binding, but the role of the cytoplasmic N-terminal domain has not yet been characterised. The aim of this study was to investigate the role of the cytoplasmic domain in cell adhesion and motility, ligand binding and uptake.

Two truncated forms of human MARCO were used in this study: N- MARCO lacks the entire cytoplasmic domain; Nmyr MARCO includes the membrane-proximal putative myristoylation site, but lacks the rest of the cytoplasmic domain. The constructs were expressed in transiently transfected HEK 293T cells. The Nmyr MARCO mutant had reduced cell surface expression, an effect that was even more apparent in cells transfected with N-MARCO. This indicates that the putative myristoylation site is not obligatory for cell surface expression of MARCO, but increases cell surface expression of the protein. When bound to ligand, the two mutants demonstrated a significant increase in cell surface expression, while the cell surface expression of the full-length MARCO remained unchanged. This indicates that the cytoplasmic tail affects membrane trafficking and the regulation of cell surface expression.

Maleylated proteins are ligands for scavenger receptor A and other scavenger receptors. This study demonstrates that maleylated bovine serum albumin (MalBSA) is also a ligand for MARCO and this modified protein was used to study MARCO-mediated adhesion and ligand binding. The results show that the cytoplasmic domain of MARCO is not required for binding to MalBSA coated particles. Interestingly, the Nmyr MARCO mutant showed a decreased ability to bind soluble MalBSA compared to the full-length protein indicating that the cytoplasmic domain may be necessary for endocytosis. The cytoplasmic domain of MARCO appeared to be absolutely required for adhesion to tissue culture plastic, anti-MARCO antibody PLK-1 coated surfaces and suspension grade plastic, and a lesser extent to MalBSA coated surfaces. MARCO mediated adhesion was shown not to require the presence of serum and divalent cations. Studies performed with murine primary resident peritoneal macrophages on a MalBSA coated surface indicated that MARCO is responsible for increased chemokinesis.

The results of this study show that the cytoplasmic domain of MARCO affects cell surface expression, endocytosis and adhesion. Further research is needed to determine if and how the cytoplasmic domain on MARCO participates in signalling required for these processes and if it affects cell motility.

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Abbreviations

AcLDL	acetylated low density lipoprotein
AGE	advanced glycation end product
apo	apolipoprotein
BSA	bovine serum albumin
CD	cluster of differentiation
CHO	Chinese Hamster Ovary
$ChSO_4$	chondroitin sulfate
CR3	complement receptor 3
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
ddH_2O	double distilled water
DiDAcLDL	DiI-labelled acLDL
DiI	1,1'-dioctadecyl-1-3, 3'-tetramethylindocarbocyanine perchlorate
DKO	SR-A and MARCO knockout
DMEM	Dulbecco's Modified Eagle Medium
dSR-CI	*Drosophila* scavenger receptor CI
$DxSO_4$	dextran sulfate
EDTA	etylenediaminetretraacetic acid
EGF	epidermal growth factor
ER	endoplasmic reticulum
FBS	foetal bovine serum
FEEL	fasciclin, epidermal growth factor (EGF)-like, laminin-type EGF-like, and link domain-containing scavenger receptor
Fig.	figure
FITC	fluorescein isothiocyanate
g	gravity
GM-CSF	granulocyte-monocyte colony-stimulating factor
h	hour
HDL	high density lipoprotein
HEK	Human Embryonic Kidney
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfanic acid
hMARCO	human MARCO
HRP	horse-radish peroxidase
hSR-A	human SR-A
IgG	immunoglobulin G
kDa	kilodalton
KO	knockout
LCM	L-cell conditioned medium
LDL	low density lipoprotein
LOX-1	lectin-like oxidised LDL receptor-1
LPS	lipopolysaccharide
LTA	lipoteichoic acid
Mϕ	macrophage
mAb	monoclonal antibody
MalBSA	maleylated BSA

MARCO	macrophage receptor with collagenous structure
M-CSF	macrophage colony-stimulating factor
min	minute
NKT	natural killer T cells
OxLDL	oxidised LDL
pAb	polyclonal antibody
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PFA	paraformaldehyde
RPE	R-phycoerythrin
RPMϕ	resident peritoneal macrophage
RS	rabbit serum
SCARA-5	class A scavenger receptor 5
SDS	sodium dodecylsulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SR-A	scavenger receptor A
SR-BI	scavenger receptor BI
SRCL	scavenger receptor with C-type lectin
SRCR domain	scavenger receptor cysteine-rich domain
SREC	scavenger receptor expressed by endothelial cells
SR-PSOX	scavenger receptor that binds phosphatidylserine and oxidized lipoprotein
SWDSOP	Sir William Dunn School of Pathology
TDM	trehalose 6,6'-dimycolate
TLR	toll-like receptor
TRITC	tetramethylrhodamine B isothiocyanate
WT	wild type

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1 Chapter One: Introduction

1.1 Macrophages

Macrophages (Mϕs) are leukocytes of myeloid lineage. In mammals, resident Mϕs can be found in many tissues such as the brain, the liver, the spleen and the gut where they have an important role in host defence. Mϕs differentiate in the bone marrow from myeloid precursor cells. These granulocyte/monocyte precursors can also differentiate into other cell types than Mϕs, such as dendritic cells (DCs), Langerhans cells and osteoclasts depending on stimulation with growth factors (Gordon, 2005). The most important factors that mediate the development of these precursor cells into monocytes are macrophage colony-stimulating factor (M-CSF), granulocyte-monocyte colony-stimulating factor (GM-CSF) and interleukin 3 (Chitu and Stanley, 2006; Gordon, 2005; Gordon, 2007). After the monocytes leave the bone marrow, they enter the bloodstream where they circulate for several days until they enter tissues where they become resident Mϕs. In addition to resident Mϕs, during infection larger numbers of monocytes differentiate into Mϕs and migrate to infected tissue where they kill and phagocytose bacteria. These Mϕs are called recruited Mϕs and they differ from resident Mϕs in their repertoire of cell surface receptors (Gordon, 2005; Gordon, 2007). In addition to roles in innate immunity, one of the major roles of Mϕs is to clear apoptotic cells. In humans, every day about 360 billion senescent erythrocytes are phagocytosed by resident Mϕs (Bratosin et al., 1998), which means that Mϕs have to distinguish not only between self and non-self but also self and altered self. Other important functions of Mϕs include the secretion of chemokines that affect the migration of other immune cells, antigen presentation

to primed T-cells and production of many cytotoxic and regulatory molecules, such as reactive oxygen and nitrogen metabolites, tumor necrosis factor-alpha, interleukins 6 and 10 (Gordon, 2005).

1.1.1 Macrophage subpopulations

Resident Mϕs in various tissues have different properties – they express specific receptors to fulfil their roles in tissue homeostasis and host defence (Gordon, 2007). This heterogeneity is necessary for diverse Mϕ functions. For example, osteoclasts are capable of remodelling bone, alveolar Mϕs are capable of binding bacteria and environmental particles due to their high expression of phagocytic receptors, whereas gut Mϕs are exposed to products of symbiotic gut bacteria and have excellent phagocytic capacity, but are less effective in producing cytokines (Smythies et al., 2005). Monocytes, from which these cells are derived, are highly heterogeneous as they vary in size and cell surface markers, which determine their properties as Mϕs (Gordon, 2007). A list of different resident tissue Mϕ populations and their functions can be found in Table 1.1.

Table 1.1 Resident tissue Mϕ populations and their functions
Adapted from (Gordon, 2005)

Organ	Name/Site	Functions/Properties
Bone	Osteoclasts	Bone remodelling
Bone marrow	Stromal macrophages	Interacts with haematopoietic cells Interface with erythroid nuclei
Brain	Microglia, choroid plexus	Interacts with neurons Interface with cerebrospinal fluid
Endocrine organs	Adrenal glands, thyroid, pancreas, etc.	Metabolic homeostasis
Gut	Lamina propria, Peyers' patch	Endocytosis
Liver	Kupffer cells	Clearance of cells and complexes from blood
Lung	Alveolar macrophages	Clearance of particles
Lymph node	Subcapsular sinus macrophages Medullary macrophages	Interface with afferent lymph Interface with efferent lymph
Reproductive tract	Ovary, testis	Clearance of dying cells
Skin	Langerhans cells	Antigen capture
Spleen	Red pulp macrophages Tingible body macrophages Marginal zone macrophages	Clearance of senescent blood cells Phagocytosis of apoptotic B cells Interface between circulation and immune system
Thymus	Thymic macrophages	Clearance of apoptotic cells

1.1.2 The role of pattern recognition receptors in innate immunity and inflammation

Mϕs have two major functions, one of which is to recognise and destroy pathogens, such as viruses, parasites and bacteria. Mϕs are also involved in clearance of various endogenous substances such as apoptotic cells and cellular debris (Gordon, 2005). The exact mechanism by which Mϕs discriminate between self and non-self is not known, but it is proposed that receptors with broad binding specificity have a major role in this process (Gordon et al., 1988; Janeway and Medzhitov, 2002).

The innate immune system is an ancient form of host defence, as it exists in all species from invertebrates to mammals. The concept of pattern recognition was first proposed by Janeway and later by Medzhitov (Janeway and Medzhitov, 2002). The adaptive immune system uses an unlimited number of T and B cell receptors generated by somatic recombination; whereas the innate immune system uses a limited number of germline-encoded pattern recognition receptors. These receptors bind to conserved microbial, fungal and viral products, so-called pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002). These receptors are expressed on the cell surface as well as in intracellular compartments of Mϕs, DCs and neutrophils, or can be secreted into the bloodstream and tissue fluids (Taylor et al., 2005). Although the variety of receptors expressed on Mϕs is genetically limited, these receptors are capable of recognising a vast variety of ligands which includes proteins, lipids, nucleic acids and carbohydrates. The expression of pattern recognition receptors changes during Mϕ differentiation from monocytes and upon Mϕ activation and the tissue microenvironment and extracellular matrix have a profound effect on receptor expression.

The repertoire of ligands is also increased by alternative splicing to generate multiple isoforms and post-translational modifications, such as proteolysis, glycosylation and lipid modifications (Taylor et al., 2005).

The best known pattern recognition receptors are the Toll-like receptors (TLRs). This is a family of well-conserved non-phagocytic receptors that have important functions in recognising conserved pathogen structures, PAMPs, and inducing inflammatory signals (Leulier and Lemaitre, 2008; McCoy and O'Neill, 2008). Non-TLR receptors that are involved in pathogen recognition include scavenger receptors, the mannose receptor and the β-glucan receptor dectin-1 (Mukhopadhyay et al., 2004). In contrast to well-characterised Fc-receptors and complement receptors, they do not require the presence of opsonins for pathogen recognition. These receptors mediate pathogen recognition directly and mediate particle internalisation. It has been suggested that in many cases the TLRs and non-TLR receptors interact with each other and the outcome of this interaction modulates the response to pathogens (Mukhopadhyay et al., 2006; Mukhopadhyay et al., 2004). Some examples of pathogen recognition receptors and their ligands are given in Table 1.2.

Table 1.2 Selected Mϕ pattern recognition receptors and their ligands (adapted from Gordon, 2002; Taylor et al., 2005)

Receptor family	Receptor	Examples of ligands
TLR	TLR2	Peptidoglycan
	TLR4	LPS
Scavenger receptor class A	SR-A	Bacteria, apoptotic cells, modified lipoproteins
Scavenger receptor class B	CD36	Apoptotic cells, modified lipoproteins
NK-like C-type lectin-like	Dectin-1	*β*-glucan
Multiple extracellular carbohydrate binding domains	Mannose receptor	Bacteria, mannosyl, fucosyl glycoconjugates, lysosomal hydrolases

1.2 The scavenger receptor family

Mϕ scavenger receptors were first defined by their ability to bind modified lipoproteins, such as acetylated low density lipoprotein (AcLDL) and oxidised low density lipoprotein (OxLDL) (Goldstein et al., 1979). The uptake of LDL by Mϕs is thought to be the primary cause of atherosclerosis as Mϕs bind and endocytose modified lipoproteins and become foam cells. Scavenger receptors have been shown to be expressed in atherosclerotic plaques and they endocytose modified LDL. This indicates that these receptors have an important role in human disease (Suzuki et al., 1997). In 1988, the first scavenger receptor defined by molecular cloning was scavenger receptor A (Kodama et al., 1988) and in subsequent decades the family of molecules classified as scavenger receptors has become larger. The scavenger receptors are classified into eight classes: A, B, C, D, E, F, G and H, based on their structure (Krieger, 1997). These receptors are multiligand receptors – in addition to recognising modified lipoproteins they are also pattern recognition receptors. The ligand repertoire of scavenger receptors is very broad, it includes Gram-positive and –negative bacteria, apoptotic cells, microbial and viral products and a variety of natural and artificial polyanionic ligands. Most of the scavenger receptors are expressed only on myeloid cells, such as Mϕs, but some receptors are also found on epithelial and endothelial cells (reviewed in Pluddemann et al., 2006; Pluddemann et al., 2007). The ability of these receptors to recognise both endogenous and exogenous ligands suggests that scavenger receptors have a major role in innate immunity and maintenance of tissue homeostasis. Due to their ability to recognise multiple ligands, the scavenger receptors have been shown to be multifunctional: they are involved in various cellular functions such as phagocytosis, endocytosis, antigen presentation and adhesion (Platt and Gordon, 2001).

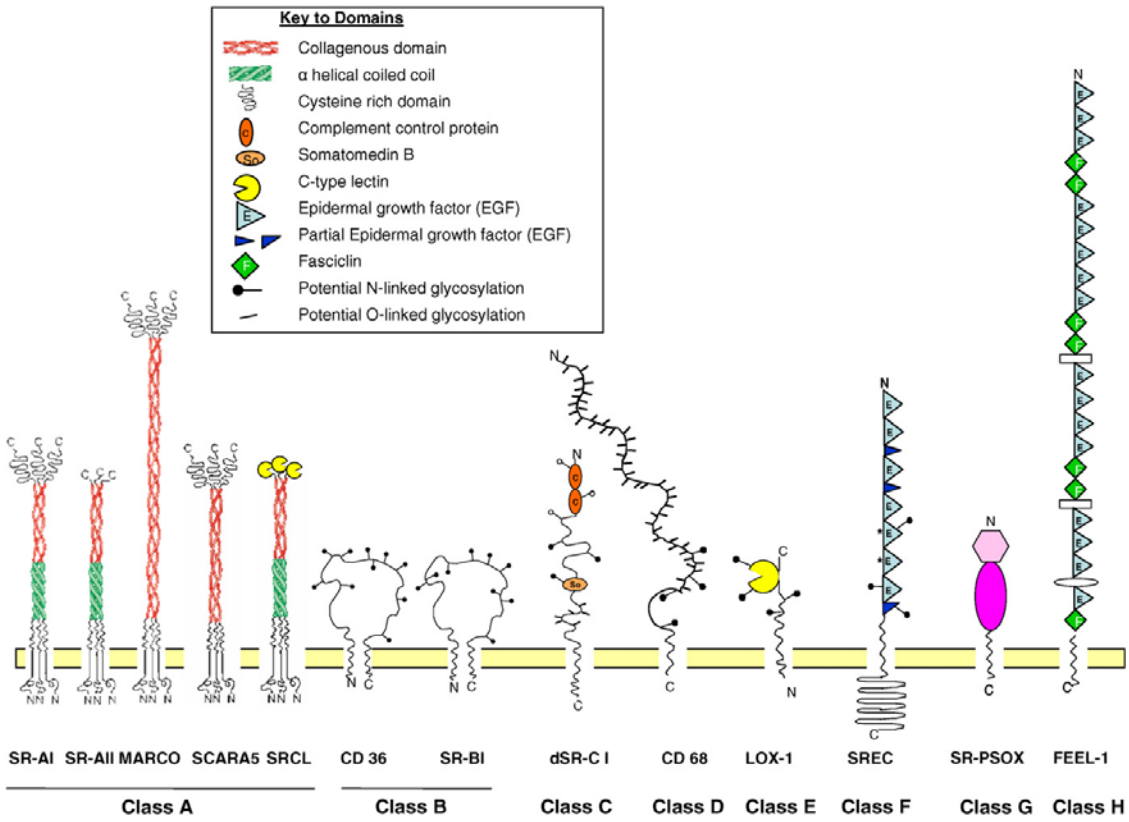


Fig. 1.1 Domain architecture of the different classes of scavenger receptors
Scavenger receptors are divided into eight classes and are structurally very diverse. The domains are indicated in the key. Taken from (Pluddemann et al., 2007).

1.2.1 The class A scavenger receptors SR-A, MARCO, SCARA5 and SRCL

Scavenger receptor A (SR-A) was the first of the class A scavenger receptors that was discovered (Kodama et al., 1988); it was followed by the structurally similar macrophage receptor with collagenous structure (MARCO) (Elomaa et al., 1995) and recently two other class A scavenger receptors, class A scavenger receptor 5 (SCARA-5) (Jiang et al., 2006) and scavenger receptor with C-type lectin (SRCL) (Nakamura et al., 2001). SR-A was first isolated from bovine messenger RNA (Kodama et al., 1990; Rohrer et al., 1990) and was later found also to be expressed in mouse and human Mϕs (Ashkenas et al., 1993; Matsumoto et al., 1990; Tomokiyo et al., 2002). SR-A is expressed in three natural isoforms that are alternative splice variants of the same gene which is located on human chromosome 8. There have been no differences described in ligand binding properties of SR-AI (Kodama et al., 1988) and SR-AII (Rohrer et al., 1990), which has a truncated scavenger receptor cysteine-rich domain (SRCR domain), but their expression levels can differ during monocyte differentiation (Geng et al., 1994). SR-III also has a truncated SRCR domain but in contrast to SR-AI and SR-AII it lacks the two key cysteines that are important for disulphate bond formation. It is thought that these changes in the SRCR domain cause of misfolding of the protein and its retention in the endoplasmic reticulum (ER) due to different intracellular processing (Gough et al., 1998). The schematic structure of each isoform is depicted in Fig. 1.2.

In addition to binding to various endogenous ligands, such as AcLDL (Kodama et al., 1988), OxLDL (Freeman et al., 1991), apoptotic cells (Platt et al., 1996), modified extracellular

matrix proteins (Santiago-Garcia et al., 2001) and advanced glycation end products (AGE) (Araki et al., 1995), SR-A has been shown to have an important role in host defence (reviewed in Peiser et al., 2002b), presumably because it binds many microbial products, such as lipopolysaccharide (LPS) from Gram-negative bacteria (Hampton et al., 1991) and lipoteichoic acid (LTA) from cell walls of Gram-positive bacteria (Greenberg et al., 1996). It also binds to intact Gram-negative bacteria such as *Neisseria meningitidis* and *Escherichia coli* (Peiser et al., 2000) and Gram-positive bacteria, such as *Listeria monocytogenes* and *Staphylococcus aureus* (Dunne et al., 1994). It has been demonstrated that the scavenger receptor mediated binding to LTA depends on the negative charge of the molecule (Greenberg et al., 1996), but not all polyanionic substances are ligands for SR-A (for example, polyinosinic acid but not polycytidylic acid) (Kodama et al., 1990), therefore a certain conformation might be required for binding. Recent research also indicates that SR-A might have an important role in antiviral immune responses as it has been identified as a cell surface receptor for double-stranded RNA (Limmon et al., 2008). The lysine cluster in the C-terminal end of the collagenous domain has been implicated to be responsible for binding of AcLDL (Yamamoto et al., 1997). SR-A has also been shown to mediate calcium and magnesium independent adhesion to modified extracellular matrix components (el Khoury et al., 1994; Fraser et al., 1993; Gowen et al., 2000; Santiago-Garcia et al., 2003). SR-A ligands for adhesion include serum coated tissue culture plastic (Fraser et al., 1993) and recently it was found that SR-A recognises apolipoprotein (apo) A-I, the main component of high density lipoprotein, and apoE (Neyen, 2008). SR-A expression is regulated by M-CSF which also enhances SR-A mediated adhesion and modified LDL uptake (de Villiers et al., 1994).

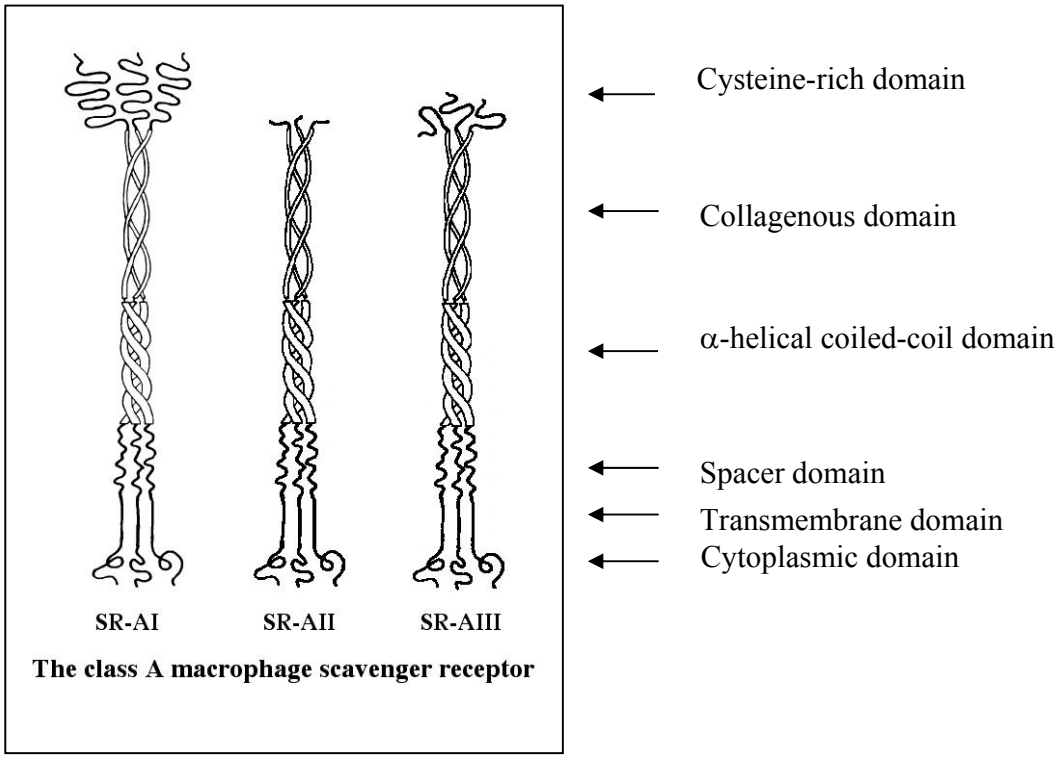


Fig. 1.2 The three isoforms of SR-A. SR-AII is identical to SR-AI, except that the SRCR domain is truncated. SR-AIII is a non-functional isoform, that is trapped in the ER and is defective in ligand binding. SR-AIII lacks a segment of the SRCR domain that is important for disulphide bond formation (Gough et al., 1998). Adapted from (Gough et al., 1998; Rohrer et al., 1990).

The two newest members of the class A scavenger receptor family are SRCLI/II and SCARA-5. SRCLI/II is expressed only by vascular endothelial cells (Nakamura et al., 2001) and SCARA-5 is restricted to mucosal epithelial cells (Jiang et al., 2006). SCARA-5 has also been shown to bind to *Escherichia coli* and *Staphylococcus aureus* but not modified lipoproteins (Jiang et al., 2006). SCARA-5 is structurally very similar to SR-AI and MARCO. MARCO is the main subject of this thesis and will be discussed in detail in subsequent sections.

1.2.1.1 Scavenger receptor cysteine-rich domain superfamily

SRCR domain receptors form a superfamily of structurally related transmembrane glycoproteins. There are two structurally different variants of the SRCR domain based on which these receptors can be divided into two groups, A and B. Group A molecules contain three disulfide bonds and six cysteines, while group B molecules contain four bonds between eight cysteine residues (Fabriek et al., 2005). The group A SRCR molecules include MARCO and SR-AI. SRCR domain is the ligand binding region of MARCO and will be discussed in more detail in section 1.3.3.4.

CD163 is one of the few group B SRCR receptors that is selectively expressed on monocytes and Mϕs (Kristiansen et al., 2001). CD163 binds to hemoglobin:haptoglobin complexes and therefore protects tissues from free hemoglobin-mediated oxidative damage (Fabriek et al., 2005; Kristiansen et al., 2001). CD163 on Mϕs has been identified as an adhesion receptor for erythroblasts and it has been suggested that it promotes erythroblast proliferation or survival (Fabriek et al., 2007).

1.2.2 The class B scavenger receptors CD36 and SR-BI

Both CD36 (Endemann et al., 1993) and scavenger receptor BI (SR-BI) (Acton et al., 1994) are type III transmembrane proteins with two short cytoplasmic tails and an extracellular loop. The central extracellular loop is thought to be important for ligand binding (Greenwalt et al., 1992). Both CD36 and SR-BI can form trimers and are often glycosylated (Thorne et al., 1997). CD36 was the first of the class B scavenger receptors described and it is expressed in many different cell types, such as Mϕs, platelets, adipocytes and some endothelial cells (Greenwalt et al., 1992). First it was described as a major receptor for OxLDL (Endemann et al., 1993), but many other ligands for this receptor have also been described, including apoptotic cells (Ren et al., 1995), thrombospondin (Savill et al., 1992), *Plasmodium falciparum* parasitized erythrocytes (Oquendo et al., 1989) and anionic phospholipids (Rigotti et al., 1995). Recent unpublished studies by Dr. Laura Helming indicate that CD36 is involved in Mϕ fusion. Class B scavenger receptors differ from all other scavenger receptors by their ability to recognise a variety of native lipoproteins, such as very low-density lipoprotein (VLDL), LDL and high density lipoprotein (HDL) (Calvo et al., 1998; Murao et al., 1997; Rigotti et al., 1997). Therefore, these receptors have an important role in lipid metabolism.

SR-BI was the first scavenger receptor that was found to bind with high affinity to HDL (Acton et al., 1996), and therefore is responsible for the delivery of cholesterol to cells. SR-BI expression has been reported on Mϕs and DCs and their precursor monocytes, but also on hepatocytes, adrenal glands and fat tissue (Acton et al., 1996; Acton et al., 1994; Terpstra et al., 2000). SR-B has two alternative splice variants, SR-BI (Acton et al., 1994) and SR-BII

(Webb et al., 1997), but SR-BII is four times less effective in transporting cholesterol to cells, therefore its biological significance is not known (Webb et al., 1998). SR-BI has also been shown to bind LPS (Vishnyakova et al., 2003), but the role of this receptor in host defence is unclear.

1.2.3 The class D scavenger receptors CD68/macrosialin and lysosomal membrane glycoproteins

Class D scavenger receptors include CD68 in humans and its murine homologue macrosialin (Ramprasad et al., 1995). In addition, lysosomal membrane glycoproteins Lamp-1, Lamp-2 and Lamp-3 have been added to this group (de Saint-Vis et al., 1998). These receptors are all intracellular and can be found on the endosomal compartment of monocytes and monocyte derived cells, including Mϕs (Jiang et al., 1998; Ramprasad et al., 1995). The major known ligand for CD68 is OxLDL (Ramprasad et al., 1995). Originally it was suggested that murine macrosialin also localises to cell surface where it recognises OxLDL, but this may be an artefact, since no binding was found in transfected cells (de Beer et al., 2003). Due to the mainly intracellular localisation of CD68, it is not likely that these receptors have a role in host defence or the formation of atherosclerotic plaques, their role is more likely to be the intracellular processing of OxLDL.

1.2.4 The class E scavenger receptor LOX-1

The only member of the class E scavenger receptors is lectin-like oxidised LDL receptor-1 (LOX-1) (Sawamura et al., 1997). LOX-1 is similar to the class A scavenger receptors in that

it is a type II transmembrane protein, but in contrast, it has a highly conserved extracellular C-type lectin domain, which mediates ligand binding (Nagase et al., 1997; Sawamura et al., 1997; Shi et al., 2001). This receptor binds to OxLDL, but not AcLDL and was first identified in vascular endothelial cells (Sawamura et al., 1997), but was later also found on Mϕs and smooth muscle cells (Draude et al., 1999). In endothelial cells the expression of LOX-1 is inducible by tumor necrosis factor-alpha (Kume et al., 1998) and by hypertension (Nagase et al., 1997). Similar to other scavenger receptors, its ligands also include apoptotic cells (Oka et al., 1998) and AGE (Jono et al., 2002).

1.2.5 The class C, F-G scavenger receptors

The only class C scavenger receptor *Drosophila* scavenger receptor CI (dSR-CI) is expressed in the fruit fly *Drosophila melanogaster* and a homologue in other eukaryotes has not been found (Pearson et al., 1995). The expression of this receptor is restricted to Mϕs/haemocytes during embryonic development. Its ligand binding properties have been reported to be very similar to that of SR-A, as it binds to AcLDL (Pearson et al., 1995) and both Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli*), and therefore acts as a pattern recognition receptor in *Drosophila* (Ramet et al., 2001). However, dSR-CI is structurally very different from all other scavenger receptors and it contains multiple domains, not found in other scavenger receptors.

Another structurally distinct class of scavenger receptors, class F, consists of SREC (scavenger receptor expressed by endothelial cells) type I (Adachi et al., 1997) and II (Ishii et al., 2002). In contrast to LOX-1, SREC preferably binds and internalises AcLDL rather than

OxLDL. SREC is a type I transmembrane protein that has five EGF domains (Adachi et al., 1997). However, in contrast to SREC-I, the structurally similar SREC-II has a reduced ability to internalise modified LDL (Ishii et al., 2002). Although these receptors were first found on endothelial cells, the expression of SREC has also been reported on Mϕs, where the uptake and degradation of AcLDL can be enhanced by stimulation with LPS (Tamura et al., 2004).

