CHARACTERIZATION OF MARCO-MEDIATED ENDOCYTOSIS

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By

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ABSTRACT

Class A scavenger receptors are multifunctional transmembrane glycoproteins that mediate macrophage functions like phagocytosis and endocytosis. The macrophage receptor with collagenous structure (MARCO) is one such receptor. It has been shown that the extracellular cysteine-rich domain of MARCO is responsible for ligand binding, but the role of the cytoplasmic domain in ligand uptake is unclear. The aim of the studies presented in this thesis is to characterize the role of the cytoplasmic domain of MARCO and to characterize the molecular pathway of MARCO-mediated endocytosis.

Full-length human MARCO (hMARCO) and Δ1-34hMARCO, which lacks the first thirty-four amino acids were created in order to determine whether amino acids 1-34 contained residues required for receptor internalization and surface expression. The constructs were stably expressed in HEK293T cells and found to have similar levels of surface expression and same rate of internalization without ligand. Interestingly, hMARCO, but not Δ1-34hMARCO, surface expression was up-regulated upon ligand incubation.

In order to ascertain the importance of clathrin, dynamin and actin in MARCO-mediated endocytosis, specific endocytic inhibitors were used. MARCO-mediated ligand uptake was inhibited when clathrin and actin polymerization and, dynamin functions were impaired by these inhibitors. Furthermore, ligand uptake by Δ1-34hMARCO-expressing HEK293T was insensitive to inhibitors of clthrin and dynamin but not inhibitors of actin.

In conclusion, MARCO mediates endocytosis via a clathrin-mediated, dynamin-dependent pathway that involves actin. Amino acids 1-34, are required clathrin and dynamin but not actin functions during MARCO-mediated endocytosis. Additionally, amino acids 1-34 might also be important for MARCO recycling but not receptor internalization or surface expression.

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Finally, I should also thank my parents for their blind faith in me.

Abbreviations

AcLDL	acetylated low density lipoprotein
AGE	advanced glycation end product
AF488	Alexa Fluor 488
AP-2	adaptor protein-2
APC	allophycocyanin
ARF6	ADP-ribosylation factor 6
BSA	bovine serum albumin
BDB	bisdiazonium-benzidine
CCV	Clathrin-coated vesicle
CME	Clathrin-mediated endocytosis
CpG	-C-phosphate-G-
CPZ	chlorpromazine
CTxB	Cholera toxin subunit B
CytoD	Cytochalasin D
ddH_2O	double distilled water
dSR-CI	*Drosophila* scavenger receptor CI
DTT	dithiotheitol
EDTA	etylenediaminetretraacetic acid
EPGR	epidermal growth factor receptor
ELM	eukaryotic linear motif
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
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfanic acid
hMARCO	human MARCO
HRP	horse-radish peroxidase
IgG	immunoglobulin G
kDa	Kilodalton
KLH	keyhole limpet hemoocyanin
KO	knockout
LDL	low density lipoprotein
LOX-1	lectin-like oxidised LDL receptor-1
LPS	lipopolysaccharide
LTA	lipoteichoic acid
Mϕ	macrophage
MalBSA	maleylated BSA
MARCO	macrophage receptor with collagenous structure
min	minute
mRNA	messenger RNA

NZW	New Zealand white
OxLDL	oxidised LDL
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PFA	paraformaldehyde
PtdIns3P	Phophatidylinsitol-3-phosphate
RPMϕ	resident peritoneal macrophages
RS	rabbit serum
SCARA5	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	scavenger receptor cysteine rich
SREC	scavenger receptor expressed by endothelial cells
SR-PSOX	scavenger receptor that binds phosphotidylserine and oxidized lipoprotein
TDM	trehalose 6,6'-dimycolate
TLR	toll-like receptor
TRITC	tetramethylrhodamine B isothiocyanate
WT	wild type

TABLE OF CONTENTS

1 Chapter 1: Introduction

1.1 Macrophages and their functions

Macrophages are white blood cells of myeloid lineage. They are migratory cells deriving from bone marrow precursors and are found in all human tissues and play a crucial role in host defence (Gordon and Taylor 2005). Macrophages in different tissues have different names historically; for instance, microglial cells in the neural tissue and Kupffer cells in the liver. In general, these cells are referred to as mononuclear phagocytes.