SR-PSOX (scavenger receptor that binds phosphatidylserine and oxidized lipoprotein) is the sole member of class G scavenger receptors (Shimaoka et al., 2000). This receptor shows ligand binding activity similar to that of LOX-1, as it binds to OxLDL but not to AcLDL. It is expressed mainly on resident Mϕs and DCs (Shimaoka et al., 2000). In addition, SR-PSOX has been shown to be the chemokine ligand for a CXC chemokine receptor 6 that is expressed on activated T cells and natural killer T cells (NKT). Therefore it is proposed that SR-PSOX attracts T cells and NKT cells towards Mϕs and DCs and functions as a cell adhesion molecule (Shimaoka et al., 2004).

FEEL (fasciclin, epidermal growth factor (EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor) belongs to the class H scavenger receptors (Adachi and Tsujimoto, 2002). Two FEEL receptors, FEEL-1 and FEEL-2, have been described that are encoded by paralogous genes. They are both very large type I transmembrane proteins of similar structure, except that FEEL-2 has one EGF domain less than FEEL-1 (Adachi and Tsujimoto, 2002). FEEL-1 and FEEL-2 are highly expressed in various endothelial cells, but only FEEL-1 is expressed on monocytes and Mϕs (Adachi and Tsujimoto, 2002). Both FEELs bind to AcLDL and Gram-positive and –negative bacteria, which indicates that these receptors may have an important role in host defence (Adachi and Tsujimoto, 2002). FEEL-1

is involved in intracellular trafficking pathways and may be involved in adhesion and transmigration in various cell types (Kzhyshkowska et al., 2006).

1.3 Macrophage scavenger receptor MARCO: structure and expression

MARCO is a member of scavenger receptor class A, and is structurally very similar to SR-A. While SR-A was first described in 1988 (Kodama et al., 1988), MARCO was characterised seven years later in 1995 by Elomaa and colleagues (Elomaa et al., 1995). SR-A and MARCO were first grouped together because they share similar domain structure and ligand binding properties. Until recently, all described ligands for MARCO were also ligands for SR-A, which led to the assumption that these two receptors may be functionally redundant. Recent findings indicate that there are differences in ligand recognition (Elomaa et al., 1998; Plüddemann et al., 2008) and in other cellular functions which are the subject of this thesis.

1.3.1 Molecular characterisation of human MARCO

SR-A exists in three forms SR-AI (Kodama et al., 1988), SR-AII (Rohrer et al., 1990) and SR-AIII (Gough et al., 1998), which are generated by alternative splicing of a single gene. The gene encoding SR-A consists of 11 exons and is located on human chromosome 8 (Matsumoto et al., 1990). The gene *MARCO* is localised to human chromosome 2 and mouse chromosome 1 (Kangas et al., 1999). Thus far, only one isoform of MARCO has been described, but recent findings indicate that an alternative splice variant of the same gene exists, that similarly to SR-AII has a truncated SRCR domain (Bowdish et al., 2008b). The

closely related SRCL gene is on human chromosome 18 and can also be spliced into 2 isoforms. All three genes encode a collagen-like domain which indicates that those three genes arose from a common ancestral gene that duplicated and dispersed through the human genome during evolution (Murphy et al., 2005).

1.3.2 MARCO is a type II transmembrane glycoprotein

All glycoproteins are synthesised in the ER and transported to the plasma membrane from the Golgi complex. These glycoproteins contain specific sequences that determine in which orientation the protein is inserted into the membrane – these sequences are called topogenic sequences. The largest class of transmembrane proteins have a single α-helix spanning the phospholipid bilayer, the C-terminus in the cytosol and an extracellular N-terminus. These proteins are called type I transmembrane proteins. These proteins have two kinds of topogenic sequences, an N-terminal cleaved signal sequence and stop-transfer membrane-anchor sequence. Examples of type I transmembrane proteins include the LDL receptor and CD68 (Lodish et al., 2003).

Type II transmembrane proteins also span the membrane once, but their N-terminus is cytosolic and their C-terminus is extracellular. Such proteins are anchored to the membrane by a hydrophobic sequence in the middle of the protein. These proteins are not cleaved during their synthesis; the hydrophobic stop-transfer membrane anchor sequence also functions as an internal signal sequence. This hydrophobic stop-transfer membrane sequence does not significantly differ from that of type I transmembrane proteins, but the amino acids

that are close to this sequence determine the orientation of the protein in the membrane because the side that has more positively charged amino acids remains in the cytosolic face of the membrane (Lodish et al., 2003). Examples of type II transmembrane include the transferrin receptor and class A scavenger receptors.

1.3.3 The structure of MARCO

MARCO is a 210 kDa (519 amino acids) transmembrane protein, that like SR-A forms homotrimers. It consists of three ~52 kDa subunit monomers that each spans the membrane (Elomaa et al., 1995). MARCO consists of five domains: I cytoplasmic tail, II transmembrane domain, III spacer region, IV collagenous domain and V C-terminal SRCR domain (Fig. 1.3). Additionally, SR-A expresses an α-helical coiled-coil domain between the spacer region and the collagenous domain, however MARCO lacks this coiled-coil domain and its collagenous domain is three times as long as that of SR-A (Elomaa et al., 1995).

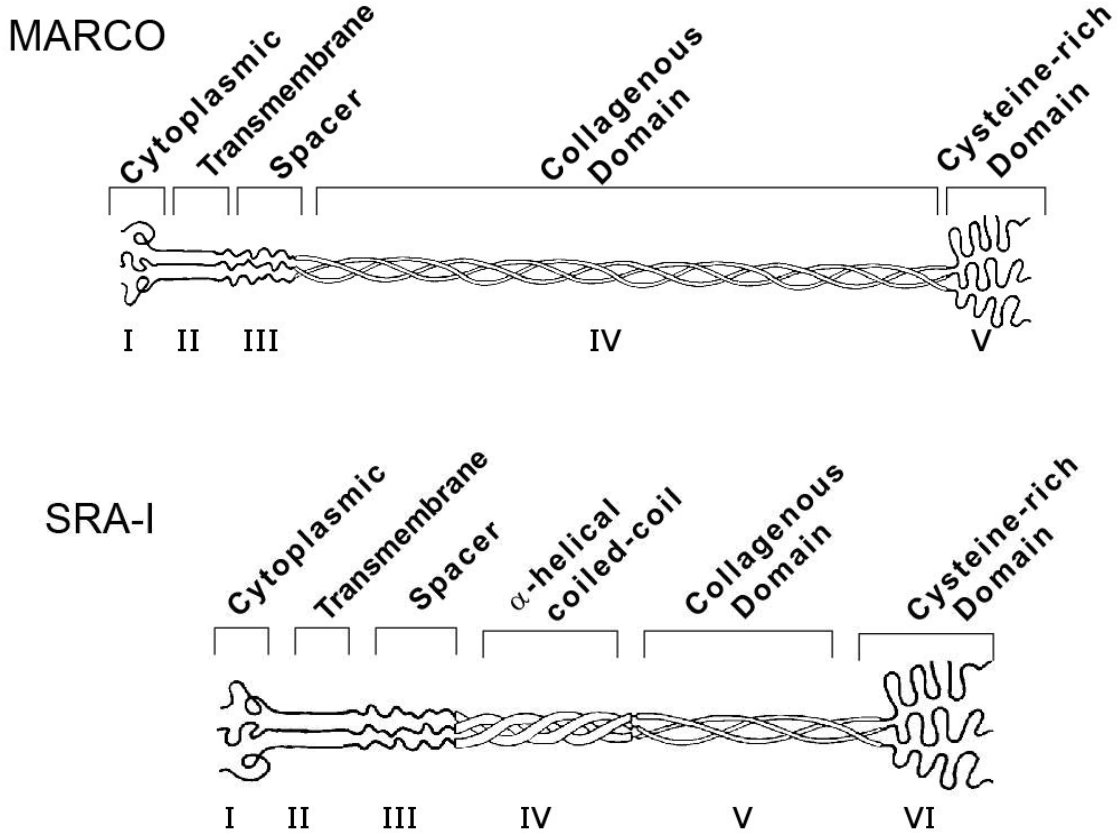


Fig. 1.3 The structure of MARCO and SR-AI. MARCO consists of a I cytoplasmic tail, II transmembrane domain, III spacer region, IV collagenous domain and V C-terminal SRCR domain. SR-AI also has an α-helical coiled-coil region. Adapted from (Elomaa et al., 1995; Rohrer et al., 1990).

1.3.3.1 Domain I: the N-terminal cytoplasmic domain

The function of the cytoplasmic domain of MARCO has not been characterised, but for SR-A it is known to mediate several important cellular functions. It has been shown that the six membrane-proximal amino acids are required for the cell surface expression of SR-A (Fig. 1.4) (Kosswig et al., 2003). Kosswig and colleagues also demonstrated in the same study that the cytoplasmic tail mutant of SR-A, which lacks the whole cytoplasmic domain except those six amino acids, is overexpressed on the cell surface of transfected Human Embryonic Kidney (HEK) 293 cells (Kosswig et al., 2003).

Receptor-mediated endocytosis requires specific internalisation motifs located in the cytoplasmic tail. For SR-A, a crucial sequence for efficient endocytosis and receptor internalisation is the VXFD motif between amino acids 18 and 27 from the N-terminus (Morimoto et al., 1999). Phosphorylation sites and di-leucine motifs in human SR-A have also been shown to be important for SR-A mediated internalisation of AcLDL (Chen et al., 2006b; Fong, 1996; Fong and Le, 1999). However, HEK 293 cells that express the cytoplasmic tail mutants of SR-A adhere and spread equally well in culture as full length SR-A transfected cells. This indicates that the cytoplasmic tail of SR-A does not contain sequences for cell spreading and adhesion (Kosswig et al., 2003).

The function of the cytoplasmic domain of MARCO has not been described thus far. The amino acid sequence of the cytoplasmic domain of MARCO is different from that of SR-A and the same signalling motifs are not present. Bioinformatic analysis revealed that the membrane proximal part of the cytoplasmic domain of MARCO contains a putative

myristoylation site. N-myristoylation is an acylation of the amino acid glycine and it is considered as one of the first steps by which the protein associates with the membrane (Boutin, 1997). However, the putative N-myristoylation motif in the cytoplasmic domain of MARCO does not show a high correlation with other known myristoylation motifs (Boutin, 1997), nor has it experimentally been shown that the glycine actually gets myristoylated. A comparison of the cytoplasmic amino acid sequences of human MARCO and SR-A, and signalling motifs is shown in Fig. 1.4.

1.3.3.2 Domains II and III: the transmembrane domain and the spacer domain

Domain II forms a 24-amino acid hydrophobic transmembrane domain that is thought to contain sequences for membrane anchoring. It is followed by 75 amino acid hydrophilic spacer domain, which contains two cysteines and two putative N-glycosylation sites. These cysteines are thought to be involved in the linkage of the monomers through formation of interchain disulfide bonds (Elomaa et al., 1995).

1.3.3.3 Domain IV: the collagenous domain

The collagenous domain is a well conserved structure that is present in many proteins. All collagenous transmembrane proteins are type II proteins and besides the class A scavenger receptors, this group of biologically versatile molecules includes collagens XIII, XVII, XXIII, XXV and ectodysplasin A (Franzke et al., 2003). The collagenous domain of MARCO consists of 270 amino acids that form 89 G-X-Y triplets that is interrupted with A-

E-K sequence which is suggested to be the hinge region in the triple helix (Elomaa et al., 1995).

1.3.3.4 Domain V: the cysteine-rich domain

The cysteine-rich domain is highly conserved and can be found on many proteins including SR-AI. The SRCR domain consists of about 100 amino acids and contains six cysteine residues that form intrachain disulfide bonds (Kraal et al., 2000). By expressing full-length and truncated forms of MARCO in transfected cells, it was found that the bacterial binding region of MARCO was located in the cysteine-rich region of the COOH-terminal of the protein (Elomaa et al., 1998). A study with chimaeric scavenger receptors indicated that even minor sequence changes in the MARCO SRCR domain have an effect on endocytosis. The cysteine-rich domain of mouse MARCO contains a basic and an acidic cluster (Ojala et al., 2007). The acidic cluster was also reported to be required for adhesion to gelatine (Ojala et al., 2007). These results demonstrate that it is the major ligand-binding domain in MARCO (Chen et al., 2006a).

The crystal structure of the monomeric form of the cysteine-rich domain of the mouse receptor revealed that it has a compact globular fold with a twisted five-stranded antiparallel β-sheet and a long loop covering a single α-helix. The dimer consists of β-strand-swapped two monomers. The monomer has a dipolar charge distribution. The β-sheet region contains several arginines forming a basic cluster. Dimerisation brings the positively charged clusters together, while negative clusters point in separate directions. It is proposed that the presence of the positively charged cluster explains the binding to negatively charged molecules (Ojala

et al., 2007). This correlates with the fact that the arginine residues (especially the RXR motif) in the SRCR domain have been shown to be critical for binding to bacteria (Brannstrom et al., 2002). The acidic cluster also seems to be responsible for Ca^{2+}, Mg^{2+} dependent adhesion gelatin (Ojala et al., 2007).

SR-A

MEQWDHFHNQQEDTDSCSESVKFDARSMTALLPPNPKNSPSLQEKLKSF

internalisation sequence di-leucine motif cell surface expression

phospho-site endocytosis

MARCO

MRNKKILKEDELLSETQQAAFHQIAMEPFEINVPKPKRRNGVNFSLAVV

di-leucine motif N-myristoylation site

Fig. 1.4 The amino acid sequences of the cytoplasmic domain of human SR-A and MARCO. The signalling motifs are highlighted.

The VXFD motif was the first signalling motif to be determined in the cytoplasmic domain of SR-A. COS-7 cells were transfected with bovine SR-A, that had mutations in certain amino acids. Subsequently, cell surface expression and the ability to internalise AcLDL were measured. As a result, it was reported that Val21, Phe23 and Asp24 are responsible for efficient internalisation and efficient cell surface expression of SR-A (Morimoto et al., 1999). In human MARCO these sites have not been experimentally confirmed, but the VXFD motif is highly conserved between species.

Using mouse SR-A, it was shown that the elimination of Ser20 increases cell surface expression and consequently endocytosis of AcLDL in transfected Chinese Hamster Ovary (CHO) and COS7 cells. In contrast, the elimination of Ser48 did not reduce cell surface expression but reduced ligand internalisation (Fong and Le, 1999).

Kosswig and colleagues showed that mouse SR-A, that had amino acids 1-49 deleted, had reduced ability to endocytose AcLDL but cell spreading was not affected compared to full-length receptor. It was also demonstrated that deletion of those six membrane proximal amino acids significantly reduced cell surface expression (Kosswig et al., 2003). This amino acid sequence is conserved among species.

More recently, it was determined that in human SR-A the di-leucine motif contributes to SR-A-mediated endocytosis. These authors showed that CHO cells that expressed the di-leucine mutant SR-A, showed decreased uptake but unchanged binding to AcLDL. This suggests that the di-leucine motif acts as a signal sequence to mediate SR-A into the cell (Chen et al., 2006b).

In the cytoplasmic tail of MARCO the signalling motifs are only putative, as their function has not been experimentally proven.

1.3.4 Homology between human and mouse MARCO

Human *MARCO* gene encodes a 519-amino acid protein which shares 68.5% identity with murine MARCO (Elshourbagy et al., 2000). The most similar domains are the collagenous domain and the cysteine-rich domain (Table 1.3). In contrast, the cytoplasmic domain is only 51% similar between mouse and human MARCO. There are also differences in the cysteine residues. Outside the cysteine-rich domain, mouse MARCO has one cysteine in the cytoplasmic domain, two in the spacer domain. In humans, on the other hand, there are no cysteines outside the cysteine-rich domain (Elomaa et al., 1998). In mouse, the cysteine residues of the spacer domain are involved in formation of disulfide bonds that link the three monomers. This indicates that in man the monomers are bound only through helix formation from the collagenous domain (Kraal et al., 2000).

Table 1.3 Amino acid sequence identity and similarity between human and mouse MARCO receptors. Adapted from (Elomaa et al., 1998).

Domain	Identity %	Similarity %
I Cytoplasmic domain	51	51
II Transmembrane	69	88
III Spacer	57	67
IV Collagenous	75	78
V Cysteine-rich	74	80

1.3.5 The expression profile

Both MARCO and SR-A are expressed primarily on myeloid cells. SR-A is expressed on Mϕs and DCs, but not their precursor monocytes (Becker et al., 2006). Recently SR-A was also reported to be expressed on mast cells (Brown et al., 2007), in addition to selected endothelial and smooth muscle cells (Naito et al., 1992). However, SR-A is constitutively expressed on most tissue resident Mϕs, whereas the expression of MARCO in the mouse is limited to subpopulations of Mϕs in the marginal zone of the spleen, the medullary cord of lymph nodes and a subpopulation of resident sentinel Mϕs. The expression of MARCO can be upregulated in other Mϕ populations in response to bacterial infection and LPS (Elomaa et al., 1995; van der Laan et al., 1999), but not by the major pro-inflammatory and anti-inflammatory cytokines, such as interferon γ, interleukin 1, interleukin 6 and tumor necrosis factor α (van der Laan et al., 1997). Therefore MARCO can be regarded as a marker for innate immune activation. Conversely, in humans, RNA blot analysis indicated that lung and liver Mϕs express high levels of MARCO (Elshourbagy et al., 2000). This indicates that the expression profile and the regulation of MARCO are somewhat different in humans compared to mice.

1.4 Phagocytosis

The most characteristic function of the Mϕ is its ability to perform phagocytosis. Phagocytosis is a process by which Mϕs and neutrophils ingest and clear large particles with a diameter more than half a micrometre (Groves et al., 2008). These particles can include

bacteria, apoptotic cells, cellular debris and environmental particles. Phagocytosis was first described at the end of 19th century by a Russian scientist Ilya Metchnikoff, who noticed that amoeboid-like cells in transparent sea star larvae contained ingested cells (Metchnikoff, 1905). He hypothesised that these cells with phagocytic ability were capable of ingestion and might play a key in host defence and tissue homeostasis. Later in the 20th century he was found to be correct as this process is very tightly linked with inflammation in mammals. The process of phagocytosis is complex and there is no single model of its mechanism.

A general characteristic of phagocytosis is that it depends on the cooperation of the actin cytoskeleton with microtubules (Groves et al., 2008). Particle internalisation is initiated by the interaction of specific receptors with their ligand on the particle. After actin has polymerised at the site of ingestion, the particle is internalised. Next actin is shed from the phagosome, and the phagosome matures by a series of fusion and fission events until a mature phagolysosome is formed (Huynh et al., 2007). During maturation the pH drops and pathogen killing mechanisms are activated (Aderem and Underhill, 1999; Groves et al., 2008).

1.4.1 Phagocytic receptors

The Mϕ phagocytoses via expression of cell surface receptors that bind and internalise pathogens. These phagocytic receptors expressed by Mϕs can be divided into two broad categories based on whether they require opsonisation of the particle.

One group of the best characterised opsonic phagocytic receptors are Fc-receptors which bind to the Fc-domain of immunoglobulin molecules. Fc-receptors, which are expressed on the surface of phagocytic cells, such as Mϕs, neutrophils and DCs, mediate phagocytosis of immunoglobulin coated particles and cells. The mechanism of Fc-receptor mediated phagocytosis is referred to as the zipper model because the phagocytic cup covers the particle by local sequential responses through interaction with ligand coated on the particle (Swanson, 2008). Complement proteins, which include C3, are present in serum and opsonise bacteria for phagocytosis by the complement receptors on leukocytes (van Lookeren Campagne et al., 2007).

Phagocytosis mediated by Fc-receptors and complement receptor 3 (CR3) have been shown to have different mechanisms (Allen and Aderem, 1996). Fc-receptors are constitutively active for phagocytosis (Ravetch, 1994), while CR3 cannot ingest particles in the absence of activation or a second signal, such as adhesion (van Lookeren Campagne et al., 2007; Wright et al., 1983). Fc-receptors also require small GTPases Rac and Cdc42 and tyrosine kinases, while CR3-dependent phagocytosis needs protein kinase C, the tyrosine kinase Syk, microtubules and GTPase Rho for action (Caron and Hall, 1998; van Lookeren Campagne et al., 2007). Other examples of opsonins include thrombospondin, fibronectin, LPS binding protein, and the collectins (Aderem and Underhill, 1999; Groves et al., 2008).

A second group of receptors do not need opsonins and directly recognise structures on the particle surface. These non-opsonic receptors include the mannose receptor, CD36 and the class A scavenger receptors whose role in phagocytosis will be discussed in the next section.

1.4.2 The role of MARCO and SR-A in pathogen recognition and phagocytosis

MARCO and SR-A have been shown to have very similar ligand binding properties as they both are capable of recognising self and non-self ligands. They both recognise microbial products, such as LPS and LTA, intact bacteria, apoptotic cells and environmental particles (Pluddemann et al., 2006; Pluddemann et al., 2007).

SR-A has been shown to phagocytose apoptotic thymocytes (Platt et al., 1996). In addition, the expression of SR-A in normally non-phagocytic cells can make these cells capable of binding and internalising apoptotic cells (Platt et al., 1998). Recently it was shown that MARCO also recognises apoptotic cells as it clears apoptotic B cells in the marginal zone of the spleen (Wermeling et al., 2007). The same authors also showed that SR-A and MARCO knock-out (KO) mice display lower threshold to autoantibody responses which can be predisposing factors in autoimmune diseases, such as systemic lupus (Wermeling et al., 2007).

Studies in transfected cells and SR-A and MARCO KO mice have shown that both of those receptors mediate phagocytosis of Gram-positive and –negative bacteria. For example, SR-A has been shown to recognise the Gram-negative bacteria *Neisseria meningitides* independently of LPS (Peiser et al., 2002a). MARCO is also a receptor for *Neisseria*. MARCO expression in transfected CHO-KI cell line, which is not normally phagocytic, makes these cells capable of binding and internalising *Neisseria meningitidis* (Mukhopadhyay et al., 2006). In an *in vivo* mouse model, MARCO and SR-A recognise different *Neisseria* cell surface antigens (Plüddemann et al., 2008), which indicate that a fine

specificity in ligand recognition exists between those two class A scavenger receptors. Furthermore, recent studies revealed that MARCO is the main receptor for mycobacterial trehalose 6,6'-dimycolate (TDM), a component of the *Mycobacterium tuberculosis* cell wall, whereas SR-A binds with relatively low affinity to particles coated with this agent (Bowdish et al., 2008a). These experiments demonstrated that MARCO is required for the activation of TLR 2 and CD14 to form a functional receptor complex for TDM (Bowdish et al., 2008a).

The role of MARCO in clearance of environmental particles has been demonstrated many times. First, it was shown that in hamster alveolar Mϕs, MARCO is the main receptor for binding to unopsonised environmental particles, such as TiO_2, Fe_2O_3, and latex beads (Palecanda et al., 1999). Later this observation was confirmed by Arredouani and colleagues in mouse (Arredouani et al., 2004) and human alveolar Mϕs (Arredouani et al., 2005). Recently it was demonstrated that MARCO expression in alveolar Mϕs is downregulated by interferon γ which reduces the clearance of bacteria in the lung (Sun and Metzger, 2008).

1.5 Endocytosis

In addition to phagocytosis, the Mϕ also performs receptor-mediated endocytosis and pinocytosis. Phagocytosis and endocytosis differ in their mechanism. In contrast to phagocytosis, endocytosis is generally independent of actin and requires smaller invaginations of the plasma membrane. Receptor-mediated endocytosis uses specific receptors, collected in micro-domains of the plasma membrane called coated pits to bind macromolecules and concentrate them inside the cell (Mellman, 1996). For endocytosis, the internalised particles or macromolecules have to be smaller than 0.2 μm in diameter to be

transported in small vesicles which are coated with proteins, such as clathrins, that facilitate internalisation (Swanson, 2008).

In contrast to phagocytosis and endocytosis, pinocytosis occurs with little or no involvement of receptors. Pinocytosis is the random sampling of the extracellular fluid and occurs by the random invagination and pinching off plasma membrane, which results in vesicle formation. Macropinocytosis refers to a similar mechanism as pinocytosis, except that self-organised structures, called macropinosomes, which form as a result of internalisation of extracellular fluid, can vary from 0.2-10 μm in diameter. Ruffles on the cell surface close to engulf fluid and actin polymerisation and the activation of small GTPases and several kinases (e.g. PI3K, PI5K, PAK1) are required. Mϕs use pinocytosis, macropinocytosis and receptor-mediated endocytosis to take up soluble molecules, viruses and small particles (Mellman, 1996).

Some receptors that perform phagocytosis, also perform endocytosis (e.g. the class A scavenger receptors), whereas other receptors are specialised for a specific function (e.g. the LDL receptor) (McKnight and Gordon, 1998). Endocytic abilities of cells can be regulated by interaction with various stimuli (e.g TLR ligands) and during cell development. For example, one of the characteristics of DC maturation is reduction in endocytosis and antigen sampling (Burns and Thrasher, 2004).

1.5.1 Scavenger receptor mediated endocytosis

SR-A mediates endocytosis of modified LDL by first binding to the ligand on the cell surface. Subsequently, the receptor-ligand complexes are concentrated in clathrin-coated pits

and the complexes are transported to the endosomes. Due to the low pH, the ligand is released in the endosome and the receptor is presumably recycled back to the cell surface through the Golgi network (Mori et al., 1994).

Many studies on scavenger receptors have concentrated on the uptake of endogenous ligands such as modified LDL. SR-A endocytoses a variety of endogenous ligands which form in humans by covalent modification of proteins and LDL (Fukuda et al., 1986). AcLDL and OxLDL are also ligands for MARCO (Elomaa et al., 1995), although MARCO is much less efficient in endocytosis of AcLDL than SR-A (Plüddemann et al., 2008). The physiological role of MARCO-mediated endocytosis *in vivo* is not known.

1.6 Role of class A scavenger receptors in macrophage adhesion

Cell adhesion is a critical process in determining cell shape and function. Adhesion is mediated via divalent cation dependent (e.g by integrins and cadherins) or independent (e.g. by SR-A) mechanism. Integrins are cell surface receptors that are obligate heterodimers. One of their most important roles is to interact with extracellular matrix and mediate Mg^{2+} dependent adhesion. Integrin ligands include extracellular matrix proteins, such as fibronectin, laminins, collagens and vitronectin (Hynes, 2002).

Unlike many other cell types, the Mϕ is able to adhere to surfaces in the absence of divalent cations (Fraser et al., 1993). In order to determine the molecule that is responsible for Ca^{2+}, Mg^{2+} independent adhesion to serum coated tissue culture treated plastic, a monoclonal antibody, 2F8, was generated that blocked the adherence of Mϕs in the absence of divalent

cations. This adherence molecule was determined to be SR-A (Fraser et al., 1993). Recently it was determined that SR-A ligands in the serum are apoA-I, the main component of high density lipoprotein, and apoE (Neyen, 2008).

Although tissue culture plastic is not a physiological ligand, scavenger receptors are thought to bind to this surface due to the variety of anionic functional groups present on this surface. Several components of the extracellular matrix, such as proteoglycans (Santiago-Garcia et al., 2001), heat denatured collagen I (Gowen et al., 2000) and glycated collagen IV (el Khoury et al., 1994) have been shown to be ligands for SR-A. SR-A expression in transfected cells has shown that SR-A enhances cell spreading and alters cell morphology by formation of filopodia-like projections and focal adhesion complexes (Post et al., 2002). SR-A mediated initial attachment and spreading are shown to be distinct processes. The initial attachment depends on the activation of $G_{(i/o)}$ proteins but this protein does not affect the spreading of adhered cells (Nikolic et al., 2007a; Post et al., 2002). Following initial attachment, Src kinase Lyn and PI3-kinase are sequentially activated during SR-A mediated spreading (Nikolic et al., 2007a). The signalling mechanism of SR-A mediated adhesion is shown to require calcium-independent $iPLA_2$ catalysed arachidonic acid release and subsequent processing by the 12/15-lipoxygenase enzyme. This in turn has been shown to activate Rac and Cdc42 for SR-A mediated adhesion (Nikolic et al., 2007b). Several signalling motifs, such as the di-leucine motif, the VXFD motif and two phosphorylation sites, that are located in the cytoplasmic domain of SR-A have been shown to affect ligand internalisation (Chen et al., 2006b; Fong and Le, 1999; Morimoto et al., 1999). It was thought that SR-A mediated adhesion is an attempt to internalise an immobilised ligand; therefore, it was hypothesised that the same sequences would be required for adhesion. Using a SR-A cytoplasmic tail

mutant, it has been determined that scavenger receptor-mediated adhesion and internalisation require different domains as transfected cells expressing SR-A cytoplasmic domain mutant were capable of adhering just as well as cells that expressed the full-length protein (Kosswig et al., 2003). This demonstrated that SR-A mediated adhesion and internalisation require distinct domains.

The role of MARCO in Mϕ adhesion has not been as precisely described as that of SR-A. In contrast to SR-A, mouse MARCO mediated adhesion to gelatine requires divalent cations (Ojala et al., 2007). Using transfected cells, Pikkarainen and colleagues have demonstrated that the induction of MARCO can induce the formation of long dendritic processes and lamellipodia (Pikkarainen et al., 1999). In addition, it has been demonstrated that resident peritoneal Mϕs (RPMϕs) from MARCO KO mice spread less when plated on glass coverslips than WT Mϕ and the absence of MARCO leads to changes in organisation of the marginal zone of the spleen (Chen et al., 2005). MARCO-mediated adhesion has been shown to have a role in DC and microglia development as it mediates actin cytoskeleton rearrangements that are required for the morphology of mature cells (Granucci et al., 2003). MARCO has been implicated to be involved in chemotactic responsiveness to serum (Chen et al., 2005). These data indicate that MARCO may be involved in the positioning and differentiation of Mϕs.

1.7 The involvement of scavenger receptors in disease

Scavenger receptors, particularly the most investigated class A scavenger receptors are known to be involved in many diseases. Due to their ability to recognise many endogenous

ligands and mediate adhesion, these receptors have been connected with atherosclerosis, infectious diseases, diabetes and cancer.

1.7.1 Atherosclerosis

Atherosclerosis causes heart attacks and strokes and is the primary cause of death in Western countries (Ross, 1993). It is a difficult disease to study due to its slow, progressive nature and chronicity. The Mϕ is present throughout the progression of the disease. Mϕs in lesions have been shown to express at least six different classes of scavenger receptors for uptake of modified lipoproteins. The earliest atherosclerotic lesion is called the fatty streak and its main component is cholesterol-laden Mϕ foam cells. Scavenger receptors contribute to removal of modified lipoproteins and thus pathological cholesterol loading and foam cell formation, the hallmark of atherosclerosis. For this reason the uptake of modified lipoproteins by the Mϕ has been extensively studied (Moore and Freeman, 2006).

While the role of the class A scavenger receptors in lipid accumulation in the Mϕ is evident *in vitro*, the results that have been obtained in *in vivo* experiments are controversial. Most of the research on atherosclerosis is done using mouse-models of the disease. Mice that are deficient in apoE have elevated plasma cholesterol levels and spontaneously develop severe atherosclerosis on low-fat diets (Nakashima et al., 1994). To determine, which genes have a role in the development of atherosclerosis, mice have been developed which are doubly-deficient in both apoE and the gene of interest. The role of a particular gene in increasing the susceptibility to atherosclerosis is assessed by measuring the lesion size in apoE single and double knockout mice. Some experimental evidence suggests, that the class A scavenger

receptors, SR-AI and SR-AII (Suzuki et al., 1997) and a member of the B class scavenger receptors, CD36 (Febbraio et al., 2000) increase atherosclerotic lesion development, but in contrast SR-BI protects against atherosclerosis in apoE deficient mice (Braun et al., 2002). Another publication also reported that apoE and SR-A double knockout (KO) mice display a reduction in peritoneal Mϕ lipid accumulation (Moore et al., 2005). On the other hand, different mouse models show that SR-A does not affect lesion size, but the lesions of SR-A KO are more severe (de Winther et al., 1999). This means that the role of SR-A in atherosclerosis is complex. Although MARCO has been found in atherosclerotic lesions of apoE KO mice it may not contribute to the disease. The expression of MARCO was not found on Mϕ in the atherosclerotic plaque in humans (Elomaa et al., 1998).

Most studies of scavenger receptors have concentrated on their ability to endocytose modified lipoproteins, but another important characteristic of scavenger receptors that has not been investigated so intensively, is adhesion. SR-A KO Mϕs are less adherent and remain more rounded (Suzuki et al., 1997). The role of scavenger receptor mediated adhesion is evident *in vitro*, as it has been demonstrated that peritoneal Mϕs from SR-A overexpressing mice display more spreading and adhesion in culture (Daugherty et al., 2001). In addition, more Mϕs accumulated in carageenan-induced granulomas *in vivo*, than in WT control mice (Daugherty et al., 2001). This suggests that scavenger receptor mediated Mϕ adhesion can play an important role in various inflammatory processes, for instance, to cause Mϕ retention at the sites of tissue injury.

Other scavenger receptors, particularly CD68, LOX-1, SREC and SR-PSOX have been implicated in progression of the disease because they have an ability to bind modified LDL

and are expressed in atherosclerotic lesions, but more *in vivo* research is required to determine the role of these receptors in atherosclerosis.

1.7.2 Infection

The class A scavenger receptors bind to a broad range of ligands with high affinity which suggests their role in innate immunity. Similar to SR-A, the ligands of MARCO also include Gram-positive and –negative bacteria (Gough and Gordon, 2000; Pluddemann et al., 2006). MARCO KO mice display reduced bacterial clearance from lung tissue and increased bacterial infection (Arredouani et al., 2004). MARCO enhances recognition and uptake of several pathogens such as *Neisseria meningitidis* (Mukhopadhyay et al., 2006). MARCO has also been suggested to have a role in recognition of *Mycobacterium tuberculosis*, the causative agent of human tuberculosis (Bowdish et al., 2008a). Thus MARCO contributes to host defense against infection.

1.7.3 Other diseases

Deletions and mutations in the SR-A gene have been associated with human prostate cancer (Xu et al., 2002). SR-A has also been reported to bind β-amyloid fibrils (El Khoury et al., 1996) which indicates that it might have a role in Alzheimer's disease. MARCO and SR-A have both been suggested to play a role in autoimmune diseases such as systemic lupus because the SR-A and MARCO KO mice display lower threshold to autoantibody responses (Wermeling et al., 2007). In addition, autoantibodies against those scavenger receptors were found in the blood of SR-A and MARCO KO mice before the onset of systemic lupus

(Wermeling et al., 2007). It has also been reported that MARCO-mediated clearance of cellular debris may protect from cerebral ischaemia (Milne et al., 2005).

1.8 The aims of this thesis

There are many questions regarding the role of MARCO that are still unanswered. The functions of SR-A and the role of various domains of SR-A in mediating those functions are fairly well characterised. In contrast, relatively little is known about the function of these domains in MARCO. Recent evidence indicates that these two receptors differ in their regulation, expression and ligand specificity and are far from redundant (Plüddemann et al., 2008).

Although the functions of the cysteine-rich domain as the ligand binding region of MARCO have been described (Brannstrom et al., 2002; Elomaa et al., 1998; Ojala et al., 2007; Pikkarainen et al., 1999), the role of the cytoplasmic domain has not yet been characterised. Research done on the functions of the cytoplasmic domain of SR-A has indicated that this domain may fulfil similar functions in MARCO as well. Therefore, it was hypothesised that the cytoplasmic domain of MARCO is important for cell surface expression, uptake and adhesion.

Chapter Three describes the differences in cellular localisation of SR-A and MARCO, highlighting that their expression profile is different. I also investigated the role of the cytoplasmic tail in cellular localisation of MARCO. In Chapter Four, the ligand for MARCO is characterised and the role of the cytoplasmic domain in binding particulate ligands and

uptake of soluble ligands is investigated. The aim of Chapter Five was to characterise MARCO-mediated adhesion and determine the role of the cytoplasmic tail in this function of MARCO. Lastly, the results are summarised and their implications in the biology of MARCO are discussed in Chapter Six.

2 Chapter Two: Materials and Methods

2.1 Materials

2.1.1 Antibodies and other reagents

Rat monoclonal anti mouse MARCO clone ED31 (van der Laan et al., 1997) and mouse monoclonal anti human MARCO clone PLK-1 (Arredouani et al., 2005) were grown and maintained as described in section 2.3.1. R-phycoerythrin (RPE) conjugated ED31 was purchased from AbD Serotec. RPE conjugated rat immunoglobulin G 1 (IgG1) isotype control was obtained from BD Biosciences. Rat IgG2b isotype control was obtained from AbCam. Mouse IgG3 isotype control OX61 was a generous gift from Dr. Marion Brown (Sir William Dunn School of Pathology, University of Oxford, (SWDSOP)). Rat anti mouse SR-A monoclonal antibody (mAb) 2F8 (Fraser et al., 1993), mouse anti human SR-A antibody C6 (Tomokiyo et al., 2002) and mouse IgG1 isotype control D1.3 were provided by the Gordon lab (SWDSOP). Rabbit anti human MARCO polyclonal antibody (pAb) 977 was a generous gift from Dr. Timo Pikkarainen (Karolinska Institutet, Sweden). Alexa488 donkey anti-mouse, Alexa488 goat anti-rat, Alexa488 goat anti-rabbit, Alexa647 goat anti-rabbit and Alexa647 goat anti-mouse $F(ab')_2$ antibodies (all Molecular Probes) were used as secondary antibodies for immunofluorescence microscopy and flow cytometry. Alexa488 protein conjugation kit was obtained from Molecular Probes. Fluoresbrite® Yellow Green 2.00 μm microspheres and polyethylenimine were purchased from Polysciences. Fluorescent cell mounting medium was purchased from DakoCymation. Sterile phosphate buffered saline (PBS), Optimem media, TrypleExpress (recombinant enzyme replacing animal trypsin), bovine fibronectin, DiI (1,1'-dioctadecyl-1-3, 3'-tetramethylindocarbocyanine perchlorate)

cell-labelling solution and DiI-labelled acetylated LDL (DiIAcLDL) were obtained from Invitrogen. Collagen I from rat tail was obtained from BD Biosciences. Protein assay BCA kit and dialysis cassettes were obtained from Pierce. Glass coverslips for microscopy were purchased from VWR. Glass bottom dishes for live cell imaging were purchased from MatTek Corporation. All other tissue culture plates were purchased from BD Biosciences. Other reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

2.1.2 Plasmids

The plasmid containing human MARCO (hMARCO) was a kind gift from Dr. Timo Pikkarainen (Karolinska Institutet, Sweden). The plasmid containing human SR-AI has been previously described (Gough et al., 1998). Constructs of hMARCO that lack the cytoplasmic tail (N- hMARCO and Nmyr hMARCO) were previously created by Dr. Dawn Bowdish by designing primers that amplified the transmembrane region of hMARCO and contained restriction enzyme sites. After amplification and restriction digest, the amplified region was subcloned into pcDNA3.1 (Invitrogen).

2.1.3 Animals

SR-A I/II/III KO (Suzuki et al., 1997) and MARCO KO (Arredouani et al., 2004) mice, both on the C57BL/6 background, were generous gifts from Dr. Karl Tryggvason **(**Karolinska Institutet, Sweden) and were bred and housed at the University of Oxford under pathogen-free conditions. Mice deficient in both SR-A and MARCO (SR-A and MARCO knockout (DKO)) were obtained by cross-breeding the single knockouts. Wild type (WT) C57Bl/6

mice were purchased from either Taconic or Harlan. Experiments used age- and sex-matched mice, between 5 weeks and 3 months of age. Experiments were performed in accordance with local ethical guidelines.

2.2 Cell culture

2.2.1 General conditions for mammalian cell culture

The HEK 293T and HeLa cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM medium) supplemented with 10% heat inactivated foetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin (all PAA), unless otherwise indicated. Antibiotics were excluded during transfection experiments. All cells were incubated at 37 ^{o}C in a 5% CO_2, 95% humidity incubator. Cells were detached by incubation with TrypleExpress for 5 min at room temperature. The cell line was routinely sub-cultured between 1:6 and 1:10, twice weekly.

2.2.2 Isolation of resident murine peritoneal Mϕs

Mouse RPMϕs were obtained by lavaging the peritoneal cavity with 10 ml PBS and resuspending the peritoneal cells in complete growth medium (RPMI supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin) and allowing the Mϕs to adhere overnight. Non-adherent cells were then rinsed off before RPMϕs were used for experiments.

2.2.3 Isolation of murine bone marrow derived Mϕs

Femurs and tibias were dissected from two euthanased mice and were sterilised by incubation in 70% ethanol for one minute. The ends of the bones were removed and bone marrow cells were flushed with PBS using a syringe and 26G needle. These plugs were mechanically disrupted by pipetting through a 19G needle prior to centrifugation at 400 x g for 5 min. The bone marrow cells were resuspended in 15 ml R10 medium (RPMI supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 10 mM HEPES buffer), containing 15% L-cell conditioned medium **(**LCM) (see 2.2.3.1). The bone marrow cells were cultured on 15 cm bacterial plastic Petri dishes containing 20 ml R10-LCM medium. The Mϕs were allowed to differentiate for 7 days until they were harvested and used for experiments.

2.2.3.1 L-cell conditioned media

Supernatant was collected from the L929 fibroblast cell line (Cell Bank, SWDSOP) cultured in DMEM supplemented with 10% FBS, 20 U/ml penicillin, 0.02 mg/ml streptomycin and 2 mM L-glutamine for 10 days and then filtered through a 0.45 μm filter.

2.2.4 The isolation and culture of human monocytes from peripheral blood

The isolation and culture of human monocytes from peripheral blood were performed by a modified protocol described by Montaner and colleagues (Montaner et al., 1996). Mononuclear cells were obtained by Ficoll-Hypaque density sedimentation and washed 4-5

times with PBS to remove platelets. Autologous plasma was recovered from the upper phase after Ficoll-Hypaque centrifugation, heat-inactivated for 30 min at 56 ^{o}C prior to cooling on ice and centrifugation at 3000 x g to remove fibrin and passed through a 0.22 μm syringe filter. Cells were resuspended in X-VIVO 10 (Cambrex) containing 1% autologous human plasma and plated on 150 mm cell culture dishes that had been previously coated with 2% gelatine solution. After 1 h, when the monocytes had adhered, the nonadherent lymphocytes were removed by washing the dishes with RPMI-1640. The monocytes were incubated overnight with X-VIVO 10 containing 1% autologous plasma. On the next day the cells were detached with PBS containing 4 mg/ml lidocaine-HCl and 10 mM EDTA. The cells were resuspended in X-VIVO 10 supplemented with 1% autologous human plasma and plated in 24-well plates containing glass coverslips at a density of 2.5 x 10^5 cells per well. After replating the cells, the monocytes differentiated into mature Mϕs, gaining a flattened adherent phenotype, and becoming terminally differentiated after approximately 5-7 days in culture. Previous studies in the laboratory have shown that this protocol yields a >95% pure Mϕ population (Montaner et al., 1996).

2.3 Methods

2.3.1 Antibody production and concentration

Rat monoclonal anti mouse MARCO clone ED31 and mouse monoclonal anti human MARCO clone PLK-1 hybridomas were grown in DMEM and RPMI-1640 medium respectively, supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin (all reagents PAA). Fresh media were added every

other day until the volume 4.8 l was obtained. After the maximum cell density possible was obtained, the cells were allowed to produce antibody in the medium until approximately 80% of the cell population became non-viable, and the medium containing the antibody was collected. The collected medium with the cells was spun down at 1000 x g for 10 min. To remove the remaining cell debris, the supernatant was filtered through a 0.22 μm membrane into a sterile bottle. The filtered supernatant was concentrated by ammonium sulfate precipitation overnight by adding an equal volume of 50% (w/v) ammonium sulfate solution (Jakoby, 1971). The precipitate was dialysed overnight back into PBS using a dialysis cassette. The protein content was determined using BCA protein assay reagent. The purity of the concentrated antibody solution was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining (Fig. 2.1).

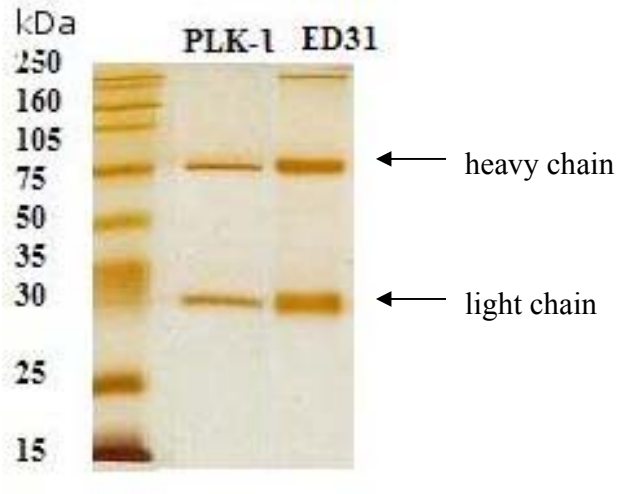


Fig. 2.1 The purity of ammonium sulfate precipitated antibodies. 1 µg of protein in a reducing loading buffer was loaded per lane of a 12% SDS-PAGE gel. The proteins were visualised by silver staining. Two major bands that appeared were approximately the size of the heavy and light chain of IgG, which showed the purity of the concentrated hybridoma supernatants. Molecular size markers are shown in the first lane.

2.3.2 Transfection of HEK 293T and HeLa cells

HEK 293T cells were seeded, 3 x 10^5 cells in 1 ml DMEM medium, in 12-well plates or 6 x 10^5 cells in 2 ml DMEM medium in 6-well plates, or 10 x 10^6 cells in 150 mm tissue culture dishes overnight. For microscopy, HEK 293T and HeLa cells were seeded, 8 x 10^4 cells in 1 ml DMEM medium on glass coverslips in 24-well plates. The following day the medium was changed to Optimem and the cells were transfected using 8 μg of linear polymeric transfection agent polyethylenimine per 1 μg of DNA. The cells were used for analysis 48 h later.

2.3.3 Maleylated bovine serum albumin (MalBSA)

MalBSA has previously been reported to be a SR-A ligand (Takata et al., 1989). Maleylation of BSA was carried out by adding maleic anhydride (final concentration 0.1 M) to reaction buffer (0.2 M Na_2CO_3 pH 8.5) containing 10 mg/ml BSA. The pH was readjusted by adding 5 M NaOH keeping the reaction pH above 7.5. The aliquots were stored at -20 ^oC (adapted from Butler and Hartley, 1972). Protein content was determined using BCA protein assay reagent. Protein modification was confirmed by SDS-PAGE and silver staining (Fig 2.2). MalBSA was conjugated to Alexa488 according to the manufacturer's instructions.

2.3.4 SDS-Polyacrylamide Gel Electrophoresis

Polyacrylamide separating gels of 8% and 12% (pH 8.8) and stacking gels of 4% (pH 6.8) were used to resolve proteins approximately according to molecular size. Cell lysates and

protein solutions were loaded mixed with 5 x SDS-PAGE loading buffer (312.5 mM Tris base, 10% glycerol, 11.5% SDS, with or without a reducing agent 500 mM dithiothreitol, bromophenol blue 0.1%, pH 6.8). Gels were cast and run using a Mini-PROTEAN III gel system (Biorad). Gels were electrophoresed at 100 mV per 1.5 mm thick gel in buffer containing 25 mM Tris and 90 mM glycine, until the dye front had run off the end of the separating gel. Molecular weights were estimated by comparison with standards ranging from 14.3 to 220 kD (Rainbow marker, Invitrogen).

2.3.5 Silver staining

Silver staining was performed at room temperature with gentle agitation. First, the gel was soaked in the fixing solution that contained 50% methanol, 40% double distilled water (ddH_2O) and 10% acetic acid for 1 h. Next, it was washed 2 x 10 min in 50% ethanol. The gel was then pretreated by soaking 1 min in 0.02% sodium thiosulfate solution in ddH_2O. Before adding the silver nitrate solution (0.2% $AgNO_3$, 0.028% formaldehyde) for 10 min, the gel was rinsed 3 x 30 seconds in ddH_2O. After staining the gel, it was also rinsed 2 x 20 seconds in ddH_2O. Finally the developer (6% Na_2CO_3, 0.0185% formaldehyde, 0.4 mg sodium thiosulfate) was added and the gel agitated gently. When the bands seemed sufficiently bright, the fixing solution was added to stop the reaction.

2.3.6 Western blotting

The proteins were transferred from SDS-PAGE gels to Hydrobond C+ nitrocellulose membrane (GE Healthcare) at 100 V for 90 min in western blot transfer buffer (30 g/l Tris,

14.4 g/l glycine, 20% methanol in H_2O) (Towbin et al., 1979). The membrane was then blocked overnight at 4 ^oC in blocking buffer (4% fat-free milk powder (Marvel), 0.1% Tween in PBS) and then incubated for one hour in blocking buffer containing 20 μg/ml polyclonal rabbit anti human MARCO antibody 977. The membrane was washed three times in 0.1% Tween in PBS, before incubation with horse-radish peroxidise (HRP)-conjugated anti-rabbit secondary antibody (dilution 1:2000) for further one hour. Finally, the membrane was washed three times in 0.1% Tween in PBS and developed using the ECL Western blotting detection system (GE Healthcare) onto Kodak film.

2.3.7 Immunofluorescence and microscopy

HEK 293T and HeLa cells were seeded onto coverslips at 8 x 10^4 cells per well in DMEM and transfected as described in section 2.3.2. Human monocytes and bone marrow Mϕs from C57BL/6 mice and RPMϕs were seeded onto coverslips at 2.5 x 10^5 cells per well in 500 μl of RPMI. For immunofluorescence staining the cells were fixed with 2% paraformaldehyde (PFA) for 25 min and then blocked with PBS containing 1% BSA and 5% heat inactivated goat serum with or without a permeabilising agent, 0.25% saponin. After blocking, the primary antibody was added at 5 μg/ml in blocking mixture. After 1 h the cells were washed 3 times and an appropriate secondary antibody was added (dilution 1:500 for anti-rat and anti-mouse; 1:2000 for anti-rabbit) in blocking mixture. The cells were incubated with the secondary antibody for 1 h and washed 4 times with PBS. To stain the actin cytoskeleton and the nuclei, tetramethylrhodamine B isothiocyanate (TRITC) labelled (phalloidin-TRITC) or fluorescein isothiocyanate (FITC) labelled phalloidin (phalloidin-FITC) 25 μg/ml and 4',6-diamidino-2-phenylindole (DAPI), 1 μg/ml, were added to cells for 45 min. The coverslips

were washed with PBS and mounted in fluorescent mounting medium. In experiments where PLK-1 antibody was used, the cells were fixed and permeabilised after incubation with the secondary antibody because this antibody is incapable of binding to its antigen in fixed cells. The images were captured by Zeiss fluorescence light microscope or Zeiss confocal laser scanning microscope.

2.3.8 Immunochemical labelling of cells and flow cytometry

Cells for flow cytometry and immunofluorescence were labelled with antibodies using the same protocol. For flow cytometry, the cells were harvested by incubation with PBS containing 4 mg/ml lidocaine-HCl and 10 mM EDTA. The cells were fixed with 2% PFA for 25 min on ice and then blocked with 1% BSA and 5% heat inactivated goat serum in FACS Wash solution (5 mM EDTA, 2 mM NaN_3, 0.5% BSA in PBS). Primary antibodies were diluted to 5 μg/ml in blocking mixture. After 1 h the cells were washed 3 times in FACS Wash solution and an appropriate secondary antibody was added (dilution 1:2000). Finally, the cells were washed 4 times with FACS Wash solution and analysed on a FACSscan (BD Biosciences). Data were analysed using CellQuest software (BD Biosciences).

2.3.9 MalBSA coating of latex beads

Fluorescent polystyrene latex microspheres were passively coated with either MalBSA or normal BSA as a control, by incubating for 1 h at room temperature in 1 ml of BSA or MalBSA (1.5 mg/ml). In both cases, the beads were centrifuged and resuspended in 1 ml of PBS containing no Ca^{2+} or Mg^{2+} (PBS, Ca−, Mg−). To estimate the resulting concentrations

of beads per ml, the beads were first sonicated to produce single-bead suspensions, and an aliquot was then counted with the use of a haemocytometer. To control whether the beads actually had BSA or MalBSA on them, an aliquot of 30 µl was boiled for 5 min in 5 x SDS-PAGE loading buffer. Next the beads were spun down and the supernatants were run on a 12% SDS-PAGE gel with MalBSA and BSA as a control. The proteins were visualised by silver staining (Fig. 2.2).

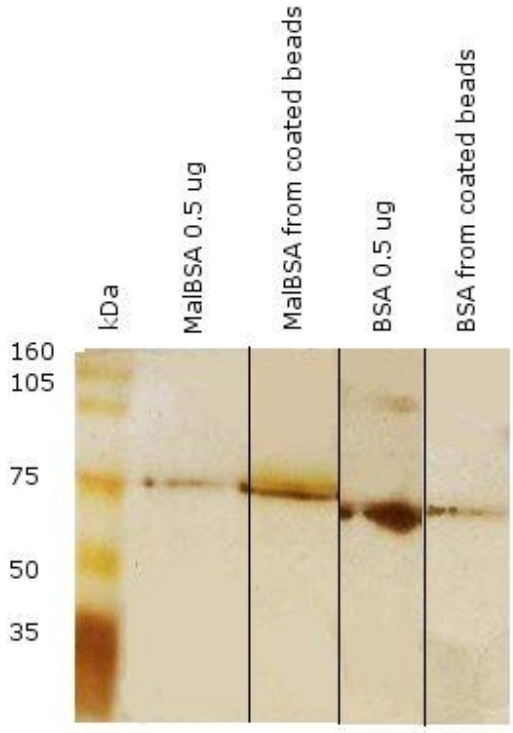


Fig. 2.2 BSA modification and passive coating of fluorescent beads. The solutions in non-reducing loading buffer were loaded on a 12% SDS-PAGE gel and the proteins were visualised by silver staining. The molecular weight of MalBSA is a couple of kDa higher than that of BSA due to covalent modification. MalBSA and BSA were also detected on beads that were passively coated with the protein. The samples were run on a same gel and selected lanes are shown. Molecular size markers are shown in the first lane.

2.3.10 Binding assays performed with RPMϕs

To determine whether MalBSA is a ligand for MARCO, RPMϕs were first blocked for 20 min at 37 ^{o}C with 1% BSA, 5% goat serum in Optimem media. Next MalBSA or BSA coated beads were added (10 beads per cell) and the cells were incubated at 37 ^{o}C for 20 min. After incubation, the cells were washed with PBS and fixed with 2% PFA for 20 min. DNA and actin cytoskeleton were stained with 1 μg/ml DAPI and 25 μg/ml TRITC-phalloidin, respectively, in blocking solution which also contained a permeabilising agent, saponin (0.25%). The coverslips were washed with PBS and mounted in fluorescent mounting medium. Using fluorescence light microscopy, pictures of about 80 cells per coverslip were taken and the number of bead bound cells was counted.

2.3.11 Binding and uptake assays performed with transfected HEK 293T cells

HEK 293T cells were transfected as described in section 2.3.2 and seeded on 12-well tissue culture plates or on coverslips that were previously treated with poly-D-lysine. When immunofluorescence staining was analysed by microscopy, the plasma membrane was first labelled with DiI, according to the manufacturer's instructions. The cells were blocked with Optimem containing 1% BSA and 5% goat serum for 20 min on ice. To test if the binding of the beads was scavenger receptor dependent, a general scavenger receptor inhibitor, dextran sulfate ($DxSO_4$) (500 μg/ml), was added to the blocking mixture. For control, chondroitin sulfate ($ChSO_4$), which has a similar structure, was added at the same concentration. Next, the beads (10 beads per cell) or soluble Alexa488 labelled MalBSA (2.5 μg/ml) or

DiIAcLDL (2.5 μg/ml) were added and the cells were placed on ice for 10 min. Subsequently, the plates were placed in the incubator at 37 ^{o}C. The cells were then washed with PBS and fixed with 2% PFA for 20 min. Subsequently, the cells were stained for MARCO or SR-A. The results were analysed by laser scanning microscopy or flow cytometry.

2.3.12 Transfected HEK 293T cell adhesion experiments

HEK 293T cells were grown and transfected on 150 mm cell culture dishes and then seeded on 24-well tissue culture or suspension grade plastic that was previously coated with 400 mg/ml MalBSA, BSA or 60 μg/ml of PLK1, C6, IgG1, IgG3 or collagen I at 37 ^{o}C for 2 h. The cells were allowed to adhere for 24 h and then washed 4 times with a warm PBS 1% BSA solution. The remaining adherent cells were stained with crystal violet solution (crystal violet dye 0.2%, ethanol 2% and ddH_2O 97.8%) for 20 min, washed 10 times with water and to quantify the number of adherent cells, 1% SDS solution was added to wells and the plates were incubated overnight at 4 ^{o}C. The next day the number of adherent cells was quantified by measuring optical density at 550 nm wavelength. Unwashed cells were used as a control to which the results were compared. To test whether MARCO mediated adhesion is divalent cation independent, the transfected cells were grown to confluence on 6-well plates, washed with PBS, Ca−, Mg− to remove traces of media. PBS containing 10 mM EDTA was added to wells for selected periods of time. The number of adherent cells was quantified by the crystal violet method, as described previously.

2.3.13 Live cell imaging

RPMϕs (10^5 cells in 70 μl of RPMI) were seeded on the glass microwell of 35 mm glass bottom culture dishes and left to adhere for 15 min. Then, 3 ml of media were added and the cells cultured overnight. The following day, nonadherent cells were removed by washing and imaging of live cells was performed at interval of 1 min during a 2 h period using a Zeiss laser scanning microscope with CO_2 incubator. Cell movement was then analysed by MetaMorph software (Molecular Devices).

2.3.14 Statistical analysis

Most experiments were performed in triplicate, unless noted otherwise. Differences between the means of amongst samples and sample populations were analysed using the Student's T-test. Differences were considered significant when $p \leq 0.05$. The statistical analysis was performed using Microsoft Excel.

3 Chapter Three: Expression profile and cellular localisation of MARCO and SR-A

3.1 Introduction

The studies presented in this chapter investigate the differences in localisation within the cell between the two class A scavenger receptors, MARCO and SR-A. It has been shown that cellular localisation has a profound effect on the function of scavenger receptors. For example, SR-AIII, which differs from SR-AI only in the cysteine-rich domain, is misfolded and trapped in the ER and is shown to be defective in endocytosis (Gough et al., 1998). To date, we do not know whether regions of MARCO other than the transmembrane domain are important for cell surface expression and trafficking within the cell.

Both SR-A and MARCO have a short N-terminal cytoplasmic tail of 55 and 49 amino acids, respectively. Besides the hydrophobic transmembrane region, SR-A has been shown to require the cytoplasmic six membrane-proximal amino acids for cell surface expression (Kosswig et al., 2003). The amino acid sequence of the cytoplasmic tail of MARCO is considerably different, but similar to SR-A, it may contain an element that can affect cell surface expression of the protein. Bioinformatic analysis revealed that the membrane-proximal region contains a putative myristoylation site (Fig. 3.1A). Therefore, the six membrane-proximal amino acids might be important for cell surface expression because myristoylation has been shown to be involved in intracellular trafficking and cell surface expression of many membrane proteins (Boutin, 1997). To investigate the role of the cytoplasmic tail in cellular localisation of MARCO, two cytoplasmic tail mutants were used:

the Nmyr MARCO cytoplasmic tail mutant contains the myristoylation site but lacks rest of the cytoplasmic tail; N- MARCO lacks the entire cytoplasmic tail (Fig. 3.1B).

A.

SR-A
MTKEMTENQRLCPHEREDADCSSESVKFDARSMTASLPHSTKNGPSVQE**KLKSFK**
Cell surface expression

MARCO
MRNKKILKEDELLSETQQAAFHQIAMEPFEINVPKPKRRN**GVNFSL**AVV
Putative N-myristoylation site

B.