Macrophages are mainly involved in engulfing and killing invading microorganisms and other insoluble material, initiating the innate immune responses and, subsequently, helping to orchestrate adaptive immune responses (Murphy 2007). For example, in the case of pathogen invasion, macrophages bind and ingest the pathogen. Ingested pathogens are found within a membrane-enclosed vesicle called the phagosome, which is a specialized endocytic vacuole. In macrophages, there are intracellular vesicles that contain enzymes and toxic peroxides that are detrimental to the pathogens called lysosomes. The pathogen-containing phagosome fuses with one or more lysosomes to form a phagolysosome. Consequently, pathogens are destroyed by the lysosomal contents released into the phagolysosome (Murphy 2007).

In addition to recognizing and destroying pathogens, macrophages also play a homeostatic role by removing apoptotic cells from the human body. It is estimated that

360 billion senescent red blood cells are cleared by macrophages every day (Bratosin *et al.* 1998).

Another important function of macrophages includes cytokine and chemokine production during inflammation, which activates or helps recruit other immune cells to fight infection. Additionally, like dendritic cells, macrophages can also present antigens to T cells, albeit less efficiently (Gordon and Taylor 2005).

1.1.1 Pattern recognition receptor and macrophage function

There are two majors functions carried out by macrophages: recognition and destruction of invading foreign pathogens and clearance of endogenous cellular debris and apoptotic cells (Gordon 2002). Thus it is important for macrophages to discriminate between self and non-self. It is proposed that cell surface receptors with broad specificity are important during this process (Janeway and Medzhitov 2002).

Unlike the adaptive immune system, the innate immune system is a universal and ancient form of host defense against infection. While the adaptive immune system uses a virtually unlimited number of B and T cell receptors via somatic recombination, innate immune recognition relies on a limited number of germline-encoded receptors that evolved to recognize conserved microbial products called pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov 2002), but not by the host. These receptors are termed pattern recognition receptors (PRRs) (Janeway and Medzhitov 2002). Recognition of PAMPs allows the immune system to distinguish non-self or modified-self from self. PRRs are localized to the cell surface or are intracellular and are capable of recognizing a

variety of ligands including lipopolysaccharides, proteins and double-stranded RNA. Table 1.1 provides a non-exhaustive summary of PRRs and their cognate ligands.

To date, the best-characterized PRRs are the Toll-like receptors (TLRs). They function as signalling receptors to induce pro-inflammatory signals upon recognition of PAMPs (Takeuchi and Akira 2010). The endocytic or phagocytic PRRs that are mainly involved in engulfing and destroying pathogens (Gordon 2002). Scavenger receptors are such endocytic PRRs. Scavenger receptors (SRs) were originally defined by their ability to bind modified low-density lipoprotein (LDL) (Goldstein *et al.* 1979) and will be discussed in detail in 1.2. It is worth mentioning that there has been an increasing appreciation that the signalling PRRs cooperate with the endocytic PRRs to combat infections. For example, SR-A, a class A SR attenuates TLR4-induced NF-κB activation by directly inhibiting ubiquitination of tumor necrosis factor receptor-associated factor 6, which attenuates its pro-inflammatory signalling activity (Yu *et al.* 2011).

Table 1.1 Summary of selected macrophage pattern recognition receptors and their ligands. (Adapted from Gordon 2002 and Takeuchi and Akira 2010)

PRRs	Localization	Ligand	Origin of the Ligand
Toll-like receptors			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa
RIG-I-like receptors			
RIG-I	Cytoplasm	Short dsRNA, 5'triphosphate dsRNA	RNA viruses, DNA virus
MDA5	Cytoplasm	Long dsRNA	RNA viruses (Picornaviridae)
LGP2	Cytoplasm	Unknown	RNA viruses
NOD-like receptors			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria
C-type lectin receptors			
Dectin-1	Plasma membrane	β-Glucan	Fungi
Dectin-2	Plasma membrane	β-Glucan	Fungi
MINCLE	Plasma membrane	SAP130	Self, fungi
Scavenger Receptors			
SR-AI/II	Plasma membrane	Lipid A, LTA, N. meningitidis, abestos, modified LDL, apoptotic cells	Bacteria, self
MARCO	Plasma membrane	modified LDL, LPS, environmental particles	Bacteria, self
CD36	Plasma membrane	Modified LDL, Plasmodium falciparum-malaria parasitized erythrocytes	Self