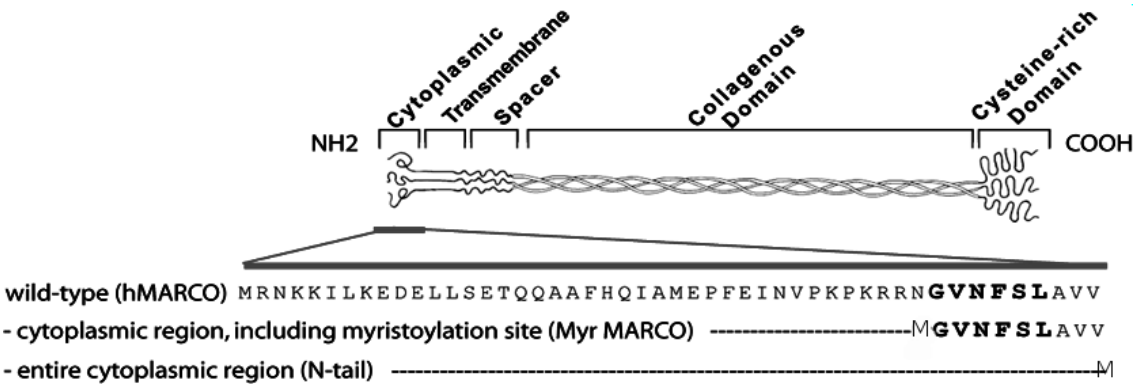


Fig. 3.1 A. **The amino acid sequences of the cytoplasmic component of SR-A and MARCO.** The six amino acid sequence, that has been shown to be important for SR-A cell surface expression (Kosswig et al., 2003), is highlighted. MARCO does not contain a similar sequence but bioinformatic analysis has revealed that the membrane-proximal region contains a putative N-myristoylation site. The role of this sequence in cellular functions of MARCO is investigated in this thesis. **B**. **Wild type hMARCO and the mutants used in this thesis.** The structure of the full-length MARCO and the comparison of the amino acid sequences of the cytoplasmic domain. The Nmyr MARCO mutant includes the myristoylation site but lacks the rest of the cytoplasmic domain, the N- MARCO mutant lacks the entire cytoplasmic tail.

3.2 The aims of this chapter

The aims of this chapter are to characterise the cellular localisation of MARCO and SR-A, and determine, if the cytoplasmic domain of MARCO has a role in the cell surface expression of the protein. The extracellular domain of MARCO, which is shown to be about 80 nm long (Elomaa et al., 1995), is relatively well characterised, but less is known about the function of the cytoplasmic domain. To demonstrate the difference in cellular localisation between MARCO and SR-A, both receptors were stained in permeabilised and non-permeabilised human monocyte derived Mϕs and mouse RPMϕs. In addition, the expression profile of MARCO and SR-A in mouse RPMϕs and bone marrow derived Mϕs was compared. I hypothesised that the N-terminal cytoplasmic tail contains sequences that are required for cell surface expression. To test this hypothesis, I utilised the HeLa and HEK 293T cell lines in which the full-length hMARCO, the Nmyr MARCO and the N-tail mutants were transiently expressed. The results indicate that the cytoplasmic tail might be important for trafficking to the cell surface.

3.3 Results

3.3.1 A higher proportion of MARCO is expressed on the cell surface compared to SR-A in primary human and murine Mϕs

Mouse RPMϕs were stained for MARCO or SR-A using the monoclonal antibodies ED31 (RPE conjugated) and 2F8, respectively. Cell surface expression of the protein was determined by fluorescence microscopy of the non-permeabilised cells. In addition, the

intracellular pool of protein was visualised in permeabilised cells. As can be seen from Fig. 3.2, the extracellular expression of MARCO is greater than that of SR-A. In contrast, the cytoplasmic expression of MARCO is less than that of SR-A, as can be seen in Fig. 3.3. A similar cellular localisation profile was evident in non-permeabilised (Fig. 3.4) and permeabilised human monocyte derived Mϕs (Fig. 3.5). These results indicate that MARCO and SR-A differ in their cellular localisation profile.

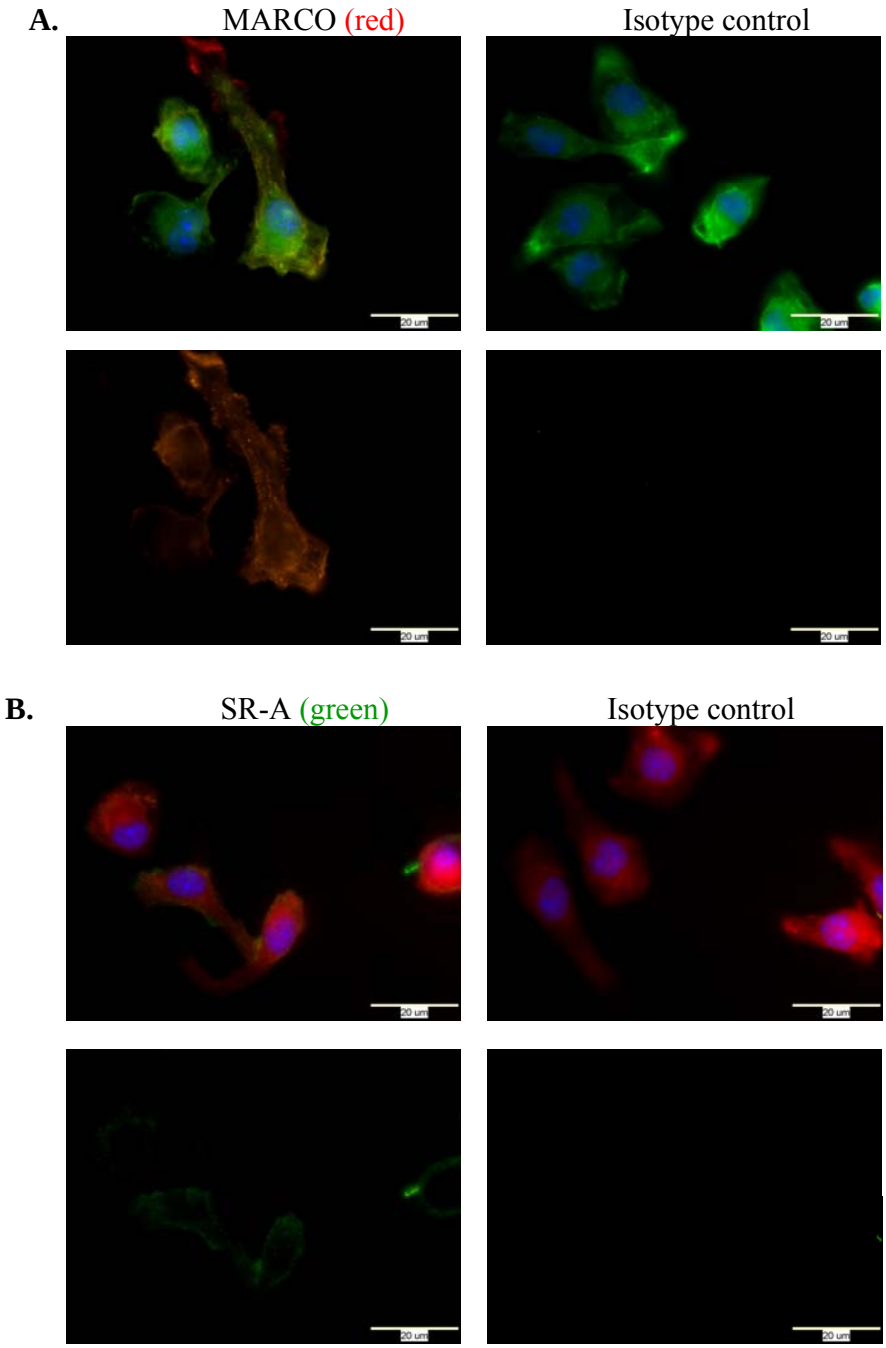


Fig. 3.2 The expression profile of MARCO and SR-A in non-permeabilised mouse RPMϕs. A. MARCO is stained in red (ED31-RPE), actin in green (phalloidin-FITC) and the nucleus in blue (DAPI). **B.** SR-A is stained in green (2F8 and anti-rat Alexa488), actin in red (phalloidin-TRITC) and nucleus in blue (DAPI). MARCO is expressed on the surface of most RPMϕs at high levels, whereas only a small proportion of SR-A is on the cell surface.

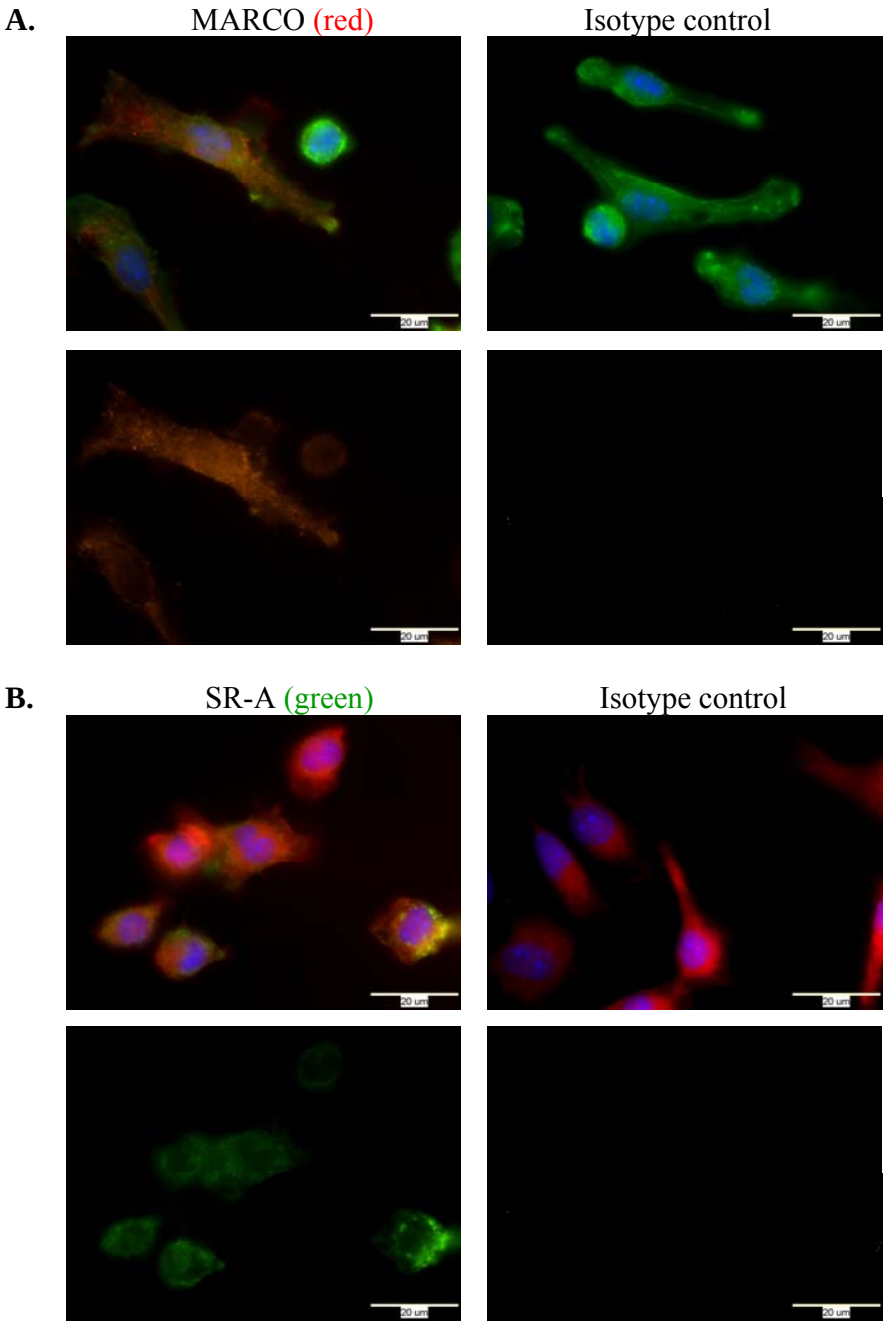


Fig. 3.3 The expression profile of MARCO and SR-A in permeabilised mouse RPMϕs. **A**. MARCO is stained in red (ED31-RPE), actin in green (phalloidin-FITC) and the nucleus in blue (DAPI). **B.** SR-A is stained in green (2F8 and anti-rat Alexa488), actin in red (phalloidin-TRITC) and nucleus in blue (DAPI). Fig. B shows that a greater proportion of SR-A is intracellular, also the expression level of SR-A in RPMϕs varies more than the expression level of MARCO. A comparison between the staining of MARCO in permeabilised (Fig. 3.3 A) and non-permeabilised RPMϕs (Fig. 3.2 A) indicates that a greater part of the protein is on the cell surface.

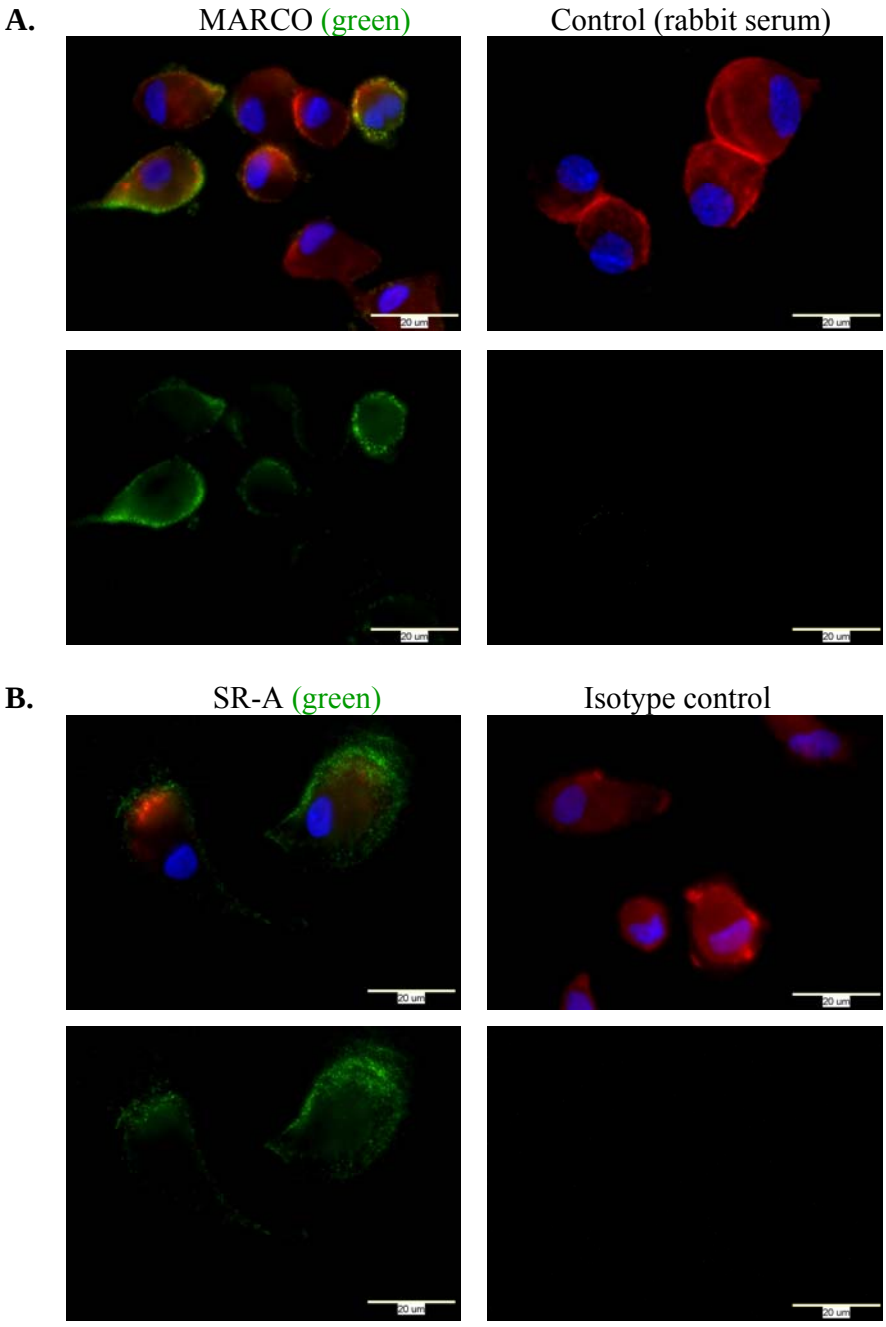


Fig. 3.4 The expression profile of MARCO and SR-A in non-permeabilised human monocyte derived Mϕs. A. MARCO is stained in green (rabbit anti hMARCO pAb 977 and anti-rabbit Alexa488), actin in red (phalloidin-TRITC) and the nucleus in blue (DAPI). **B**. SR-A is stained in green (mouse anti hSR-A mAb C6 and anti-mouse Alexa488), actin in red (phalloidin-TRITC) and DNA in blue (DAPI). MARCO is expressed on the surface of most, but not all Mϕs. Differences between MARCO and SR-A in cellular localisation are similar to those of mouse RPMϕs.

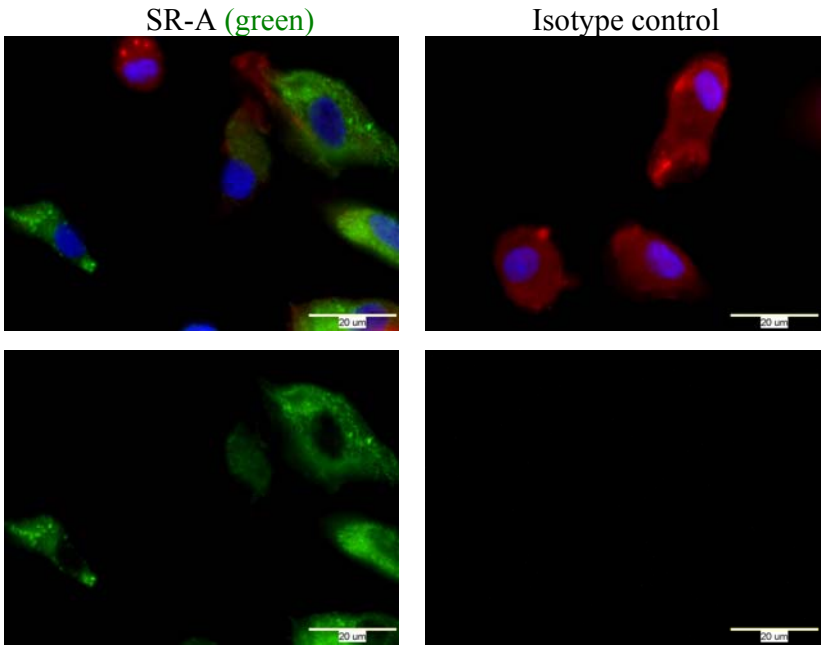


Fig. 3.5 The expression profile of SR-A in permeabilised human monocyte derived Mϕs. SR-A is stained in green (mouse anti hSR-A mAb C6 and anti-mouse Alexa488), actin in red (phalloidin-TRITC) and nucleus in blue (DAPI). The variability in expression levels of SR-A was most evident in human monocyte derived Mϕs.

3.3.2 Expression profile of MARCO and SR-A in mouse bone marrow derived Mϕs

In contrast to RPMϕs, the expression of MARCO was not detected in mouse bone marrow derived Mϕs, while the level of expression of SR-A was comparable to expression in RPMϕs (Fig. 3.6, Fig. 3.7). Consistent with previously published results, this indicates that these two receptors have different expression profiles. While SR-A is present on resident Mϕs of most tissues, MARCO expression is restricted only to certain Mϕ subpopulations in healthy animals, e.g. RPMϕs, the lymph nodes and the spleen (Elomaa et al., 1995).

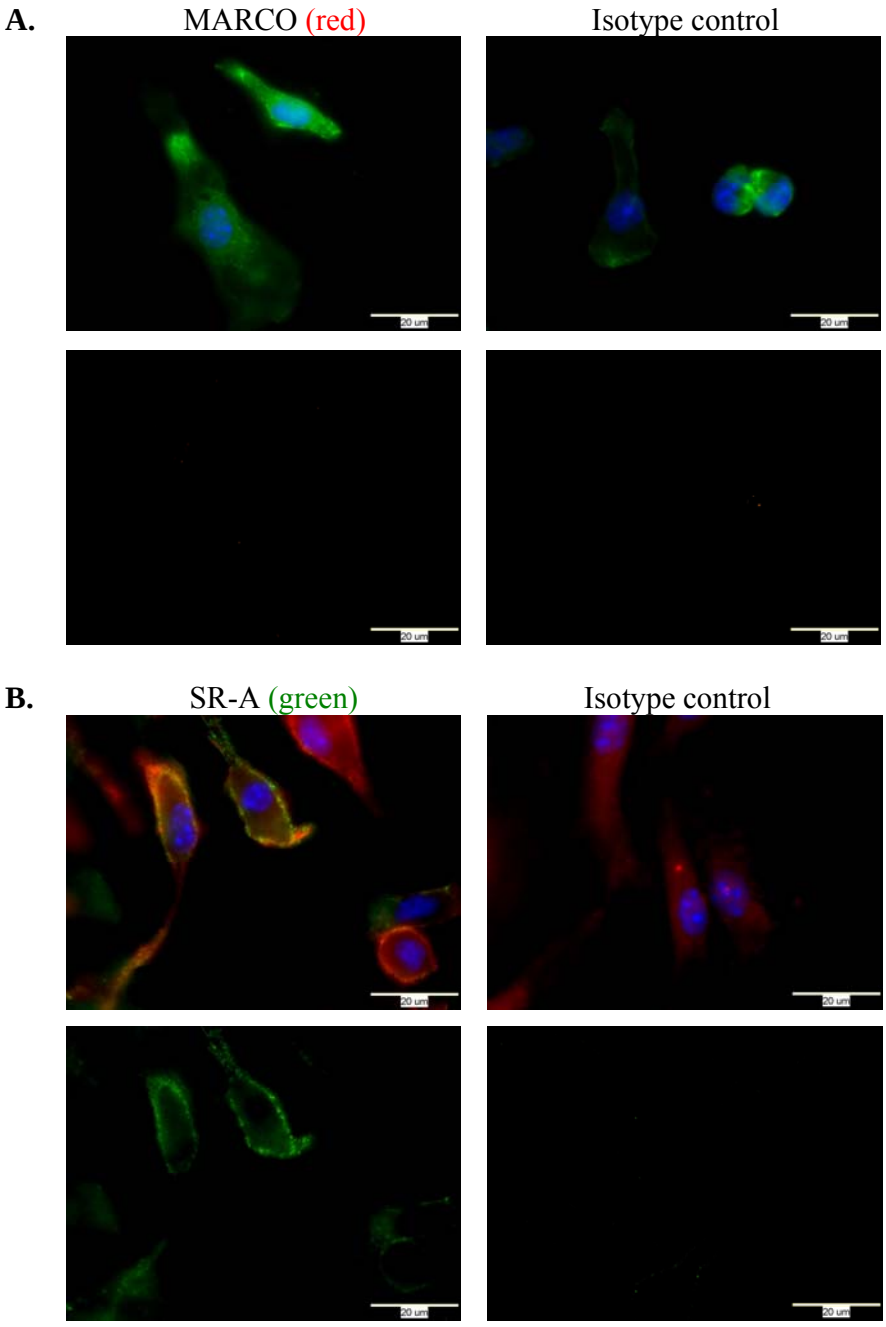


Fig. 3.6 The expression profile of MARCO and SR-A in non-permeabilised mouse bone marrow derived Mϕs. A. MARCO is stained in red (ED31-RPE), actin in green (phalloidin-FITC) and the nucleus in blue (DAPI). **B.** SR-A is stained in green (2F8 and anti-rat Alexa488), actin in red (phalloidin-TRITC) and nucleus in blue (DAPI). While the expression of SR-A is similar to its expression in RPMϕs, the expression of MARCO was not detected in bone marrow derived Mϕs. This confirms previously published results that the expression of MARCO is limited to selected resident Mϕ populations.

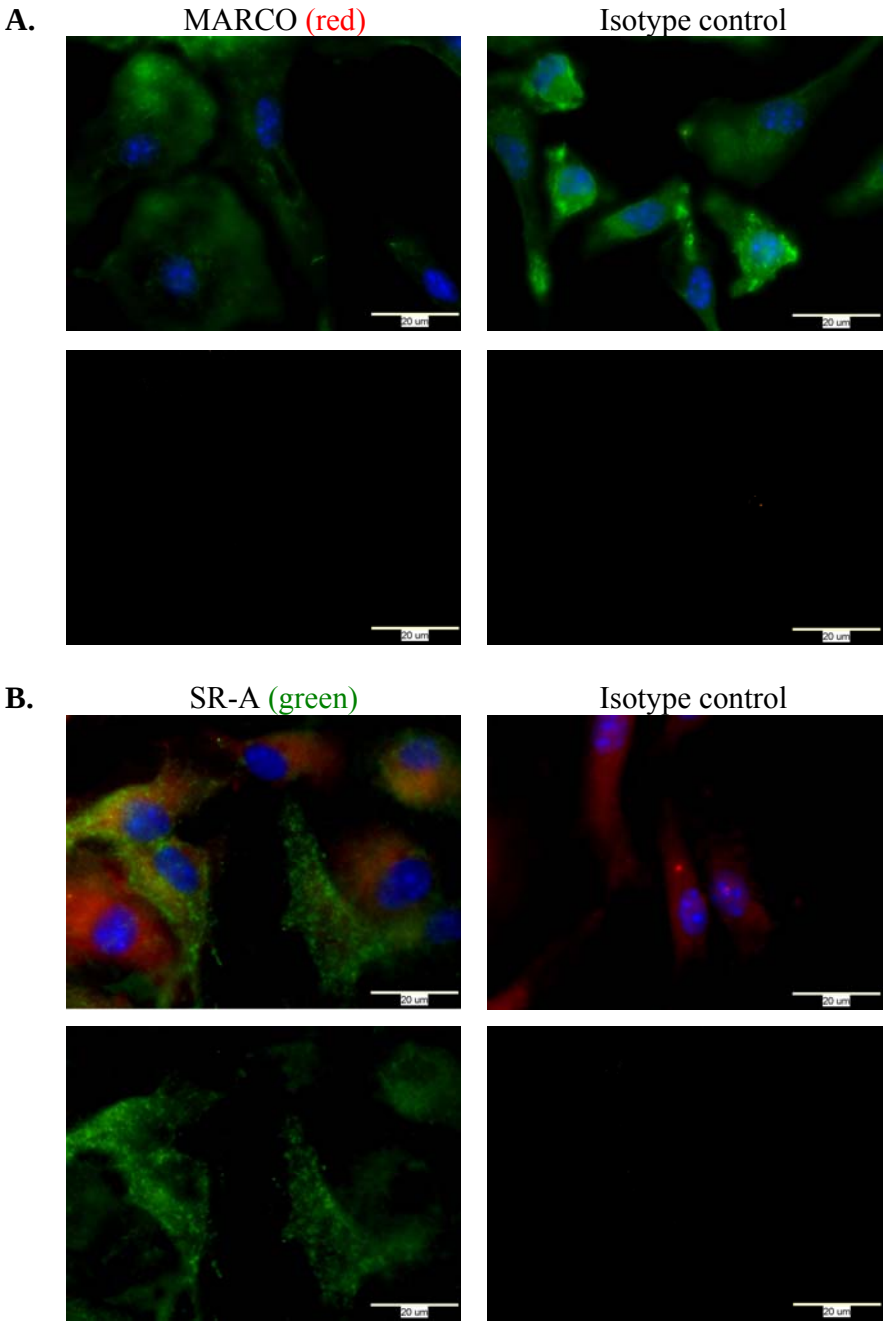


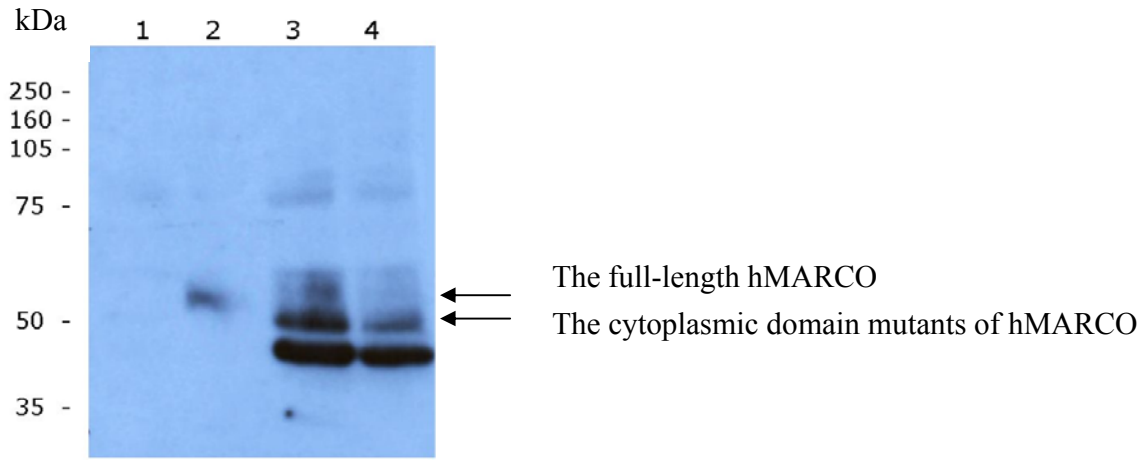
Fig. 3.7 The expression profile of MARCO and SR-A in permeabilised mouse bone marrow derived Mϕs. **A**. MARCO is stained in red (ED31-RPE), actin in green (phalloidin-FITC) and the nucleus in blue (DAPI). **B.** SR-A is stained in green (2F8 and anti-rat Alexa488), actin in red (phalloidin-TRITC) and DNA in blue (DAPI). As confirmed with RPMϕs, a greater proportion of SR-A is intracellular. MARCO is not expressed in mouse bone marrow derived Mϕs, unlike SR-A.

3.3.3 The cytoplasmic tail of MARCO is important for cell surface expression

To test the hypothesis that the cytoplasmic domain of MARCO is necessary for cell surface expression, transiently transfected HEK 293T cells that expressed either the full-length hMARCO, Nmyr MARCO or N- MARCO were used. Western blotting showed that all the constructs were expressed as monomers and the molecular weight of the cytoplasmic domain mutants was lower (~45 kDa) than that of full-length MARCO (~52 kDa) (Fig 3.8 A). Flow cytometry analysis using the rabbit anti human MARCO pAb revealed that fewer of the cells transfected with the Nmyr MARCO and N- MARCO plasmids expressed MARCO on the cell surface. In an average of 3 experiments, 58.2±10.1% of hMARCO, 36.9±4.1% of Nmyr MARCO and 27.0±7.6% N- MARCO transfected cells were found to express the protein on the cell surface (Fig. 3.8 B). Surprisingly, the percentage of positive cells was found to be much lower when anti hMARCO mAb PLK-1 was used for detection (~45% hMARCO, ~22% Nmyr MARCO and ~10% N- MARCO, data not shown), presumably because this antibody has a greater detection threshold of the antigen. Since the results for cell surface expression of hMARCO using the pAb 977 correlated more with the percentage of cells that displayed EDTA resistant adhesion (~60% of hMARCO expressing cells, see Chapter Five, Fig. 5.1) and also correlated more with staining by microscopy, these results were considered more reliable. Nevertheless, using either PLK-1 and 977 antibodies, it was found that the cell surface expression of the cytoplasmic domain mutants of MARCO was significantly reduced in transiently transfected HeLa (Fig 3.9) and HEK 293T cells (Fig. 3.10). Analysis of MARCO staining in both non-permeabilised and permeabilised transfected HEK 293T cells

revealed that the cytoplasmic domain mutants were mostly expressed intracellularly and displayed reduced cell surface localisation (Fig. 3.10).

A.



B.

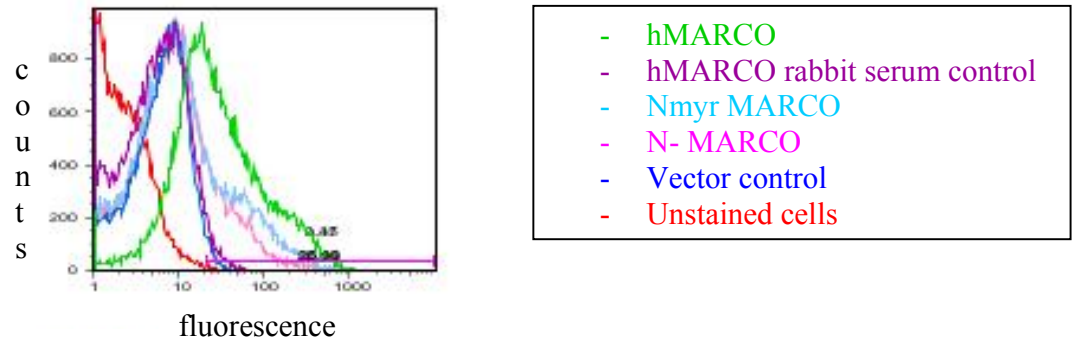


Fig. 3.8 Expression of hMARCO and the cytoplasmic domain mutants in HEK 293T cells. **A.** Cell lysate (35 µg of protein) was resolved on a non-reducing 8% SDS-PAGE gel and MARCO expression was detected with rabbit anti-hMARCO pAb 977 and anti-rabbit-HRP secondary by Western blotting. Lanes: 1. vector control; 2. hMARCO; 3. Nmyr MARCO; 4. N- MARCO. No bands were detected in the vector control lane or in a control membrane, which was incubated with rabbit serum and anti-rabbit-HRP (data not shown). MARCO is expressed as a monomer in HEK 293T cells of ~52 kDa molecular weight, the N-tail mutants are slightly smaller, ~45 kDa molecular weight. The double band may indicate that the protein might be alternatively glycosylated **B.** Measurement of cell surface expression of MARCO in non-permeabilised transfected HEK 293T cells by flow cytometry. MARCO expression was detected with rabbit anti human MARCO pAb 977 and an anti-rabbit Alexa647 secondary, rabbit serum was used as a control. The graph shown is representative of 3 experiments. The cytoplasmic domain mutants of MARCO display lower cell surface expression. In an average of 3 experiments, 58.2±10.1% of hMARCO transfected cells, 36.9±4.1% of Nmyr MARCO transfected cells and 27.0±7.6% of N- MARCO transfected cells expressed the protein on the cell surface.

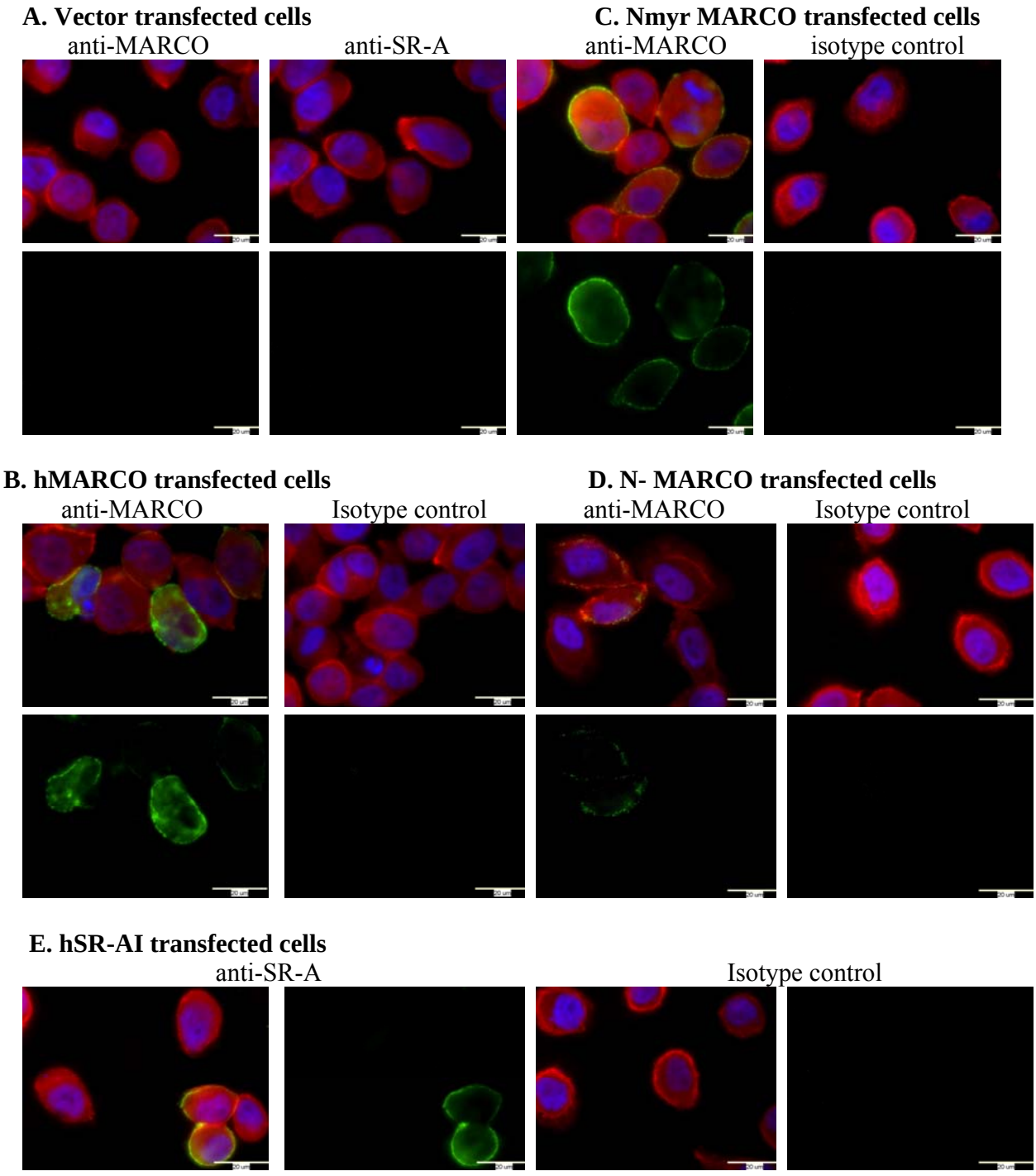


Fig. 3.9 The expression profile of MARCO and the cytoplasmic domain mutants in non-permeabilised transfected HeLa cells. B. hMARCO, **C.** the Nmyr mutant MARCO, **D.** the N- mutant MARCO and **E.** hSR-AI. Fig **A** shows the vector control. MARCO and SR-A are stained in green (PLK-1/C6 and anti-mouse Alexa488), actin in red (phalloidin-TRITC) and DNA in blue (DAPI). Transfection efficiency in HeLa cells was less than in HEK 293T cells. The expression level per cell was slightly reduced in the Nmyr MARCO expressing cells and the reduction was even more dramatic in the N- MARCO expressing HeLa cells.

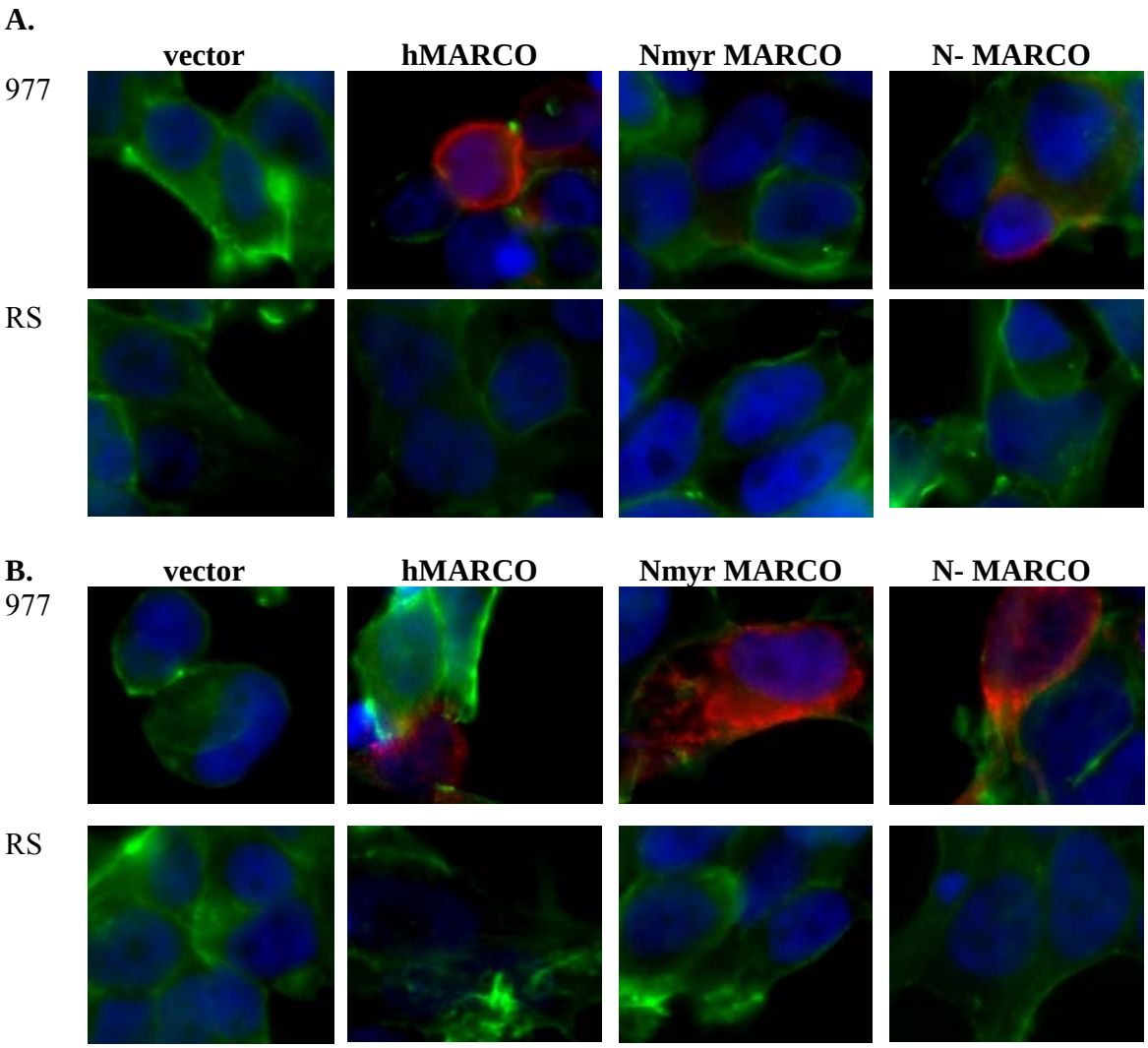


Fig. 3.10 The expression profile of MARCO and the cytoplasmic domain mutants in transfected HEK 293T cells. A. Non-permeabilised cells **B.** Permeabilised cells. MARCO is stained in red (rabbit anti hMARCO pAb 977 and anti-rabbit Alexa647), actin in green (phalloidin-FITC) and DNA in blue (DAPI). Rabbit serum (RS) was used as a staining control. The width of all pictures corresponds to 70 μm. Non-permeabilised cells in Fig. A indicate that the cytoplasmic domain mutants Nmyr and N- MARCO express lower levels of MARCO on the cell surface. Permeabilised cells in Fig. B indicate that the mutants localise more in the cytoplasm compared to the full-length MARCO.

3.4 Discussion

Although MARCO and SR-A have high homology and similar ligand binding abilities, they differ in many aspects, including their cellular localisation and expression profile. The amino acid sequences of the cytoplasmic domains of MARCO and SR-A show the smallest percentage of homology and are the least conserved between those two receptors (Elomaa et al., 1995; Elshourbagy et al., 2000). The process of how type I and type II transmembrane proteins are targeted to the ER and inserted into the membrane, is not very well understood, but it is presumed that the hydrophobic anchor sequences and amino acids proximal to that region are responsible for the correct orientation of the protein and membrane trafficking (Lodish et al., 2003). This indicates that the membrane-proximal segment of the cytoplasmic domain may affect cell surface localisation. N-myristoylation is an acylation of the amino acid glycine and it is considered to be one of the first steps by which the protein associates with the membrane (Boutin, 1997). However, the putative N-myristoylation motif in the cytoplasmic domain of MARCO does not show a high correlation with other known myristoylation motifs (Boutin, 1997), nor has it experimentally been shown that the glycine actually gets myristoylated. Therefore we cannot definitively say that surface expression is due to this putative motif. Further experiments are needed to determine how it contributes to surface expression.

The hypothesis that the N-terminal cytoplasmic tail of MARCO is required for the cell surface expression was confirmed as I showed that both the Nmyr MARCO and the N-MARCO cytoplasmic domain mutants display reduced cell surface expression (Fig. 3.8 B). In particular, it reveals that the membrane-proximal amino acids of MARCO alter cell

surface localisation, but are not obligatory for trafficking to the membrane, since 27.0±7.6% of the N- MARCO transfected cells still expressed the protein on the surface. These data contradict with what is known about the cell surface expression of SR-A, because SR-A is also shown to absolutely need the membrane proximal region consisting of six amino acids for cell surface expression. In addition, it has been published that the SR-A cytoplasmic tail mutants, that have amino acids 1-49 deleted but retain the six membrane-proximal amino acids, overexpress the protein on the cell surface (Kosswig et al., 2003), whereas the structurally similar Nmyr mutant of MARCO shows reduced surface expression compared to full-length MARCO. This means that there is a striking difference in regulation of cell surface expression between those two receptors, possibly because the cytoplasmic domain is relevant for Golgi to the ER or the ER to plasma membrane trafficking of the protein. The role of the cytoplasmic tail in cell surface expression was demonstrated in cell lines, but further experiments would be required to show if the localisation signals in the cytoplasmic tail have a role in primary cells. Other sequences in the cytoplasmic domain of MARCO that could possibly affect the cell surface expression of the protein are discussed in Chapter Six.

4 Chapter Four: The role of the cytoplasmic domain of MARCO in binding particulate ligands and uptake of soluble ligands

4.1 Introduction

Scavenger receptors are multifunctional – their functions include the uptake of endogenous soluble ligands (e.g. AcLDL, OxLDL) as well as recognition and phagocytosis of pathogens (Platt and Gordon, 2001). MARCO has also been reported to recognise AcLDL and OxLDL, but due to its restricted expression profile, the importance of MARCO as an endocytic receptor for these ligands is not clear (Elomaa et al., 1995). It has been proposed that the bacterial binding region of MARCO is in the SRCR domain (Elomaa et al., 1998); this domain of murine MARCO has also been shown to be important for adhesion to gelatine and binding to AcLDL (Ojala et al., 2007). The role of other domains in uptake of soluble and particulate ligands has not yet been established. In contrast, the collagenous and the α-helical coiled-coil domains are thought to be the bacterial binding regions for SR-A (Rohrer et al., 1990).

The cytoplasmic domain of SR-A has been shown to contain sequences for internalisation of AcLDL. A SR-A cytoplasmic tail mutant that only has the six membrane-proximal amino acids for cell surface expression in the cytoplasmic domain, is defective in endocytosis of AcLDL (Kosswig et al., 2003). It has been suggested that a single motif, VXFD is responsible for ligand internalisation (Morimoto et al., 1999). In addition, the cytoplasmic domain of SR-A contains two phosphorylation sites and a di-leucine motif, which have been

demonstrated to be required for endocytosis (Chen et al., 2006b; Fong and Le, 1999). The potential signalling sequences in the cytoplasmic domain of MARCO have not been investigated.

4.2 The aims of this chapter

I hypothesised that the cytoplasmic domain of MARCO contains sequences for efficient uptake of soluble and particulate ligands. To test this hypothesis, two cytoplasmic domain mutants of MARCO were used: Nmyr MARCO and N- MARCO (Chapter Three, Fig. 3.1 B). First, it was confirmed, that MalBSA, which is a known ligand for SR-A, is also a ligand for MARCO in primary cells. The same modified protein was then coated on particles or used in a soluble form, to determine the role of the cytoplasmic domain in uptake of particulate and soluble ligands.

4.3 Results

4.3.1 MalBSA coated beads are a specific ligand for RPMϕs

MalBSA has previously been described to be a ligand for scavenger receptors (Takata et al., 1989). To test if it is also a ligand for MARCO, MalBSA was coated on 2 μm fluorescent latex particles. As a control, unmodified BSA coated particles were used. The binding of MalBSA and BSA coated beads to WT, MARCO KO and DKO RPMϕs was assessed by fluorescence microscopy (Fig. 4.1 A). The results demonstrate that WT Mϕs bind significantly more MalBSA coated particles than BSA coated particles. The MARCO KO

and DKO Mϕs bound equal numbers of MalBSA coated beads and unlike the WT Mϕs, there was no preference for binding MalBSA beads (Fig. 4.1 B). This indicates that MARCO is a receptor for MalBSA coated particles. The most striking difference between WT, MARCO KO and DKO Mϕs was that the WT cells bound more MalBSA beads per cell (i.e. 5 or more beads per cell) than MARCO KO and DKO Mϕs (Fig. 4.1 C). This confirmed previously published observations that MARCO enhances the phagocytic abilities of Mϕs.

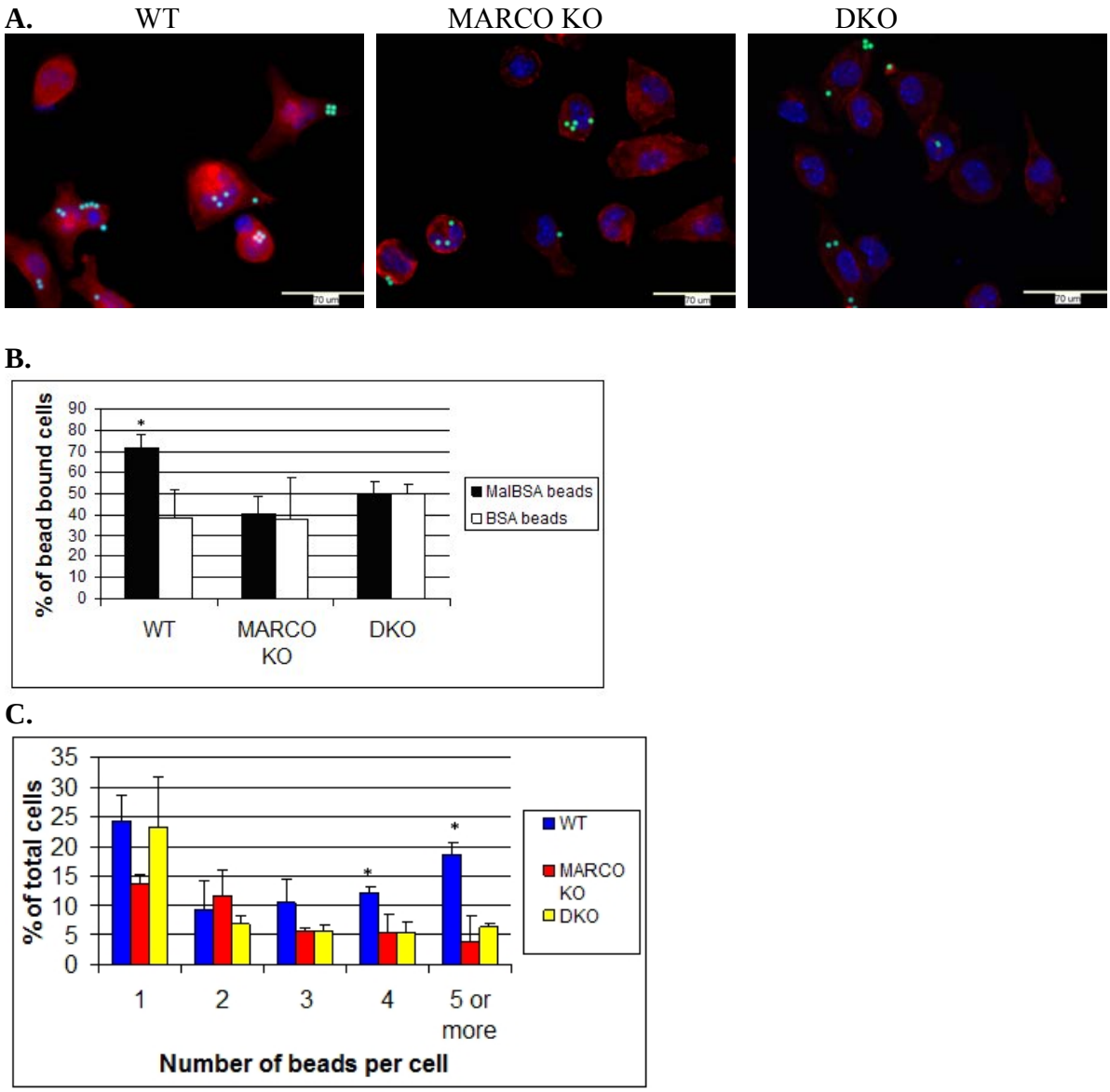


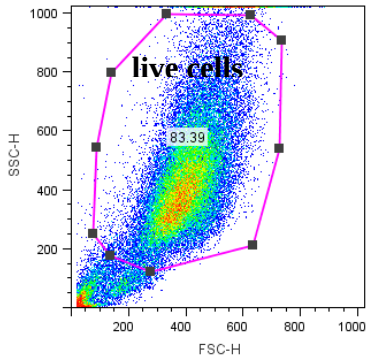
Fig. 4.1 MalBSA coated beads are a ligand for RPMϕs. A. Fluorescence microscopy of WT, MARCO KO and DKO RPMϕs binding MalBSA coated beads. One representative experiment of three is shown. Actin is stained in red (phalloidin-TRITC), DNA in blue (DAPI) and the beads are green. A higher percentage of WT Mϕs bound beads than did MARCO KO or DKO Mϕs. **B.** WT, MARCO KO and DKO RPMϕs binding MalBSA and BSA coated beads. Statistically more WT Mϕs bound MalBSA coated beads than BSA coated beads, but the difference was not evident for MARCO KO and DKO Mϕs. Bars represent the average of three experiments ± standard deviation, * indicates a statistically significant increase in binding MalBSA coated beads compared to binding BSA coated beads. **C.** More WT RPMϕs bound to 4 and 5 or more MalBSA coated beads at a time than MARCO KO and DKO RPMϕs. Bars represent the average of three experiments ± standard deviation, * indicates a statistically significant increase in bead binding of the WT Mϕs.

4.3.2 Characterisation of MARCO-expressing HEK 293T cellular binding to MalBSA coated beads

In order to confirm that MalBSA coated particles are a ligand for hMARCO expressing HEK 293T cells, binding assays were performed to compare binding of MalBSA versus BSA coated beads, as a control. The live cells were gated based on their FSC-H and SSC-H parameters (Fig. 4.2 A) and the binding of beads was assessed by fluorescence in FL1-H channel by flow cytometry (Fig. 4.2 B). In preliminary optimising experiments, it was confirmed that hMARCO transfected HEK 293T cells bound to MalBSA coated beads in a time- and dose-dependent manner (Fig. 4.3 A and B). Also, it was evident that the induction of MARCO significantly enhances the phagocytic abilities of transfected cells. hSR-AI transfected cells were used as a control, as SR-A is a known receptor for MalBSA, and the percentages of cells that bound beads were found to be similar to MARCO transfected cells. The binding of MalBSA coated beads was found to be scavenger receptor specific, as the binding was significantly reduced by a general scavenger receptor inhibitor $DxSO_4$ but not by $ChSO_4$ (Fig. 4.3 C).

Confocal laser scanning microscopy revealed that MalBSA coated beads were not internalised by transfected HEK 293T cells and were found bound to the outer membrane of the cell (Fig. 4.4). This may be due to the fact that HEK 293T cells are not professional phagocytes and they lack the machinery and compartments necessary to mediate phagocytosis.

A.



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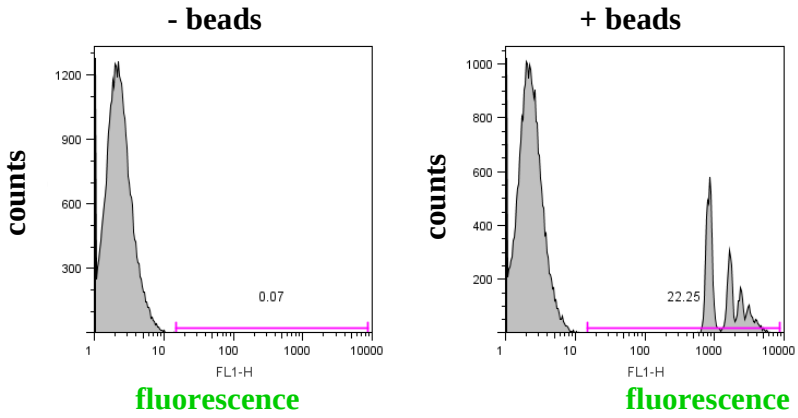
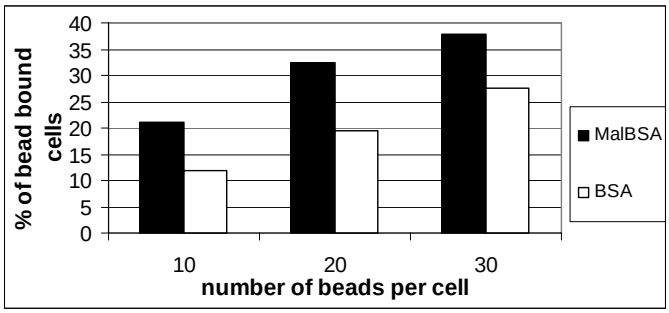


Fig. 4.2 Analysis of MARCO transfected HEK 293T cells binding to MalBSA coated beads by flow cytometry, one representative experiment of three is shown. **A.** The gating on live cells. Total 50,000 fluorescent events were analysed in each sample and typically more than 80% of the analysed cells were grouped under live cells by FSC-H and SSC-H parameters. **B**. The proportion of cells marked as associated with beads (pink line) was determined by the fluorescence in FL1-H channel. The cells bound with beads are subdivided into peaks, which probably indicate the number of beads bound per cell.

A.



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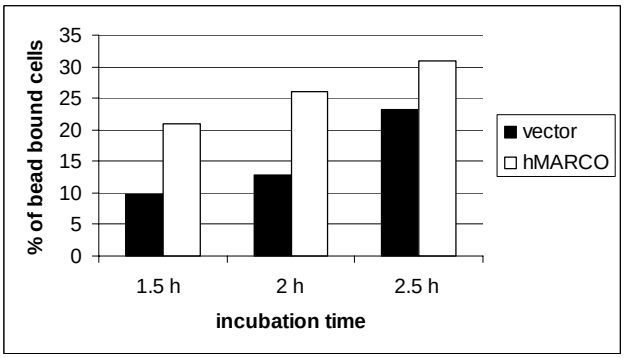

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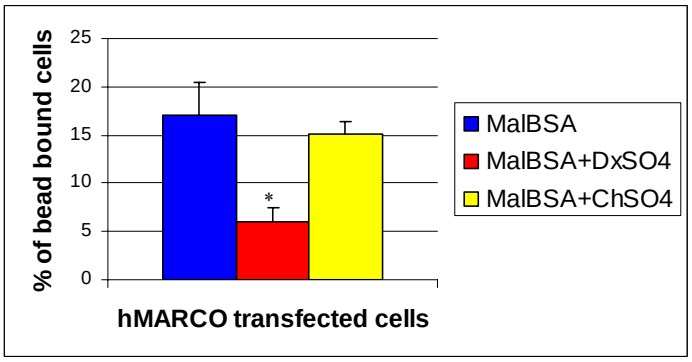


Fig. 4.3 Characterisation of hMARCO-expressing HEK 293T cellular binding to MalBSA coated beads. **A**. Dose response of binding to MalBSA and BSA coated beads, n=1. 10 beads per cell were determined to be the optimal bead concentration. **B.** The time course of MARCO and vector transfected cells binding to MalBSA coated beads, n=1. 1.5 h incubation with beads was chosen to be optimal for further experiments. **C.** The binding of hMARCO transfected cells to MalBSA coated beads is scavenger receptor dependent. A general scavenger receptor inhibitor $DxSO_4$ (500 μg/ml) inhibited about 65% of the binding to MalBSA coated beads, to about the same level as for vector control. Bead binding was not affected by $ChSO_4$ (500 μg/ml), which is not a scavenger receptor ligand. Similar results were obtained for the cytoplasmic tail mutants (data not shown). Bars represent the average of three experiments ± standard deviation,* indicates a statistically significant increase compared to cells with no inhibitor added, $p \leq 0.01$ by Student's T-test.