1.2 The macrophage scavenger receptors

In 1979, Brown and Goldstein made the initial observation that the resident mouse peritoneal macrophages were capable of taking up acetylated LDL (AcLDL) with 20 times higher the affinity than LDL (Goldstein *et al.* 1979). They termed the AcLDL binding receptors macrophage scavenger receptors for their role in scavenging modified LDL (Brown and Goldstein 1979). Since then, rapid progress had been made in discovering the physiological role of macrophage scavenger receptors in health and diseases. Work by Steinberg demonstrated that oxidized LDL is recognized by macrophage scavenger receptors and lipid-laden macrophages, or the "foam" cells, are formed (Quinn *et al.* 1987). This is thought to be the major cause of atherosclerosis (Steinberg and Witztum 2010). Thanks to the advent of molecular cloning technology, researchers were able to clone and better define the classes of scavenger receptors. Scavenger receptor A was the first receptor cloned (Kodama *et al.* 1988) and followed by many other classes of scavenger receptors. The scavenger receptors are grouped into eight classes: A, B, C, D, E, F, G and H, based on their structure (Krieger 1997). Fig 1.1 shows the domain architecture of these receptors. These receptors have broad ligand specificities that recognize a variety of ligands like lipopolysaccharide (LPS), lipoteichoic acid (LTA), apoptotic cells, microbial and viral products and polyanionic ligands in addition to recognizing modified lipoproteins (Plüddemann *et al.* 2007). Therefore, scavenger receptors are types of pattern recognition receptors. Certain Scavenger receptors (e.g. MARCO and SR-A) are expressed primarily on macrophages and dendritic cells, whereas others (e.g.SCARA3, 4 and 5) have a broader range of expression and are found on

epithelial and endothelial cells (reviewed in Pluddemann *et al.* 2006; Pluddemann *et al.* 2007). Scavenger receptors play important roles in host defence and homeostasis due to their broad ligand specificity. They contribute to macrophage functions such as endocytosis and phagocytosis (Platt and Gordon 2001).