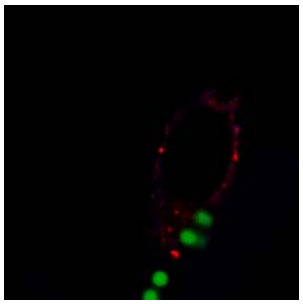


Fig. 4.4 Localisation of the MalBSA beads in hMARCO transfected HEK 293T cells. One representative confocal laser scanning image is shown. At the beginning of the experiment, the cell membranes were stained with red fluorescent DiI cell-labelling dye. After incubation with the beads (green), the cells were fixed and stained for MARCO (blue) using rabbit anti hMARCO pAb 977 and Alexa647 conjugated anti-rabbit Ab. To determine the localisation of the beads, 12 stacks were scanned by an interval of 0.5 μm. Out of 10 cells that were analysed, none of the beads was found to localise inside the cell. Similar results were obtained for other transfectants (data not shown). The width of the picture corresponds to 100 μm.

4.3.3 MARCO expression level in relation to bead binding

To determine how bead binding is correlated with the expression levels of full-length MARCO and the cytoplasmic domain mutants, the cells were stained for MARCO expression after incubating with beads. The expression levels and bead binding were determined by flow cytometry. Using unstained cells without any ligand added, the cells were divided into quadrants based on MARCO expression level and bead binding (Q1 – cells that express MARCO but have not bound to beads; Q2 – cells that express MARCO and have bound beads; Q3 – cells that do not express MARCO and do not bind to beads; Q4 – cells that do not express MARCO but have bound beads) (Fig. 4.5). As a control, stained cells without any added ligand were used (Fig. 4.6). As determined by analysis of cells in each quadrant, most of the cells that bind MalBSA (Fig. 4.7) or BSA coated beads (Fig. 4.9) expressed MARCO at the cell surface (Q2). Compared to the full-length MARCO expressing cells, a greater proportion of the Nmyr MARCO and N- MARCO expressing cells that had bound beads (Q2) displayed low cell surface expression of the protein. This is probably caused by lower level of cell surface localisation of the protein per cell.

To determine if a similar percentage of cells that express similar amount of hMARCO, Nmyr MARCO or N- MARCO on the cell surface bind beads, a narrow range of cells was gated by fluorescence in the FL4-H channel (Fig. 4.8, Fig. 4.10). In this population of cells with similar expression level of MARCO, the percentage of bead associated cells was determined. The results from three independent experiments show that there is no statistically significant difference with respect to binding MalBSA or BSA coated beads in cells expressing the full-length MARCO or the cytoplasmic tail mutants (Fig. 4.8 A, Fig. 4.10 A). In addition, it was

determined whether there were differences in the proportion of cells that had bound 2 or more beads per cell (4.8 C, 4.10 C). The results of three independent experiments indicate that cells that express similar levels of full-length MARCO bind a similar number of beads as Nmyr MARCO and N- MARCO expressing cells. The induction of MARCO also enhances general bead binding abilities of HEK 293T cells as can be seen from the increase in binding BSA coated beads in the hMARCO transfected cells (Fig. 4.9). This confirms previously published observations that scavenger receptors are associated with general bead binding (Hughes et al., 1995; Palecanda et al., 1999).

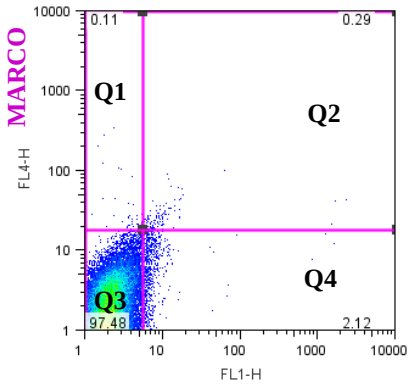
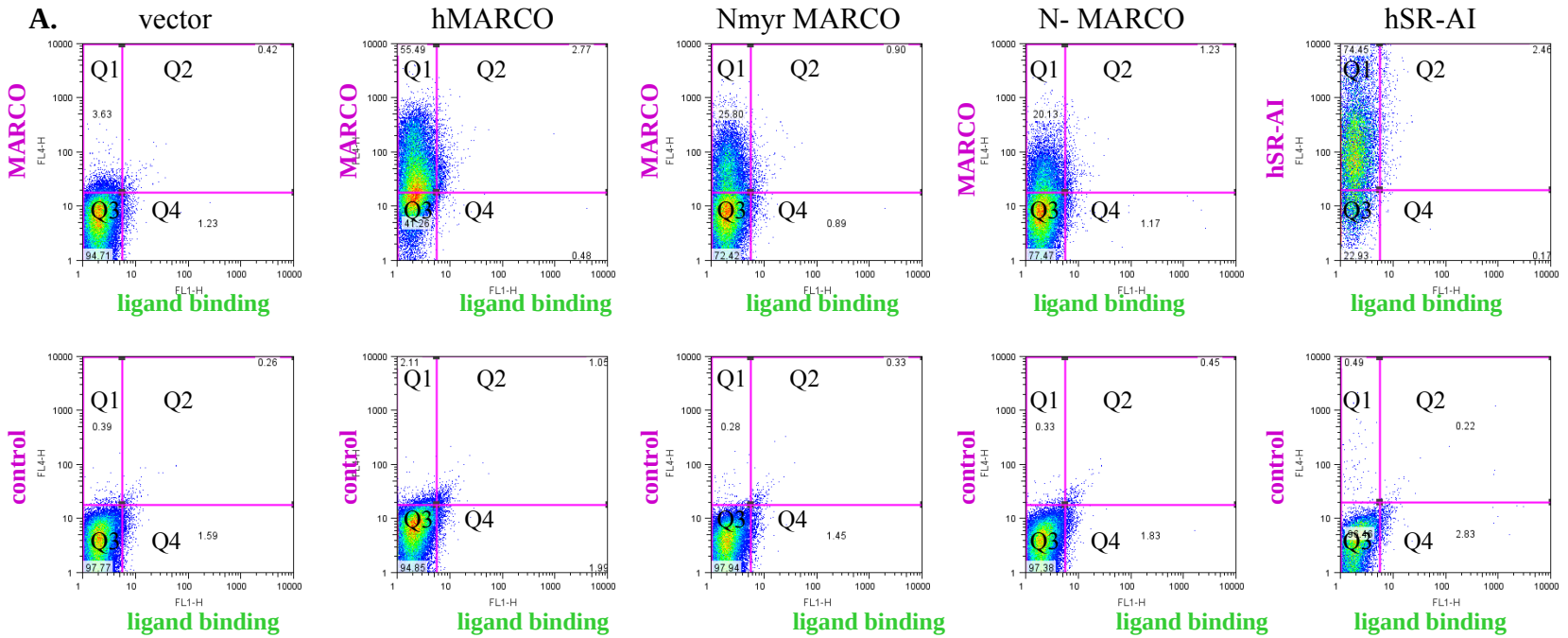


Fig. 4.5 A representative experiment showing how the gating was performed on transfected HEK 293T cells analysed by flow cytometry. In all the samples, the live cells were divided into four quadrants (Q1-4) based on their fluorescence in FL1-H and FL4-H channel. Q1 contains cells that are positive for MARCO (or SR-A) expression but have not bound ligand. Q2 contains cells that are positive for MARCO (or SR-A) expression and have bound ligand. Q3 contains cells that are not positive for MARCO (or SR-A) expression and have not bound ligand. Q4 contains cells that are not positive for MARCO (or SR-A) expression, but have bound ligand. Unstained cells shown on the graph were gated so that fewer than 5% of the cells would be considered bound to ligand or positive for MARCO expression.



B. No ligand, stained for cell surface expression

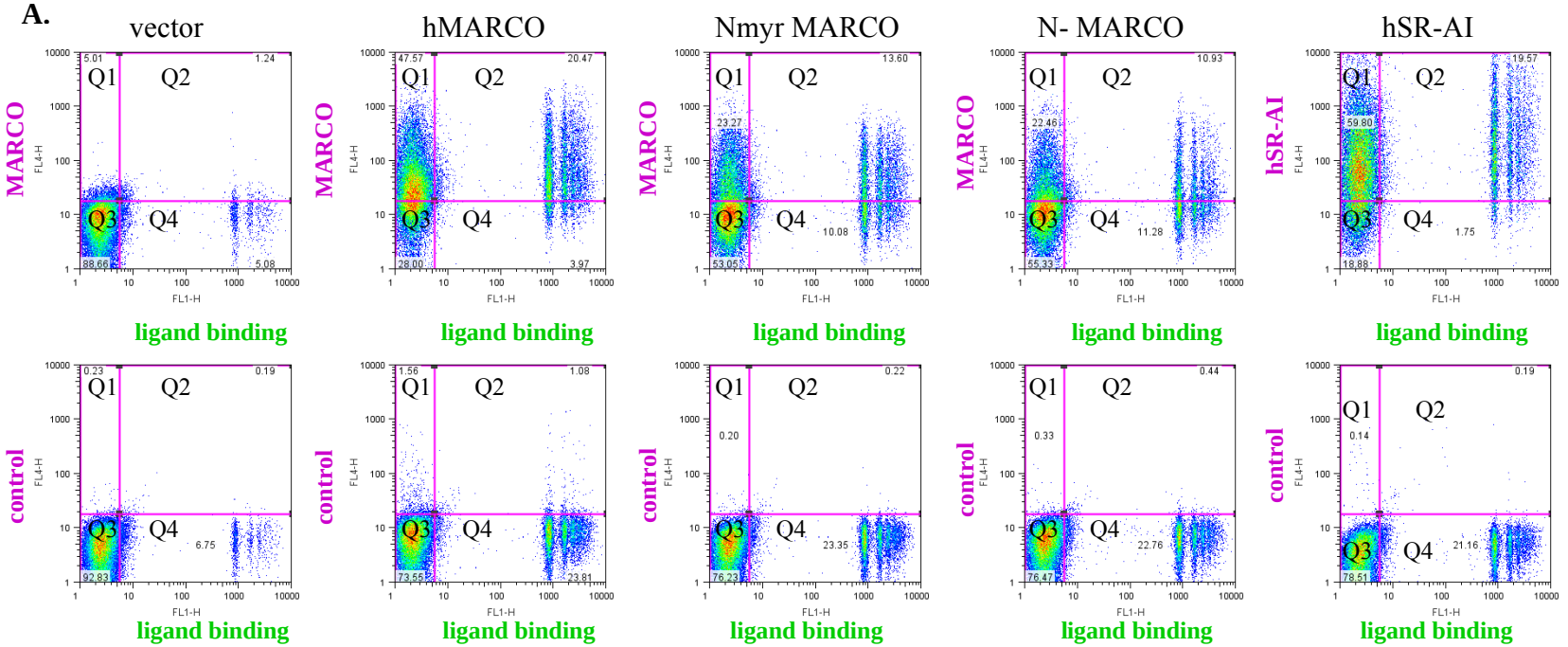
	Q1	Q2	Q3	Q4
vector	4.85±1.18	0.97±0.70	92.47±2.44	1.71±1.53
hMARCO	54.68±9.40	3.51±0.71	41.28±9.62	0.53±0.07
Nmyr MARCO	34.85±2.84	2.09±1.22	62.16±3.80	0.91±0.42
N- MARCO	23.87±5.94	3.16±1.67	71.42±6.56	1.55±1.07
hSR-AI	79.55±2.38	4.33±2.58	15.46±3.58	0.67±0.94

C. No ligand, control for cell surface expression

	Q1	Q2	Q3	Q4
vector	1.32±0.84	0.69±0.37	95.93±1.71	2.07±0.96
hMARCO	2.41±0.32	1.53±0.41	93.67±1.01	2.38±0.34
Nmyr MARCO	0.80±0.46	0.8±0.41	96.22±1.72	2.18±0.97
N- MARCO	1.09±0.78	1.21±0.67	94.23±2.81	3.47±1.85
hSR-AI	1.70±1.93	1.27±1.26	94.52±4.35	2.51±1.24

Fig. 4.6 Cell surface expression of hMARCO, Nmyr MARCO, N- MARCO and hSR-AI in transfected HEK 293T cells without ligand. A. One experiment, representative of three. MARCO expression was detected with rabbit anti-hMARCO pAb977 and anti-rabbit Alexa647. SR-A expression was detected with mouse anti-hSR-A mAb C6 and anti-mouse Alexa647. As a control, rabbit serum or mouse IgG1 isotype were used. **B.** Table shows the statistics of Q1-4 for three independent experiments where MARCO or SR-A expression was detected in the absence of ligand. **C.** Table shows the statistics of Q1-4 for three independent experiments for staining control in the absence of ligand. The cell surface expression of the cytoplasmic tail mutants was significantly reduced compared to full-length MARCO, also the level of expression per cell was reduced in the mutants.

A.



B. MalBSA beads, stained for cell surface expression

	Q1	Q2	Q3	Q4
vector	3.07±1.00	2.82±1.38	89.60±0.67	4.18±1.09
hMARCO	47.37±7.53	19.77±3.77	28.99±2.11	3.87±1.88
Nmyr MARCO	35.77±2.32	14.12±1.56	42.87±1.15	7.24±2.73
N- MARCO	29.53±10.06	10.94±2.34	51.02±3.93	8.51±5.02
hSR-AI	66.67±7.66	19.14±2.20	16.53±3.32	1.00±0.71

C. MalBSA beads, negative control for cell surface expression

	Q1	Q2	Q3	Q4
vector	0.90±0.65	0.70±0.44	91.30±1.37	7.11±0.32
hMARCO	2.33±1.09	2.00±0.80	73.17±5.00	22.50±5.95
Nmyr MARCO	1.00±0.81	0.76±0.48	80.19±5.35	18.06±6.53
N- MARCO	1.54±1.54	1.10±0.62	78.43±5.72	18.92±7.60
hSR-AI	0.88±0.95	0.46±0.24	77.56±0.83	21.10±0.79

Fig. 4.7 Transfected HEK 293T binding to MalBSA coated beads. A. One representative experiment out of three is shown. MARCO expression was detected with rabbit anti-hMARCO pAb 977 and anti-rabbit Alexa647. SR-A expression was detected with mouse anti-hSR-A mAb C6 and anti-mouse Alexa647. As a control, rabbit serum or mouse IgG1 isotype were used. Beads were detected in FL1-H channel. **B.** Table shows the percentages of cells in Q1-4 for three independent experiments where MARCO or SR-A expression and binding to MalBSA coated beads was detected. Q2 shows that most cells that have bound beads are also MARCO expressing cells. **C.** Table shows the percentages of cells in Q1-4 for three independent experiments for staining control, where binding to MalBSA coated beads was detected.

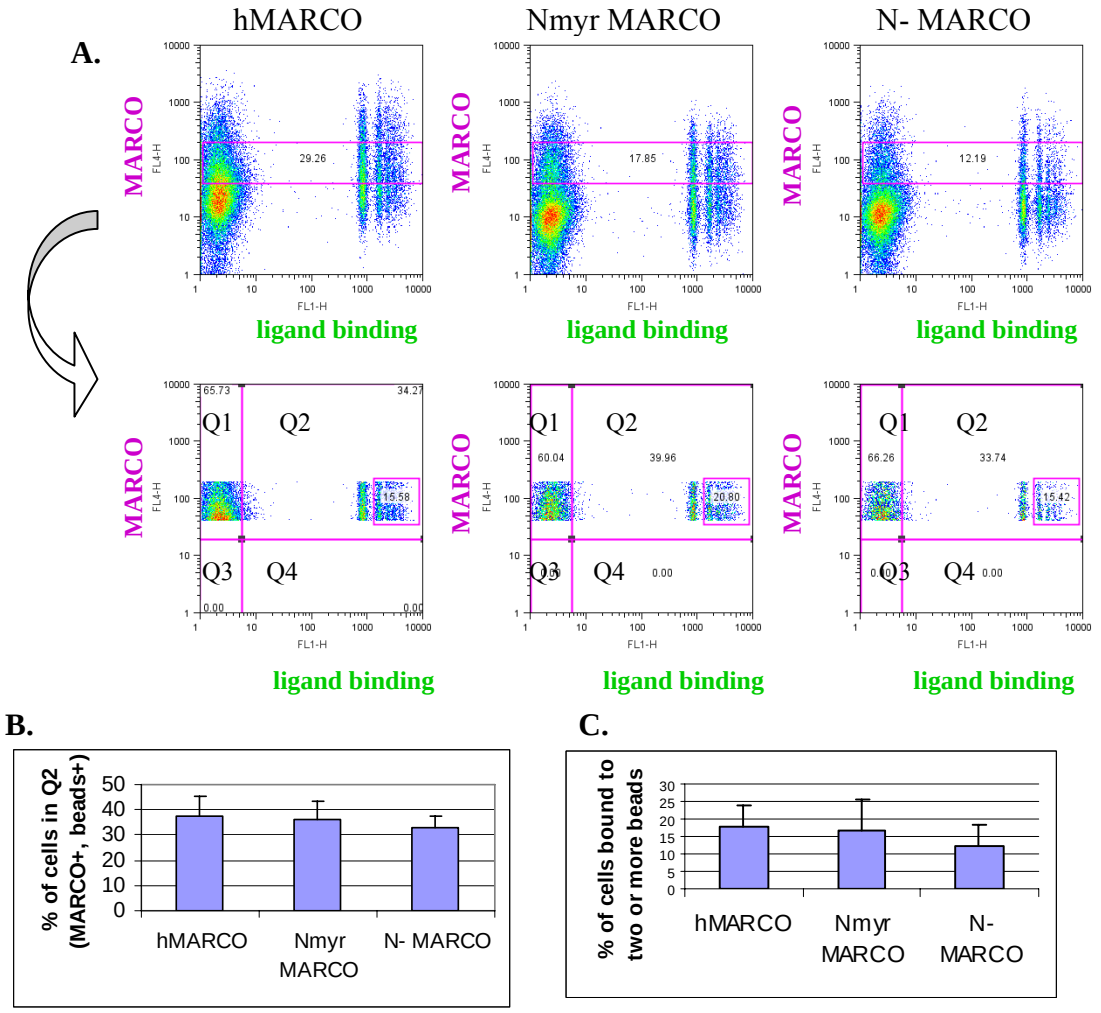
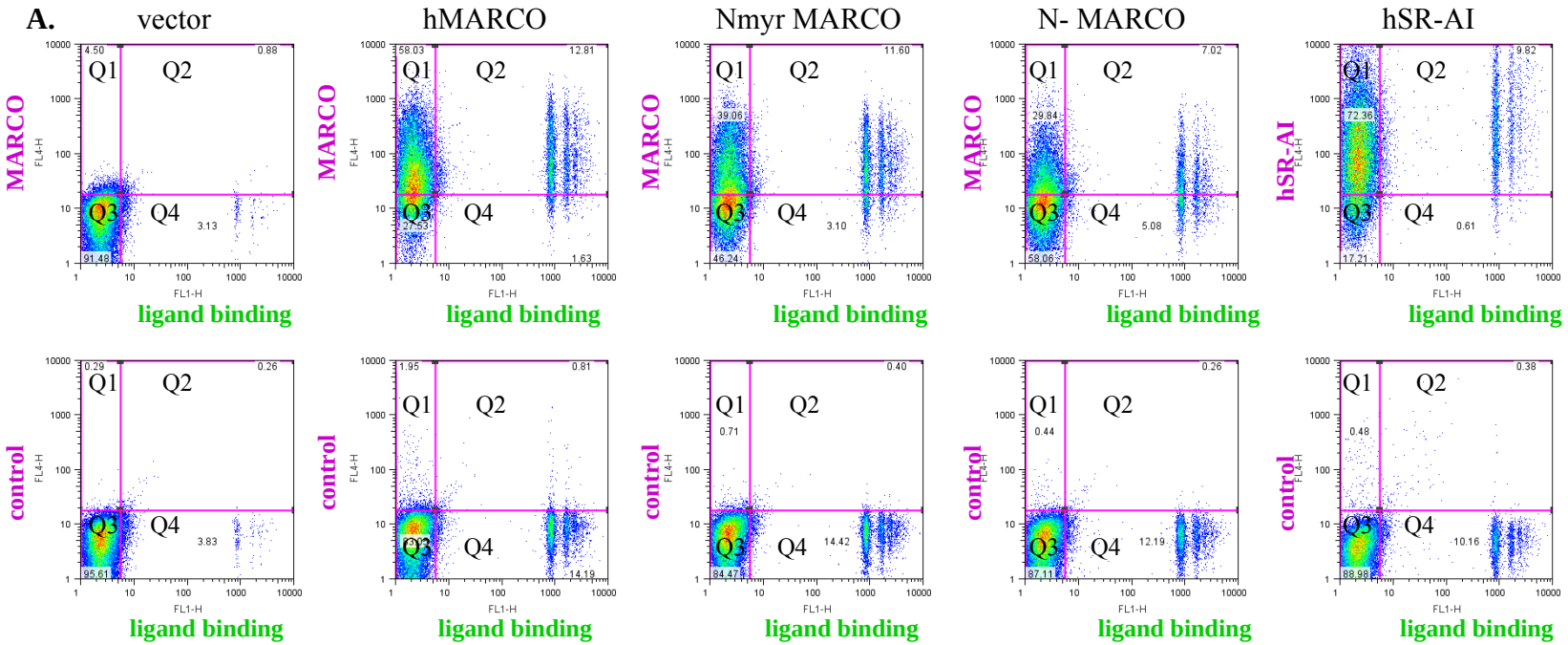


Fig. 4.8 Comparison of binding to MalBSA coated beads between full-length MARCO and the cytoplasmic tail mutants. A. A representative experiment shown. A small proportion of cells that express the same amount of receptor on the surface was gated and subsequently divided into four quadrants (by binding to MalBSA coated beads). Since all the chosen cells express MARCO, there are no cells in Q3 or Q4. Q2 shows cells that express similar amount of MARCO and are bound to beads. In addition, another gate was placed on the previously gated population. The cells in this gate had presumably bound to 2 or more beads per cell **B.** An average of three independent experiments showed that a similar proportion of cells in Q2 that express similar levels of full-length hMARCO, Nmyr MARCO or N- MARCO bind to MalBSA coated beads. **C**. An average of three independent experiments showing that the cells in Q2 that express the same amount of full-length hMARCO, Nmyr MARCO or N- MARCO bind to similar numbers of MalBSA coated beads. There was no statistically significant difference between the cells expressing full-length MARCO and the cytoplasmic tail mutants with respect to bead binding. Bars represent the average of three experiments ± standard deviation.



B. BSA beads, stained for cell surface expression

	Q1	Q2	Q3	Q4
vector	3.54±0.90	2.20±1.14	91.83±0.36	2.43±0.62
hMARCO	55.94±2.83	13.89±1.24	28.15±2.55	2.16±0.52
Nmyr MARCO	44.02±8.34	11.51±1.85	41.84±5.80	2.64±1.02
N- MARCO	34.16±3.82	8.59±1.96	52.78±5.78	4.47±1.43
hSR-AI	74.66±2.34	9.37±0.41	15.45±1.95	0.52±0.18

C. BSA beads, negative control for cell surface expression

	Q1	Q2	Q3	Q4
vector	1.47±1.48	0.67±0.40	94.01±1.87	3.84±0.02
hMARCO	2.88±0.83	1.77±0.83	80.68±2.20	14.68±1.22
Nmyr MARCO	1.42±0.61	0.89±0.45	84.22±3.26	13.48±3.20
N- MARCO	1.13±0.60	0.94±0.60	85.74±3.33	12.20±2.93
hSR-AI	0.87±0.55	1.02±0.90	85.91±4.35	12.22±2.91

Fig. 4.9 Transfected HEK 293T binding to BSA coated beads. A. One representative experiment out of three is shown. MARCO expression was detected with rabbit anti-hMARCO pAb 977 and anti-rabbit Alexa647. SR-A expression was detected with mouse anti-hSR-A mAb C6 and anti-mouse Alexa647. As a control, rabbit serum or mouse IgG1 isotype were used. Beads were detected in FL1-H channel. **B.** Table shows the percentages of cells in Q1-4 for three independent experiments where MARCO or SR-A expression and binding to BSA coated beads was detected. Q2 shows that most cells that have bound beads also express MARCO. **C.** Table shows the percentages of cells in Q1-4 for three independent experiments for staining control, where binding to BSA coated beads was detected.

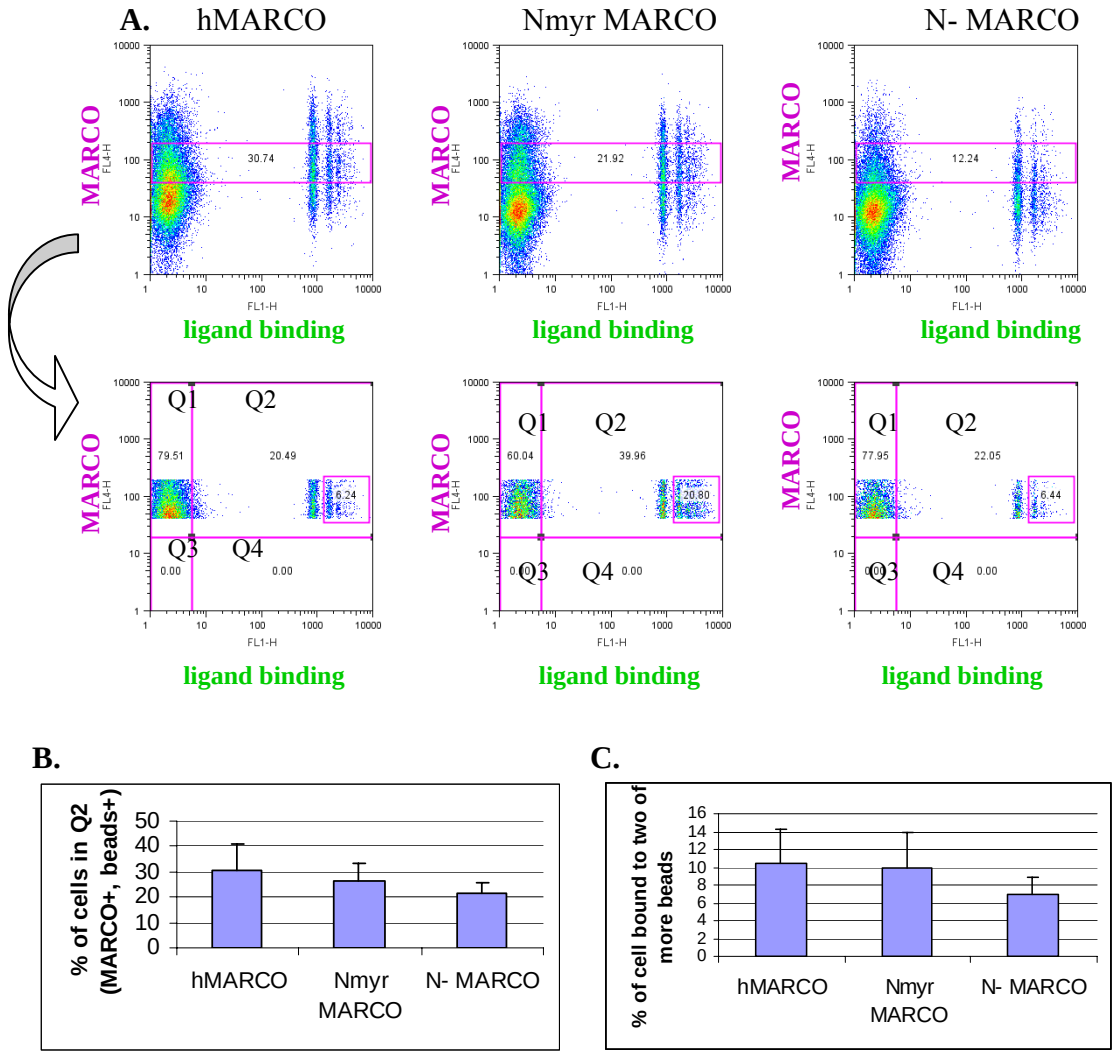


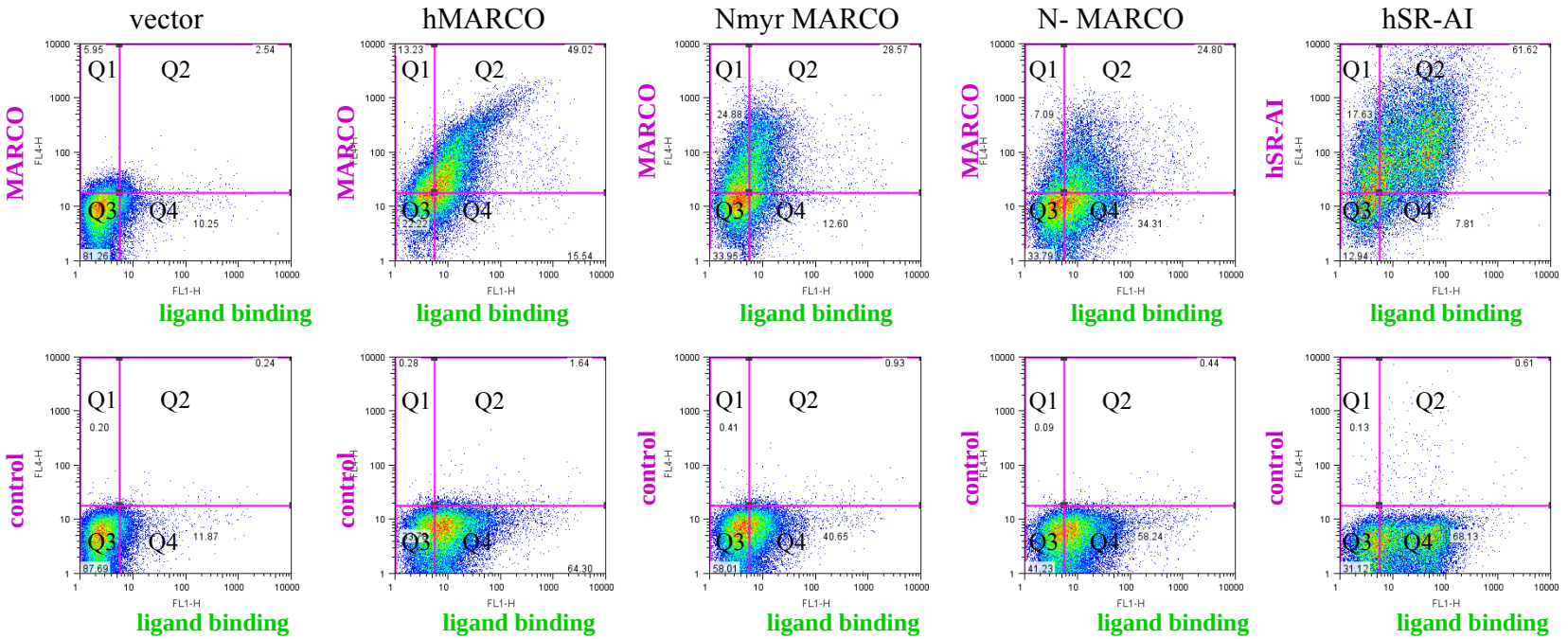
Fig. 4.10 Comparison of binding to BSA coated beads between full-length MARCO and the cytoplasmic tail mutants. A. A representative experiment shown. A small proportion of cells that express the same level of receptor on the surface was gated and subsequently divided into four quadrants by binding to BSA coated beads. Since all the chosen cells express MARCO, there are no cells in Q3 or Q4. Q2 shows cells that express similar level of MARCO and are bound to beads. In addition, another gate was placed on the previously gated population. The cells in this gate had presumably bound to 2 or more beads per cell **B.** An average of three independent experiments shows that the same proportion of cells in Q2 that express the same level of full-length hMARCO, Nmyr MARCO or N- MARCO bind to BSA coated beads. **C**. An average of three independent experiments shows that the cells in Q2 that express the same level of full-length hMARCO, Nmyr MARCO or N- MARCO bind similar numbers of BSA coated beads. There was no statistically significant difference between the cells expressing full-length MARCO and the cytoplasmic tail mutants with respect to bead binding. Bars represent the average of three experiments ± standard deviation.