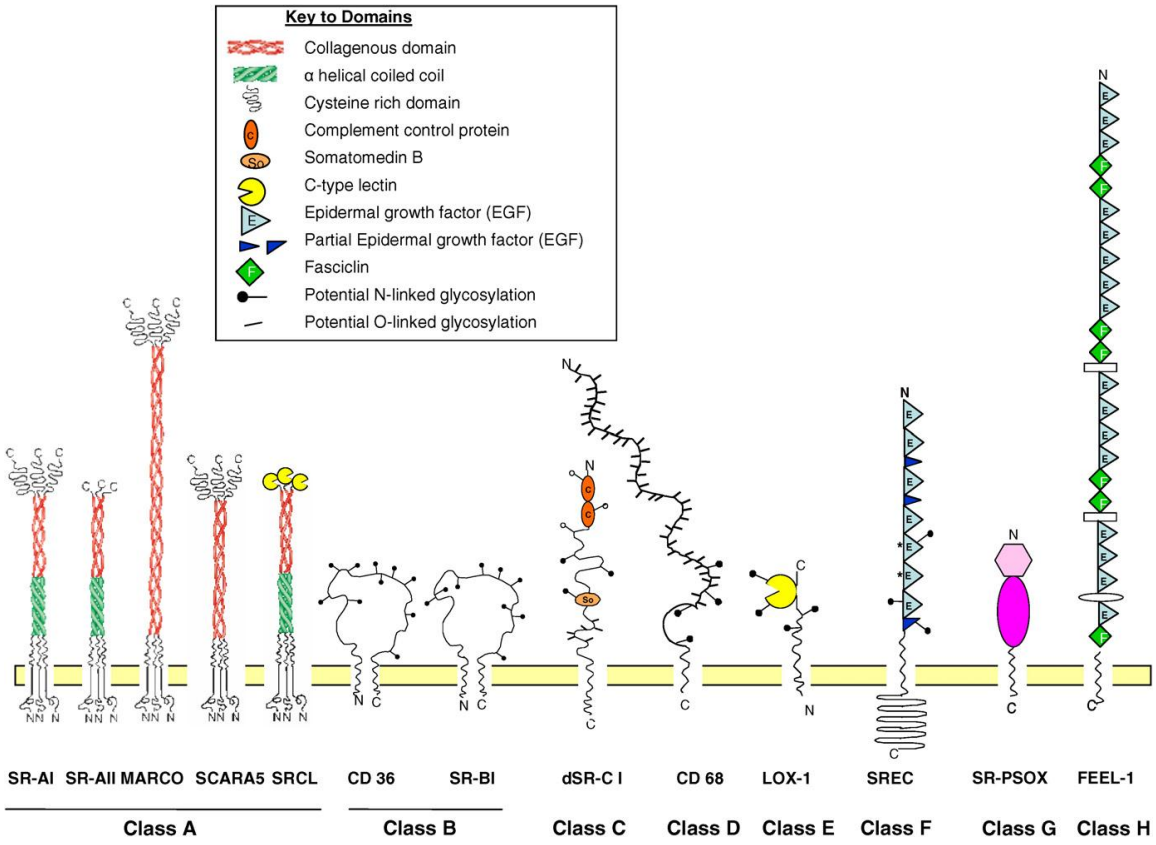


Fig. 1.1 Domain architecture of the different classes of scavenger receptors

Scavenger receptors are divided into eight classes and are structurally very diverse. The domains are indicated in the key. (Taken from Pluddemann *et al.* 2006).

1.2.1 The class A scavenger receptors

Scavenger receptor A was the first discovered (Kodama *et al.* 1988) followed by the macrophage receptor with collagenous structure (MARCO) (Elomaa *et al.* 1995). MARCO and SR-A are structurally similar and are expressed primarily on macrophages. Two other scavenger receptors that are mainly expressed on endothelial cells, class A scavenger receptor 5 (SCARA5) (Jiang *et al.* 2006) and scavenger receptor with C-type lectin (Nakamura *et al.* 2001) have also been identified.

Furthermore, SR-AI, MARCO and SCARA5 belong to a large family of receptors called scavenger receptor cycteine rich (SRCR), family proteins. All the members of this superfamily share a conserved SRCR domain that contains 100-110 amino acids (Jiang *et al.* 2006). However, a common function of the SRCR domain is not clear. Ligand binding is one of the few functions that are implicated (Sarrias *et al.* 2004). For example, the SRCR domain is the bacterial ligand binding domain for MARCO (Plüddemann *et al.* 2009) (Also see section 1.3.5.).

1.2.1.1SR-A: Structure and functions

There are three isoforms of SR-A. SR-AI (Kodama *et al.* 1988) and SR-AII (Rohrer *et al.* 1990), which has a truncated scavenger receptor cysteine-rich domain, have similar ligand binding capability despite having different expression levels during monocyte differentiation (Geng *et al.* 1994). SR-AIII is primarily localized to the ER and is not

capable of known ligand binding (Gough *et al.* 1998). Fig 1.2 depicts schematic structures of all three isoforms of SR-A.

Like most PRRs, SR-A is capable of recognizing both endogenous and exogenous ligands. SR-A has been shown to bind AcLDL (Kodama *et al.* 1988), OxLDL (Freeman *et al.* 1991), modified extracellular matrix proteins (Santiago-Garcia *et al.* 2001) apoptotic cells (Platt *et al.* 1996), and advanced glycation end products (Araki *et al.* 1995). On the other hands, SR-A has also been shown to bind components of bacterial pathogens such as LTA from the cell walls of Gram-positive bacteria (Greenberg *et al.* 1996) and LPS from Gram-negative bacteria (Hampton *et al.* 1991). Additionally, SR-A binds to intact bacteria such as *Neisseria meningitides*, *Escherichia coli* (Peiser *et al.* 2000), and *Listeria monocytogenes* and *Staphylococcus aureus* (Dunne *et al.* 1994). In additional to binding to bacterial ligands, SR-A has been shown to bind double-stranded RNA (Limmon *et al.* 2008). Furthermore, it was demonstrated that SR-A-mediated viral double stranded RNA binding and entry would lead to subsequent interferon stimulated gene induction, which is an integral part of anti-viral immunity (DeWitte-Orr *et al.* 2010), thus indicating that SR-A plays a role in anti-viral immunity.

The mechanism by which SR-A mediates ligand binding is not fully understood. 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). Additionally, it has been demonstrated that the negative charge of the SR-A within its extracellular domain is important for bacterial ligand binding (Greenberg *et al.* 1996). However, not all

polyanionic substances, such as polycytidylic acid, are ligands for SR-A (Kodama *et al.* 1990).

In addition to pathogen recognition via ligand binding, SR-A also participates in other cellular processes such as endocytosis and adhesion (Kosswig *et al.* 2003; Nikolic *et al.* 2007). The role of SR-A in endocytosis will be discussed in detail in Section 1.5.3.