4.3.4 The role of the cytoplasmic domain of MARCO in uptake of soluble ligands

To test if the cytoplasmic domain was important for endocytosis of a soluble ligand, hMARCO, Nmyr MARCO, N- MARCO and hSR-AI expressing transfected HEK 293T cells were incubated with fluorescently labelled MalBSA and stained for cell surface expression of the receptor (Fig. 4.11). Some binding to MalBSA occurred also in the vector transfected cells, possibly because HEK 293T cells express other scavenger receptors. The Nmyr MARCO expressing cells were found to be less effective in binding to MalBSA than cells that expressed a similar level of full-length MARCO, while no reduction of cell association was noticed in the N- MARCO mutant (Fig. 4.12). N- MARCO transfected cells that expressed very low levels of MARCO on the surface appeared to be efficient in binding soluble MalBSA. As a result, there was no statistically significant difference in cell association of soluble MalBSA between full-length MARCO and N- MARCO. hSR-AI expressing HEK 293T cells were used as a control, and similar levels of cell association as in hMARCO expressing cells was detected (Fig. 4.11). As the experiments were performed using a standard protocol at 37 ^{o}C, MalBSA was probably endocytosed.

In contrast, AcLDL, that has been used previously to study scavenger receptor mediated endocytosis, did not appear to be a good ligand for hMARCO. No statistically significant increase in cell association of AcLDL was detected in cells expressing full-length hMARCO or the cytoplasmic domain mutants compared to the vector control, while hSR-AI expressing cells showed a remarkable increase in binding AcLDL (Fig. 4.13). This indicates that in contrast to SR-A, hMARCO is not a receptor for AcLDL (similar results in recent studies in our group, Plüddemann et al., 2008).

A.



B. Soluble MalBSA, stained for cell surface expression

	Q1	Q2	Q3	Q4
vector	2.71±1.15	3.47±1.99	81.25±1.77	12.57±2.28
hMARCO	15.11±4.87	47.88±5.19	22.40±3.29	14.61±6.45
Nmyr MARCO	27.71±2.61	27.54±4.47	33.67±5.11	11.08±1.33
N- MARCO	10.42±3.07	27.58±2.84	33.09±0.82	28.90±4.68
hSR-AI	18.60±1.13	63.99±2.11	12.07±0.79	5.34±2.37

C. Soluble MalBSA, negative control for cell surface expression

	Q1	Q2	Q3	Q4
vector	0.54±0.40	1.16±1.15	85.75±4.38	12.55±5.19
hMARCO	0.54±0.27	3.10±1.98	36.61±2.93	59.75±5.12
Nmyr MARCO	0.61±0.22	1.50±0.56	60.80±4.11	37.08±4.44
N- MARCO	0.51±0.40	1.74±1.12	43.83±3.57	53.93±4.76
hSR-AI	0.37±0.34	2.59±2.79	37.06±8.39	60.00±11.52

Fig. 4.11 Transfected HEK 293T binding to soluble Alexa488 labelled MalBSA. A. One representative experiment out of three is shown. MARCO expression was detected with rabbit anti-hMARCO pAb 977 and anti-rabbit Alexa647. SR-A expression was detected with mouse anti-hSR-A mAb C6 and anti-mouse Alexa647. As a control, rabbit serum or mouse IgG1 isotype were used. MalBSA was detected in FL1-H channel. **B.** Table shows the percentages of cells in Q1-4 for three independent experiments where MARCO or SR-A expression and uptake of soluble MalBSA was detected. Q2 shows that most cells that associate with MalBSA also express MARCO. Some cell association of MalBSA was also detected in vector transfected cells. **C.** Table shows the percentages of cells in Q1-4 for three independent experiments for staining control, where cell association of MalBSA was detected.

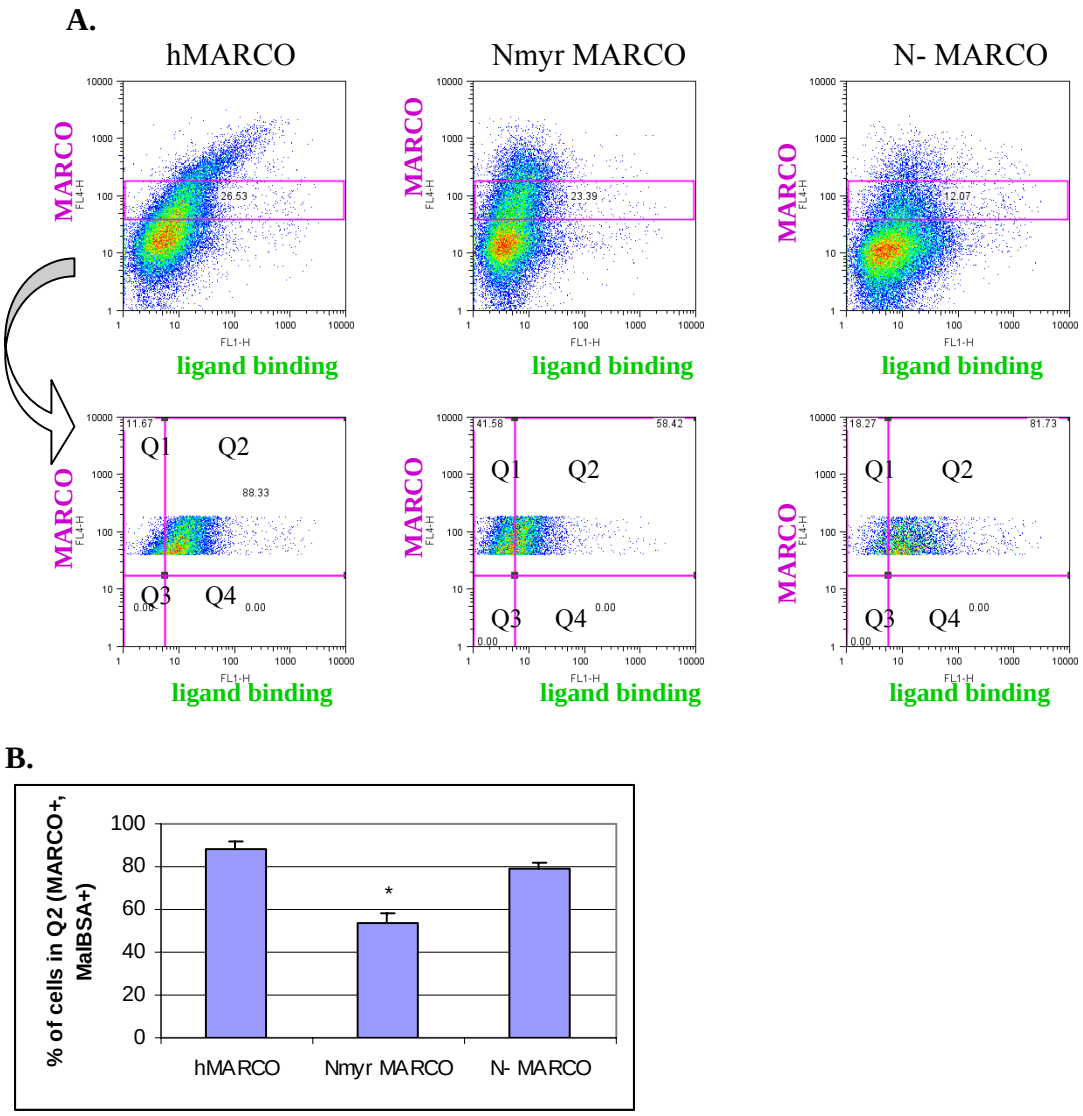
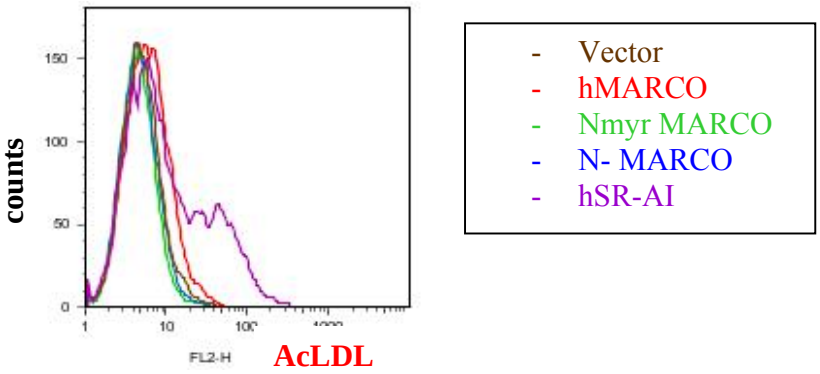


Fig. 4.12 Comparison of cell association of Alexa488 labelled soluble MalBSA between full-length MARCO and the cytoplasmic tail mutants. A. A representative experiment shown. Cells that express the same level of receptor on the surface was gated and subsequently divided into four quadrants by binding to soluble MalBSA. Since all the chosen cells express MARCO, there are no cells in Q3 or Q4. Q2 shows cells that express similar level of MARCO on cell surface and have probably endocytosed MalBSA. **B.** An average of 3 independent experiments shows the cells in Q2 that express the same level of MARCO and have probably endocytosed MalBSA. There was no statistically significant difference in endocytosis between full-length MARCO and the N- MARCO mutant, but the uptake of MalBSA was significantly reduced in the Nmyr MARCO mutant. Bars represent the average of three experiments ± standard deviation. * indicates a statistically significant decrease compared to cells expressing the full-length MARCO, * $p \leq 0.05$ by Student's T-test.

A.



B.

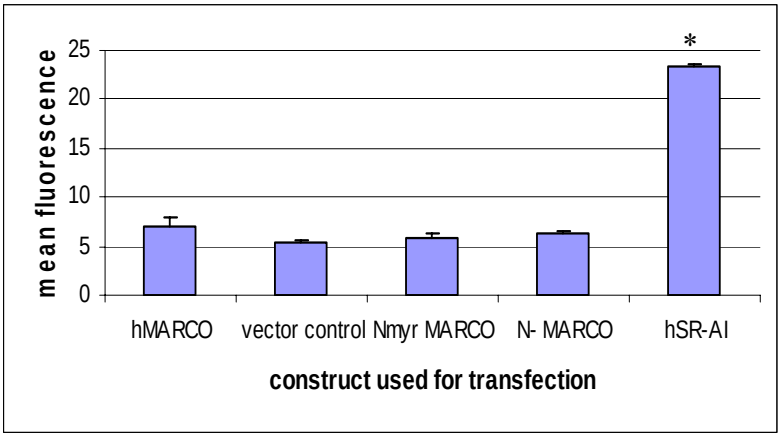
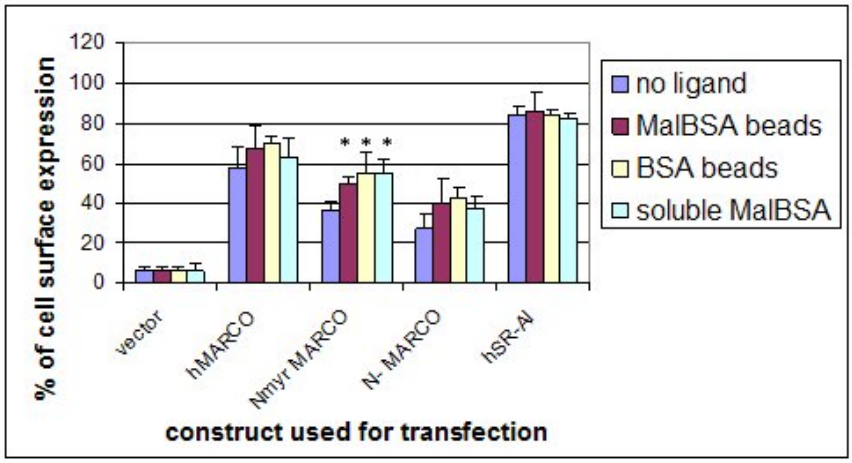


Fig. 4.13 AcLDL is not a ligand for hMARCO. A. One representative experiment out of two showing the uptake of DiI-labelled AcLDL by transfected HEK 293T cells. **B.** Average of two independent experiments showing the mean fluorescence. Only hSR-AI transfected cells endocytosed significantly more AcLDL than vector transfected cells. This indicates that, in contrast to SR-A, hMARCO is not a receptor for AcLDL. Bars represent the average of two experiments ± variability range. * indicates a statistically significant increase in uptake compared to mock transfected cells.

4.3.5 Regulation of cell surface expression

A significant increase (typically 10-20%) in the cell surface expression of the cytoplasmic domain mutants of MARCO was detected in every experiment in the presence of a ligand (beads or soluble MalBSA) compared to cells that were not incubated with ligand (Fig. 4.14). In an average of three experiments, Fig. 4.14 A shows the average cell surface expression of the protein with or without ligand. The Nmyr MARCO transfected cells showed a statistically significant increase in cell surface expression upon binding to MalBSA coated beads or soluble MalBSA. In contrast, the expression level of full-length MARCO or SR-A remained unchanged or changed very little. The increase of cell surface expression of the N-MARCO mutant is not evident on this graph due to the high variability of transfection efficiency. To show the average change in cell surface expression of the cytoplasmic domain mutants, the difference between the cell surface expression with and without ligand was calculated for all experiments and an average is presented on Fig. 4.14 B. These results indicate that the cytoplasmic domain mutants of MARCO have altered membrane trafficking and are retained at the surface when bound to ligand.

A.



B.

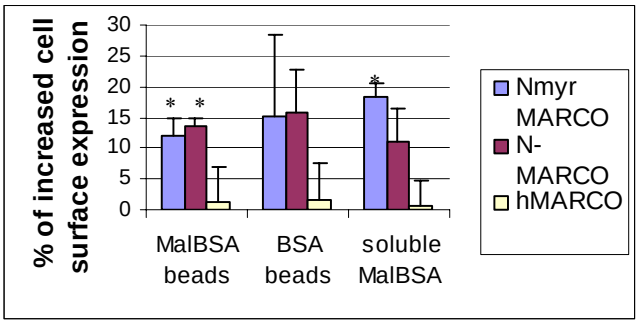


Fig. 4.14 The cell surface expression of the cytoplasmic tail mutants of MARCO is upregulated upon receptor binding to ligand. A. Cell surface expression of hMARCO or hSR-AI does not significantly change upon binding ligand. In contrast, a statistically significant increase was detected in the cell surface expression of the Nmyr MARCO transfected cells when cells were previously incubated with MalBSA coated beads, BSA coated beads or with soluble MalBSA. In individual experiments the increase in cell surface expression upon ligand binding was also evident in the N- MARCO transfected cells, but due to variability in transfection efficiency in average of three experiments the increase was not statistically significant. Bars represent the average of three experiments ± standard deviation, * indicates a statistically significant increase compared to receptor expression without ligand, $p \leq 0.05$. **B.** Upregulation of expression of the Nmyr and N- MARCO mutants was detected in all three experiments. To better illustrate the increase in cell surface expression, the difference in cell surface expression of the receptor with and without incubation with ligand was calculated. Graph shows the average percentage of increased cell surface expression upon binding to ligands in three independent experiments, ± standard deviation, * indicates a statistically significant increase compared to hMARCO transfected cells, $p \leq 0.05$.

4.4 Discussion

MalBSA has been used previously to study SR-A function, but here it was shown that MARCO transfected cells and primary RPMϕs also recognise this modified protein. This confirms previously published observations that the induction of MARCO can enhance the phagocytic abilities of a cell (Mukhopadhyay et al., 2006). In this study, it was shown that the cytoplasmic domain of MARCO does not affect ligand binding and but possibly affects internalisation of a soluble ligand.

As was discussed in the previous chapter, the cytoplasmic domain mutants of MARCO display lower cell surface expression. The cell surface expression of the protein was also tested after incubating the MARCO expressing transfected cells with a ligand at which point a significant increase in cell surface expression was recorded compared to cells that had not previously been incubated with a ligand. This observation may explain why no significant differences were found with respect to bead binding. While the cell surface expression of full-length MARCO remained unchanged, Nmyr MARCO and N- MARCO demonstrated more surface staining resulting in reduced differences in cell surface expression levels.

The mechanism by which MARCO mediates ligand internalisation remains unclear. SR-A has been shown to require a distinct motif (VXFD) in the cytoplasmic domain for efficient endocytosis but no similar motifs can be found in the cytoplasmic domain of MARCO. Indeed, there is no requirement for the cytoplasmic domain for binding particulate ligands. However, cell association of MalBSA in the Nmyr MARCO transfected cells was

significantly reduced compared to full-length MARCO expressing cells. This indicates that there may be a sequence that enhances but is not absolutely required for endocytosis.

In summary, this study showed that in contrast to SR-A, the cytoplasmic domain of MARCO does not contain signalling motifs required for ligand binding and but affects the uptake of soluble ligand. The cytoplasmic domain mutants of MARCO showed reduced cell surface expression but in contrast, in the presence of a ligand the cell surface expression was increased, while in full-length MARCO the cell surface expression remained unchanged. Scavenger receptors are constitutively recycled between the plasma membrane and endosomes (Murphy et al., 2005). These results indicate that the cytoplasmic domain may contain sequences which affect membrane trafficking and processing.

5 Chapter Five: The role of MARCO in adhesion and cell motility

5.1 Introduction

Mϕ interactions with other cells and the extracellular matrix play a key role in their differentiation, migration and interaction with endogenous ligands. In addition to integrin-mediated adhesion, Mϕs use scavenger receptors to interact with the extracellular environment. The role of SR-A in cell adhesion and spreading has been studied extensively, but less is known about MARCO-mediated adhesion.

The most striking characteristic of SR-A-mediated adhesion is its divalent cation independent nature. The ability of scavenger receptor expressing cells to adhere in the absence of calcium and magnesium demonstrates that scavenger receptor mediated adhesion is distinct from integrin mediated adhesion, which, in contrast, depends on the presence of divalent cations (Fraser et al., 1993).

SR-A recognises and subsequently adheres to many modified extracellular matrix proteins (e.g. modified collagens, proteoglycans, maleylated proteins) (el Khoury et al., 1994; Gowen et al., 2000; Santiago-Garcia et al., 2003). Many of these modifications, such as glycation and maleylation, occur naturally in the body, for example, in connection with inflammation and hyperglycaemia. For this reason, it is proposed that SR-A-mediated adhesion to modified extracellular matrix proteins plays a role in the retention or recruitment of Mϕs at sites of tissue injury. It has been demonstrated *in vitro* that Mϕs from transgenic mice that overexpress SR-A display enhanced adhesion in culture. In addition, mice overexpressing SR-A displayed increased carrageenan-induced granuloma

formation *in vivo* and increased accumulation of Mϕs within tissue compared to WT mice, processes, which are presumed to result from enhanced interactions with endogenous adhesins (Daugherty et al., 2001). SR-A-mediated adhesion has been shown to be independent of the cytoplasmic domain as full-length SR-A expressing HEK 293T cells spread equally well in culture as cells expressing the truncated form of SR-A (Kosswig et al., 2003).

The role of MARCO as a receptor for adhesion has not been previously addressed in detail. It has been demonstrated that the induction of MARCO can cause the formation of long dendritic processes and lamellipodia in transfected cells, which may increase their phagocytic abilities (Pikkarainen et al., 1999). These cell shape changes seem to depend on part of the cysteine-rich domain that is proximal to the collagenous domain of MARCO (Pikkarainen et al., 1999). In addition, it has been reported that MARCO mediates cell size changes in RPMϕs (Chen et al., 2005) and also mediates cytoskeletal rearrangement and affects DC maturation (Granucci et al., 2003). MARCO KO mice seem to have fewer resident Mϕs in the peritoneum, which may be a result of defective cell migration or reduction in cell adhesion (Chen et al., 2005). Mouse MARCO transfected cells have been shown to adhere to gelatine (Ojala et al., 2007), but other potential ligands have not yet been characterised.

5.2 The aims of this chapter

The aims of this chapter were to characterise the role of the cytoplasmic domain in MARCO-mediated cell adhesion and to determine if MARCO affects Mϕ chemokinesis. Transiently transfected MARCO expressing HEK 293T cells were used to assess cell adhesion on coated surfaces. HEK 293T cells normally adhere very loosely and they do

not naturally express other class A scavenger receptors – this makes them a good tool to study MARCO-mediated adhesion. MARCO-mediated adhesion and chemokinesis were examined on MalBSA coated surfaces; the same ligand was also used for binding and endocytosis assays to make results comparable. Primary mouse Mϕs from WT and KO mice were used to study chemokinesis. In this chapter, the role of the cytoplasmic tail in adhesion to ligands and the question of whether MARCO-mediated adhesion depends on the presence of divalent cations will be addressed in addition to the role of MARCO in Mϕ chemokinesis on modified proteins.

5.3 Results

5.3.1 MARCO-mediated adhesion is divalent cation independent and requires the cytoplasmic tail

Ethylenediaminetetraacetic acid (EDTA) is known to bind and remove divalent cations, such as Mg^{2+} and Ca^{2+} from suspension. Integrins require divalent cations as a co-factor to mediate adhesion to its ligands; therefore integrin-mediated adhesion is abolished in the presence of EDTA. However, this is not the case for the scavenger receptors. For example, SR-A binds to serum coated tissue culture plates in the presence of EDTA. This demonstrates that SR-A mediated adhesion is distinct from integrin mediated adhesion and does not require divalent cations. To mediate adhesion SR-A requires the presence of serum proteins (Fraser et al., 1993) and recently apoA-I and apoE have been identified as ligands for SR-A in human serum (Neyen, 2008).

To test if MARCO-mediated adhesion is divalent cation independent, HEK 293T cells expressing either full-length MARCO or MARCO with a truncated cytoplasmic tail, were

grown in DMEM with 10% FBS to confluence on 6-well tissue culture plates and were incubated with 10 mM EDTA in PBS (PBS-EDTA). The number of adherent cells was quantified by crystal violet staining (Fig. 5.1). Cells that were not treated with EDTA were regarded as 100% adherent. Previously published results that SR-AI mediated adhesion is divalent cation independent were confirmed as more than 50% of the cells remained adherent after 16 min incubation with PBS-EDTA. The percentage of cells expressing the full length hMARCO that displayed divalent cation independent adherence was comparable with hSR-AI transfected cells and correlated with cell surface expression of the receptor. However, most of the vector transfected cells as well as Nmyr MARCO and N- MARCO transfected cells were detached from tissue culture plastic within two minutes and were completely non-adherent by four minutes of incubation with PBS-EDTA. This experiment demonstrates that hMARCO-mediated adhesion is probably independent of divalent cations and that the cytoplasmic tail is required for adhesion.

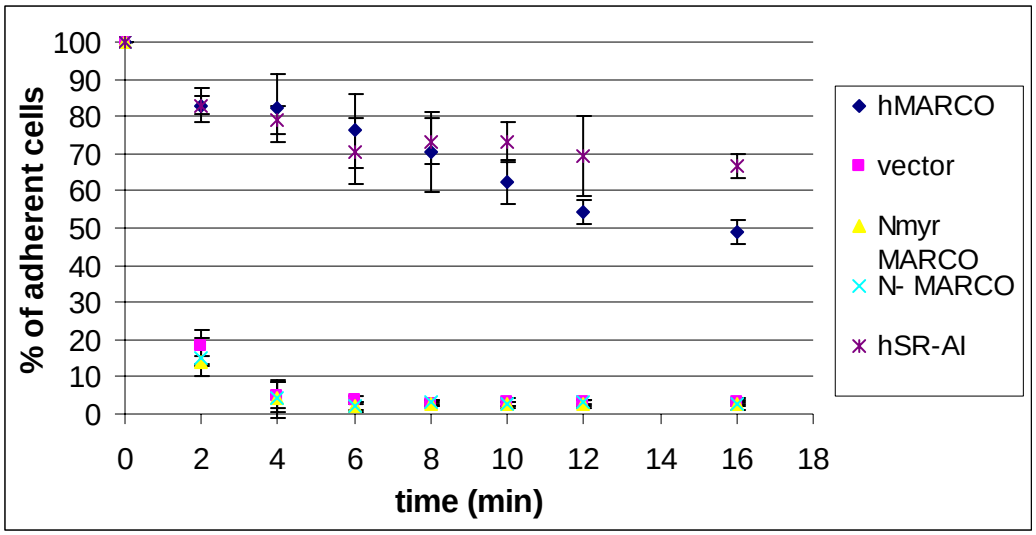


Fig 5.1 MARCO-mediated adhesion is Ca^{2+} and Mg^{2+} independent and requires the cytoplasmic domain. Transfected cells expressing either hSR-AI, full length MARCO or MARCO with a truncated cytoplasmic domain were incubated with a PBS-EDTA solution for the times indicated. Greater than 80% of the mock transfected cells, as well as cells expressing the MARCO cytoplasmic tail mutants were released from tissue culture plastic within 2 min and totally detached within 4 min of incubation with PBS-EDTA. In contrast, transfected cells that expressed the full-length hMARCO or hSR-AI remained adherent after 16 min of incubation with PBS-EDTA. The figure shows an average of three independent experiments.

5.3.2 Ligands for MARCO-mediated adhesion and the role of the cytoplasmic tail

MalBSA has been used previously to study scavenger receptor mediated binding and endocytosis (Nicoletti et al., 1999). Adhesion to MalBSA coated surfaces was compared to adhesion on anti-MARCO antibody PLK-1 coated plastic. PLK-1 mimics MARCO ligands since its binding region is mapped to the same site in the cysteine-rich domain that has been shown to be required for binding to bacteria and TiO_2 (Arredouani et al., 2005).

HEK 293T cells are loosely adherent cells that are easily detached by washing. Transfected HEK 293T cells in DMEM with 10% FBS were seeded for 24 h on tissue culture plates previously coated with MalBSA, anti-MARCO mAb PLK-1 or BSA or mouse isotype control antibody as controls. Plates coated with collagen I were used as a control to demonstrate that integrin α1β1 and α2β1 mediated adhesion, which utilises collagens as ligands in HEK 293T cells (Bodary and McLean, 1990), was not altered by the presence of MARCO. The number of adherent cells after washing plates was quantified by crystal violet staining and spectrophotometry. Unwashed plates were used as a control and regarded as 100% adherent. The summary of all results is presented on Fig. 5.2.

Uncoated tissue culture plastic was shown to be a scavenger receptor ligand in the presence of serum, as about 30% of hMARCO and hSR-AI transfected cells remained adherent after washing. In contrast, less than 5% of the vector transfected cells or Nmyr and N- MARCO transfected cells remained adherent after washing.

hMARCO transfected cells showed the strongest adherence to plastic coated with anti-MARCO mAb PLK-1 and lowest adherence to mouse isotype control IgG3 coated plastic, with 42% and 7% adherent cells, respectively. The cytoplasmic tail mutants, Nmyr MARCO and N- MARCO, as well as vector transfected cells adhered very loosely to PLK-1 coated plastic and fewer than 3% of the cells remained adherent after washing.

Cell adhesion to modified proteins was also examined. The adherence of hMARCO transfected cells to MalBSA coated plastic was comparable to adherence to PLK-1 coated plastic, but Nmyr and N- MARCO transfected cells adhered to MalBSA more strongly than to PLK-1 coated plastic, as 15% and 12% of the cells, respectively, remained adherent after washing. The percentages of adherent hSR-AI transfected cells after washing were similar to hMARCO transfected cells, except for anti-hSR-A mAb C6 isotype control IgG1 coated plastic, to which over 40% of cells adhered, possibly because SR-A mediated adherence is greater than MARCO-mediated adherence and does not depend as much on the composition of the surface. Collagen I coated plastic was used as a control for integrin-mediated adherence, and similar results for adherent cells were obtained for all transfectants, including the vector control, indicating that integrin-mediated adhesion was not affected by expression of any of the constructs.

These results indicate that the cytoplasmic tail of MARCO is required for hMARCO-mediated cell adhesion on uncoated tissue culture plastic and on antibody coated plastic but is required to a lesser extent on a MalBSA coated surface. This indicates that MARCO-mediated adhesion to MalBSA and PLK-1 or tissue culture plastic utilises different regions of the protein.

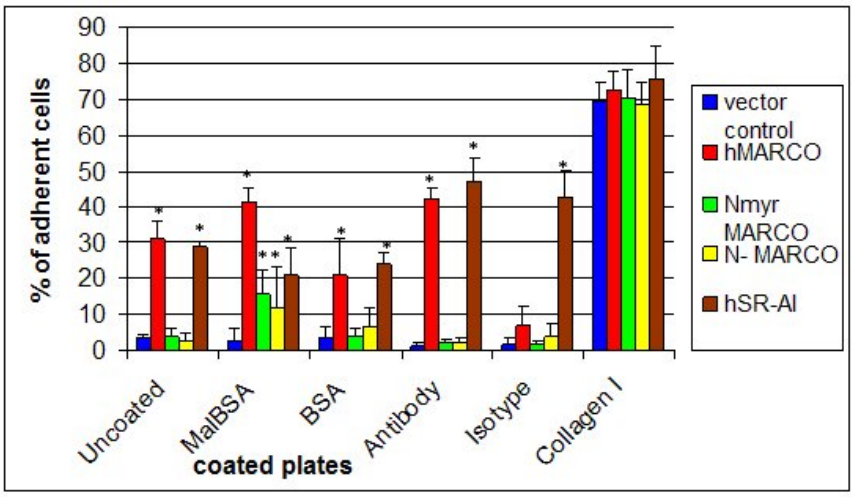


Fig. 5.2 MARCO-mediated adhesion to tissue culture plastic. The cells were allowed to adhere for 24 h in DMEM with 10% FBS and were then washed four times. The number of adherent cells was quantified by crystal violet staining and spectrophotometry. Unwashed cells were regarded as 100% adherent. Antibody coated surface refers to anti-MARCO mAb PLK-1 and anti-SR-A mAb C6 when MARCO-expressing cells or SR-A expressing cells were used, respectively. The results reveal that the cytoplasmic tail is required for adhesion to uncoated tissue culture plates, anti-MARCO antibody coated surface and to a lesser extent to a MalBSA coated surface. There was no significant difference in adhesion to an integrin ligand collagen I among all transfectants. Bars represent the average of three experiments ± standard deviation, * indicates a statistically significant increase compared to vector control, $p \leq 0.05$ by Student's T-test.

To test whether MARCO-mediated adhesion depends on the presence of any serum components, a similar experiment was performed using serum-free media and non-tissue culture treated suspension grade plastic plates. After 24 h, non-adherent cells were removed by aspiration and the number of adherent cells quantified by crystal violet staining and spectrophotometry. Fig. 5.3 shows that full-length MARCO and hSR-AI mediated adhesion in the absence of FBS to uncoated suspension grade plastic while vector transfected cells and MARCO cytoplasmic tail mutants grew in suspension and were unable to adhere. Similar results were obtained when human serum was used (data not shown). The cytoplasmic tail appeared to be necessary for adhesion to uncoated and anti-MARCO antibody coated plastic. In contrast, when plates were coated with MalBSA, Nmyr MARCO and N- MARCO expressing cells adhered more strongly, which indicates that the cytoplasmic tail is less involved in adhesion to MalBSA coated surfaces. These results correlated with previously described results for cell adhesion on tissue culture plates and show that suspension grade plastic is also a scavenger receptor ligand. These results indicate that MARCO and SR-A mediated adhesion does not require any serum components. Nevertheless, even though cells were transfected and grown in serum free conditions, we cannot eliminate the possibility of serum carryover.

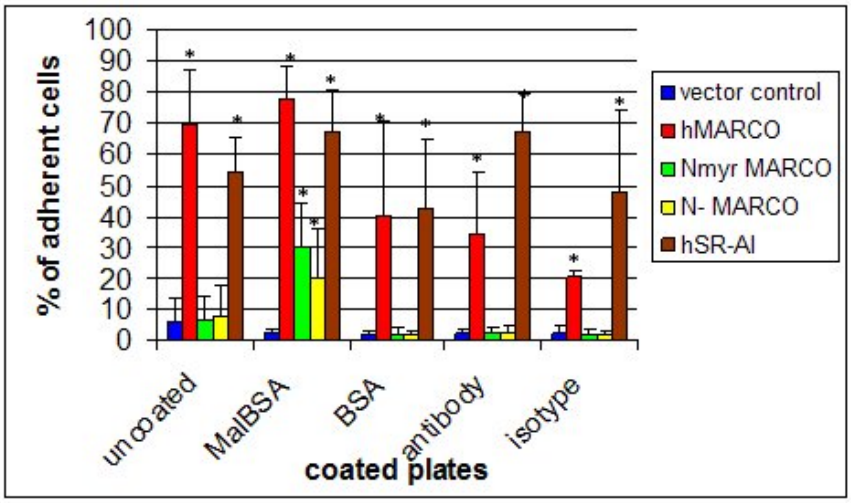


Fig. 5.3 MARCO-mediated adhesion to suspension grade plastic. The cells were allowed to adhere for 24 h in serum-free DMEM. Non-adherent cells were removed by aspiration and the number of adherent cells in each well was quantified by crystal violet staining and spectrophotometry. Antibody coated surface refers to anti-MARCO mAb PLK-1 and anti-SR-A mAb C6 when MARCO-expressing cells or SR-A expressing cells were used, respectively. All adherent cells on collagen I coated plates were regarded as 100%. The results show that MARCO mediates adhesion to uncoated suspension grade plastic in the absence of any serum components and the cytoplasmic tail is needed for adhesion. Similar results were obtained for antibody coated plastic. On MalBSA coated surface, the adhesion of Nmyr and N- MARCO mutants is significantly increased, which was not evident in any of the vector controls. hSR-AI expressing cells were used as a positive control and similar results were obtained as for hMARCO expressing cells. Bars represent the average of three experiments ± standard deviation, * indicates a statistically significant increase compared to vector control, $p \leq 0.05$ by Student's T-test.

5.3.3 The role of MARCO in Mϕ motility

Mϕs need to migrate to the source of infection in order to destroy and phagocytose pathogens. MARCO KO RPMϕs have been previously reported to have a defect in chemotaxis towards serum (Chen et al., 2005). The aim of this study was to examine the motility of RPMϕs on a scavenger receptor ligand coated surface and compare it to movement on an integrin ligand coated surface. After plating the Mϕs for 24 h on a MalBSA or a collagen I/fibronectin coated glass microwell, the movement of cells was recorded during a 2 h period and 20 randomly chosen cells were tracked in each experiment (Fig. 5.4 A). Analysis of the average distances covered by WT, MARCO KO and DKO RPMϕs on a MalBSA coated surface (Fig. 5.4 B) and on a collagen/fibronectin coated surface (Fig. 5.4 C) reveals that WT Mϕs move significantly more on the MalBSA coated surface than MARCO KO or DKO Mϕs. No statistically significant difference in the distance covered on a MalBSA coated surface during a 2 h period was recorded between MARCO KO and DKO Mϕs. There was no difference in Mϕ motility between WT, MARCO KO and DKO RPMϕs on integrin ligands collagen/fibronectin coated surface, indicating that MARCO only affects chemokinesis on a scavenger receptor coated surface. These results implicate that mainly MARCO, not SR-A, is responsible for accelerated movement of Mϕs on scavenger receptor ligands such as a maleylated protein.

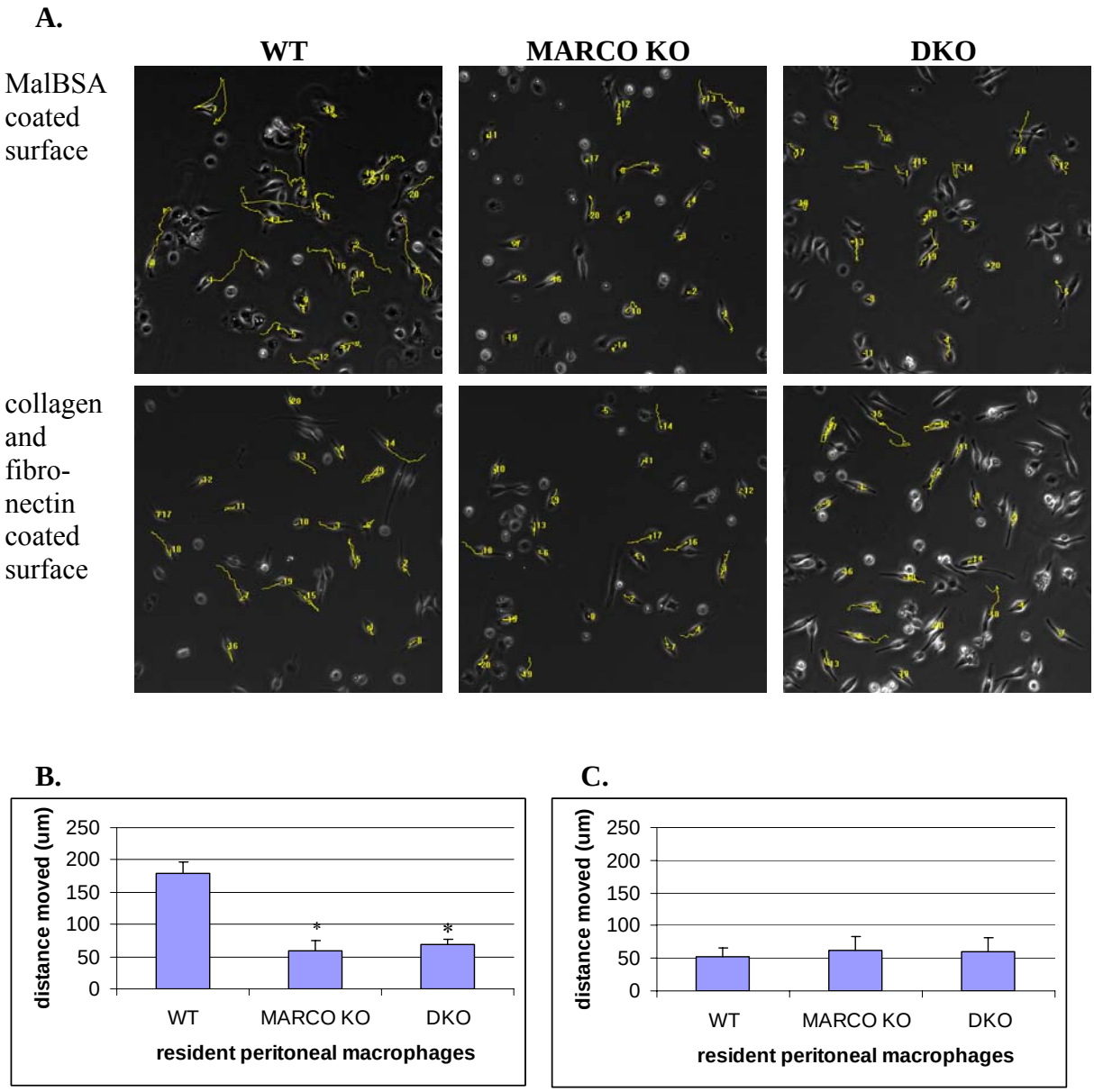


Fig. 5.4 Distance moved by RPMϕs during a 2 h period. **A**. A representative experiment showing the track of 20 randomly chosen cells. Yellow lines show the path the cells covered during 2 h. **B**. Average distance covered by RPMϕs on MalBSA coated glass microwell. Graph B demonstrates that MARCO KO RPMϕs move less on a MalBSA coated surface. No further reduction in movement was noticed in DKO RPMϕs. Bars represent the average of three experiments standard deviation, * indicates a statistically significant decrease compared to WT, $p \leq 0.05$ by Student's T-test. **C.** Average distance covered by RPMϕs on collagen I/fibronectin coated glass microwell. When cells were plated on a surface coated with an integrin ligand, no difference in movement between WT, MARCO KO and DKO was recorded. Bars indicate the range of movement between two independent experiments. The results indicate that MARCO affects Mϕ chemokinesis on a scavenger receptor ligand coated surface.

5.4 Discussion

In this study, I have shown that MARCO functions as a receptor for adhesion and cell migration on a modified protein. The results of this study show that MARCO ligands for adhesion include suspension and tissue culture grade plastic, MalBSA coated surfaces as well as anti-MARCO antibody coated surfaces. In contrast to what has been previously published about SR-A, serum components for MARCO-mediated adhesion were not required. The scavenger receptor ligands on uncoated tissue culture plastic are unknown but it is proposed that it is connected with the negative charge of the surface (Fraser et al., 1993). The serum ligand for SR-A is apoA-I and apoE (Neyen, 2008). These proteins are not ligands for MARCO (Dr. S. Mukhopadhyay, unpublished results).

Previously it has been reported that the expression of MARCO can induce the formation of processes on uncoated glass coverslips in the presence of FBS (Pikkarainen et al., 1999). This process was shown to depend on a segment of the cysteine-rich domain of MARCO (Pikkarainen et al., 1999). In this study, the role of the N-terminal cytoplasmic domain for cell adhesion was examined. The cytoplasmic tail appeared to be absolutely required for adhesion to all the coated surfaces, except for MalBSA coated surface, on which Nmyr MARCO and N- MARCO expressing cells showed increased adhesion compared to the vector control, but at reduced levels compared with cells expressing full-length MARCO. The reasons for this observation are unknown, but I hypothesise that different regions of MARCO are involved in binding to different ligands.

58.2±10.1% of hMARCO, 36.9±4.1% of Nmyr MARCO and 27.0±7.6% N- MARCO transfected cells were found to express the protein of the cell surface (Chapter Three,

section 3.3.3), suggesting that the cell surface expression of the cytoplasmic domain mutants of MARCO is normally lower than the cell surface expression of the full-length MARCO. This may suggest that there is no difference in adhesion on MalBSA coated surfaces between full-length MARCO and the mutants. On the other hand, as was shown in Chapter Four (Section 4.3.5), the cell surface expression of the cytoplasmic domain mutants increases by 10-20% upon binding to ligands, while the level of cell surface expression of the full-length MARCO remains unchanged, indicating that there might not be a relevant difference in the cell surface expression of the constructs. For this reason, the results were not normalised for cell surface expression.

Cell adhesion has been implicated in cell migration and chemotaxis. Mϕ motility on MalBSA coated glass was compared to motility on collagen/fibronectin coated glass and it was discovered that MARCO is responsible for increased motility of RPMϕs on a scavenger receptor ligand MalBSA, but not on an integrin ligand. MARCO KO and DKO Mϕs, on the other hand, had a reduced capacity for motility, which indicates that MARCO, not SR-A, is mainly responsible for faster movement on modified proteins. I hypothesise that the function of MARCO is opposite to that of SR-A, which has been implicated to be involved in retention of Mϕs on modified extracellular matrix proteins and to sites of injury, but *in vivo* experiments would be required to test this hypothesis. The role of the cytoplasmic tail in cell motility and chemotaxis is subject to further investigation.

6 Chapter Six: Summary of results, general discussion and future directions

The aim of studies presented in this thesis has been to characterise the role of the cytoplasmic domain of MARCO. By studying the cell surface expression, binding, endocytosis and adhesion of the transfected HEK 293T cells expressing the cytoplasmic domain mutants of MARCO, a variety of questions regarding the function of the cytoplasmic tail have been answered. However, there are still many areas that need further investigation, especially regarding the molecular mechanisms and the signalling required for those processes.

At the start of the studies presented in this thesis, there was no information on the function of the cytoplasmic domain of MARCO. However, research on the functions of the cytoplasmic domain of SR-A has shed some light on the role of various signalling motifs. Therefore, SR-A as one of the most closely related molecules to MARCO, was used as a model to formulate the hypothesis presented in this thesis.

6.1 Summary of main findings

First, it was hypothesised that the putative myristoylation site present in the cytoplasmic domain of MARCO would be necessary for cell surface expression of the protein. To investigate this hypothesis, the Nmyr MARCO mutant, with includes the putative myristoylation sequence, but lacks the rest of the cytoplasmic domain and the total cytoplasmic domain mutant N- MARCO were expressed in HEK 293T and HeLa cells. In both cases, the results showed that the cell surface expression of the cytoplasmic domain

mutants was reduced compared to that of the full-length MARCO. The reduction in cell surface expression was the strongest in the N- MARCO mutant, indicating that the putative myristoylation site affected cell surface expression of MARCO, but is not absolutely necessary, since 27.0±7.6% of HEK 293T cells transiently transfected with the N- MARCO, compared to 58.2±10.1% of the wild type MARCO, still expressed the protein at the surface. Presumably the other region of the molecule required for cell surface expression is the transmembrane domain.

To investigate the functions of the cytoplasmic domain in ligand binding, endocytosis and adhesion, the same ligand was used in all experiments. MalBSA, that has previously been described as a ligand for SR-A, was a good candidate because of its easy preparation, stability and adhesive properties to different surfaces. First, MalBSA coated beads were confirmed to be a specific ligand for MARCO expressing murine RPMϕs. Next, the binding of transiently transfected HEK 293T cells to MalBSA coated beads was examined. As a result, it was found that both the cytoplasmic domain mutants of MARCO showed the same level of binding to MalBSA coated beads as did the cells expressing the full-length MARCO. By staining for the cell surface expression of MARCO after removing the beads, it was confirmed that cells that expressed the same level of MARCO had bound similar number of beads.

Apart from examining the binding to a particulate ligand, cell association of MalBSA in soluble form was also examined. The binding of soluble fluorescently labelled MalBSA by transiently transfected HEK 293T cells that expressed full-length MARCO and the cytoplasmic domain mutants showed that the Nmyr MARCO mutant probably has reduced

abilities to take up soluble MalBSA compared to the full-length MARCO. On the other hand, there was no significant difference in binding between the N- MARCO and full-length MARCO transfected cells. Although it is difficult to reconcile these observations, it indicates that the cytoplasmic domain affects the uptake of soluble ligands. A stunning observation made by analysing the results of these studies was that the cell surface expression of the cytoplasmic domain mutants was significantly increased after incubation with the ligand, while the expression level of full-length MARCO remained unchanged. This shows that the cytoplasmic domain affects membrane trafficking and regulation of cell surface expression, especially upon binding to the ligand, although the exact mechanisms are yet to be determined.

MARCO-mediated adhesion is one of the least characterised functions of this receptor. The studies presented in this thesis showed that MARCO mediates adhesion that does not require the presence of divalent cations or any serum components. Also it was shown that MARCO mediates adhesion to both uncoated tissue culture plastic and to suspension grade plastic and requires the cytoplasmic domain for this function. When plastic was coated with anti-MARCO antibody PLK-1, the same results were obtained. On the other hand, when the plastic was coated with MalBSA, the adhesion of the cytoplasmic domain mutants increased. I hypothesise that different ligands bind to distinct epitopes on the protein.

MARCO was also shown to have a role in Mϕ motility on a ligand coated surface. Live imaging of WT, MARCO KO and DKO RPMϕs revealed that WT Mϕs show increased motility on a MalBSA coated surface compared to MARCO KO and DKO RPMϕs, whereas no change in chemokinesis was noticed when the RPMϕs were placed on an integrin ligand.

This observation indicates that mainly MARCO, not SR-A is responsible for increased motility on a ligand coated surface. The role of the cytoplasmic domain in this function of MARCO is yet to be examined.

To summarise, the studies presented in this thesis have demonstrated that the cytoplasmic domain of MARCO fulfils important functions, many of which contradict those of SR-A (Table 6.1). The cytoplasmic domain was found to be important for cell surface expression, cell association with a soluble ligand and for adhesion. In contrast, the binding to particulate ligands seems not to depend on the cytoplasmic domain.

Table 6.1 Comparison of the functions of the cytoplasmic domain of MARCO and SR-A

Is the cytoplasmic tail important for those functions?	MARCO	SR-A
Cell surface expression	Yes	Yes
Endocytosis	Yes	Yes
Binding to particulate ligands	No	?
Adhesion	Yes	No

? = not determined

6.2 Regulation of cell surface expression

In Chapter Three it was demonstrated that the cellular localisation of MARCO differs from that of SR-A in that a higher proportion of MARCO localises to the plasma membrane. This observation led to the hypothesis that the cytoplasmic domain of MARCO plays a role in its cellular localisation. It is generally thought that both type I and type II transmembrane proteins are targeted to the ER and are then anchored to the membrane using the hydrophobic sequences in the transmembrane domain (Lodish et al., 2003). SR-A, however, has been also shown to require the cytoplasmic domain for membrane anchoring. Besides the transmembrane region, SR-A has been shown to need the six membrane-proximal amino acids for cell surface expression (Kosswig et al., 2003). This indicates that these amino acids (KLKSFK), of which three are positively charged, are required for post-transcriptional processing and trafficking to the cell surface. Charged membrane-proximal amino acids have been shown to affect the topology of other transmembrane proteins (Spiess, 1995) and therefore it is proposed that these residues are critical for insertion to the ER in the right orientation. The structure of SR-A and MARCO is very similar, except that MARCO lacks the α-helical coiled-coil domain and its collagenous domain is much longer than that of SR-A. MARCO, on the other hand, does not contain any charged amino acids in the membrane-proximal region. Bioinformatic analysis revealed that the membrane-proximal amino acids of MARCO contain a putative myristoylation site (GVNFSL) which was proposed to be involved in cell surface expression of MARCO. Analysis of cell surface expression of the cytoplasmic domain mutants of MARCO indicated that the putative myristoylation site is not absolutely necessary for trafficking to the membrane, but enhances cell surface expression, since the Nmyr MARCO mutant showed greater cell surface expression than the N- mutant.

Therefore, it can be concluded that the putative myristoylation site as well as other sequences of the cytoplasmic domain affect cell surface expression of MARCO. This observation contradicts what is known about the regulation of cell surface expression of SR-A. Namely, the SR-A cytoplasmic domain mutant, that only has the six membrane-proximal amino acids, has been shown to have an increase in cell surface expression (Kosswig et al., 2003). This indicates that the membrane-proximal sequences of MARCO and SR-A regulate trafficking to the ER or insertion to the plasma membrane in an opposing manner. In the future, it would be worthwhile to determine whether the putative myristoylation sequence in the cytoplasmic domain of MARCO actually is myristoylated. Also there are other possible, although weak, signalling motifs present in the cytoplasmic domain of MARCO that need to be investigated (Fig. 6.1). The cytoplasmic domain of hMARCO contains a putative di-leucine intracellular sorting sequence, which may play a role in membrane trafficking (Bonifacino and Traub, 2003). Bioinformatic analysis also revealed a potential phosphorylation motif L-X-X-**S**/**T** in the cytoplasmic domain of MARCO. As a parallel, there are two phosphorylation sites in the cytoplasmic domain of SR-A, which have experimentally been shown to be important for cell surface expression (Fong and Le, 1999). In addition, two positively charged amino acids, R38 and R39, may affect membrane trafficking and cell surface expression of MARCO.

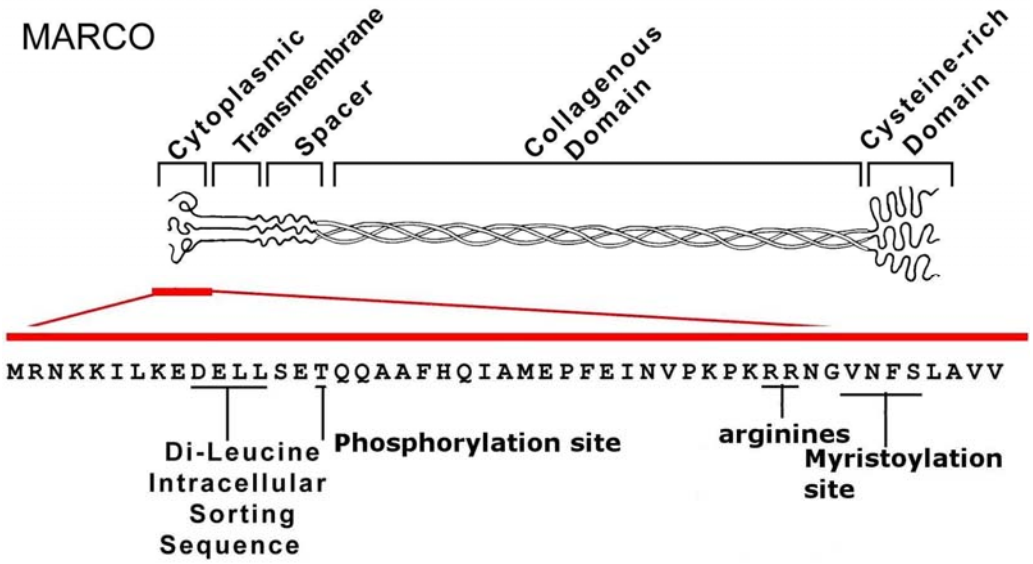


Fig. 6.1 The potential signalling motifs in the cytoplasmic domain of hMARCO. The potential myristoylation sequence was found to be involved in cell surface expression of MARCO, as was shown in Chapter Three. Other possible sequences that might affect membrane trafficking include the di-leucine motif, the T16 phosphorylation site and two polar arginines in the membrane-proximal part of the cytoplasmic domain. All signalling motifs shown on the graph are putative and their involvement in the cell surface expression and other functions of MARCO remain to be experimentally shown.

6.3 The role of the cytoplasmic domain in MARCO-mediated binding and uptake

The cytoplasmic domain mutant of SR-A has been reported to be defective in internalisation of AcLDL, possibly because of the requirement of the VXFD motif in the cytoplasmic domain (Kosswig et al., 2003; Morimoto et al., 1999). A similar sequence cannot be found in the cytoplasmic domain of MARCO. Nevertheless, it was found that the Nmyr MARCO mutant shows a reduced ability to endocytose MalBSA. In contrast, the total N- MARCO mutant displayed the same level of endocytosis of the ligand as did the full-length protein. The reason for this observation is unknown but might be due to a conformational change or changes in post-translational modifications. Therefore, both the SR-A and MARCO cytoplasmic domain mutants, that have the membrane-proximal amino acids but lack the rest of the tail, are defective in endocytosis, which indicates that the cytoplasmic tail has a role in intracellular signalling required for ligand internalisation. Since the total cytoplasmic domain mutant of SR-A is not expressed at the cell surface, a parallel cannot be drawn to compare the role of the membrane-proximal sequences of MARCO and SR-A.

The cytoplasmic tail appeared not to be required for binding to a particulate ligand (i.e. MalBSA coated 2 μm beads). Unfortunately, the MARCO transfected HEK 293T cells were not capable of internalising this ligand, possibly because these cells lack the needed machinery and cellular compartments required for this specialised function. Therefore, the role of the cytoplasmic tail in phagocytosis was not determined in studies presented in this thesis. In the future, it may be possible to express the cytoplasmic tail mutants of MARCO in

a Mϕ cell line, such as RAW, which does not normally express MARCO (data not shown), and determine if the cytoplasmic domain is needed for phagocytosis.

6.4 MARCO-mediated adhesion

Scavenger receptor mediated adhesion is biologically relevant as it has been associated with possible Mϕ retention at sites of injury or inflammation. Previous reports have indicated that MARCO may be, similar to SR-A, a receptor for adhesion (Ojala et al., 2007; Pikkarainen et al., 1999). In recent years, several studies on SR-A-mediated adhesion and signalling have been published, but not much is known about MARCO-mediated adhesion.

The studies presented in Chapter Five demonstrated that in contrast to mouse MARCO, human MARCO mediated adhesion does not require the presence of divalent cations. The crystal structure of the cysteine-rich domain of murine MARCO has been reported to contain an acidic cluster, which was found to be responsible for the requirement of divalent cations (Ojala et al., 2007). The residues in this cluster (D447, D448, E511), which were found to be directly responsible for the divalent cation dependent nature of adhesion, are also conserved in the human homologue (Ojala et al., 2007). This indicates that the crystal structure of the cysteine-rich domain of human and murine MARCO may differ in other aspects that remain to be investigated. Nevertheless, I cannot rule out the possibility that the controversial results between human and mouse MARCO may be due to the different experimental techniques used.

The fact that SR-A does not need the cytoplasmic tail for adhesion and MARCO does, may indicate that there are differences in signalling that is required to mediate adhesion. SR-A is known to require the sequential activation of Src kinase Lyn and PI3 kinase as well as arachidonic acid metabolism mediated by the calcium independent phospholipase A_2 and activity of 12/15 lipoxygenase. There are two phosphorylation sites in the cytoplasmic tail of SR-A that are apparently not required for SR-A mediated adhesion. The cytoplasmic domain of MARCO contains a potential phosphorylation site (motif L-X-X-**S**/**T**) in position T16 which may be a kinase substrate required for MARCO-mediated adhesion, since it was confirmed that MARCO requires the cytoplasmic domain. The signalling required for MARCO-mediated adhesion needs further research, which may in turn reveal the role of specific sequences in the cytoplasmic domain.

In conclusion, the studies presented in this thesis have provided information about the role of the cytoplasmic domain of MARCO in many important cellular functions of this receptor, such as binding, endocytosis and adhesion. Knowledge of the biology of this receptor can contribute to the understanding of Mϕ recruitment to sites of infection and binding to modified host ligands – processes that play a key role in many medically important diseases associated with scavenger receptors, such as cerebral ischaemia (Milne et al., 2005) and atherosclerosis (Daugherty et al., 2001).

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