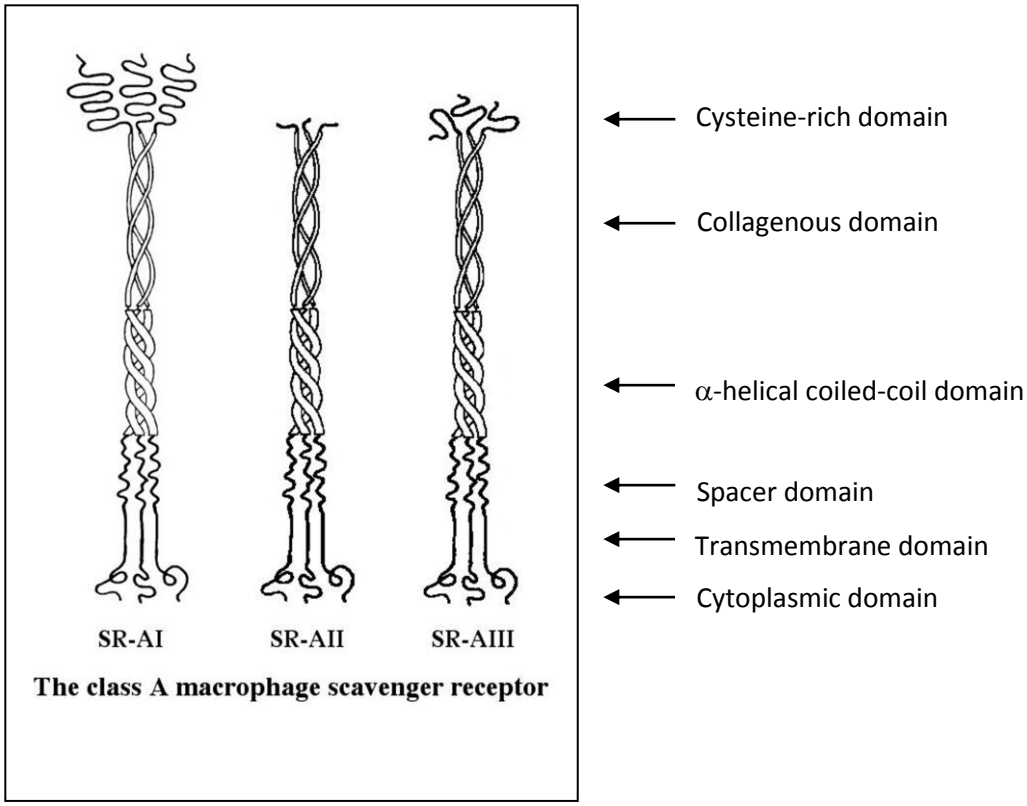


Figure 1.2 A schematic representation of the structure of SR-A. SR-AII is identical to SR-AI, except that the cysteine-rich domain is truncated. SR-AIII is a non-functional isoform, that is trapped in endoplasmic reticulum (ER) and is defective in cell surface ligand binding. SR-AII has a cysteine-rich C-terminal domain like SR-AI, but it lacks the C-terminal end of the collagenous domain, which has been implicated in the binding to polyanionic ligands (Yamamoto *et al.* 1997). (Adapted from Rohrer *et al.* 1990; Gough *et al.* 1998).

1.2.1.2 Class B scavenger receptors

Class B scavenger receptors primarily include both CD36 (Endemann *et al.* 1993) and scavenger receptor BI/II (Acton *et al.* 1994). These are type III transmembrane glycoproteins with two intracellular cytoplasmic tails and an extracellular loop. The extracellular loop is considered important for ligand binding (Greenwalt *et al.* 1992). Analogous to the Class A scavenger receptors, CD36 and SR-BI have broad ligand specificity. They bind oxLDL, 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). Unlike the class A scavenger receptors, CD36 and SR-BI also bind with high affinity to lipoproteins (Acton *et al.* 1994; Luangrath *et al.* 2008), such as very low-density lipoprotein (VLDL), LDL and high-density lipoprotein (HDL) (Murao *et al.* 1997; Rigotti *et al.* 1997; Calvo *et al.* 1998). Therefore, CD36 and SR-BI play an important role in lipid metabolism.

In addition to their important roles in lipid metabolism, CD36 and SR-BI have also been shown to bind LPS, a PAMP (Vishnyakova *et al.* 2003; Baranova *et al.* 2008). However, the role of CD36 and SR-BI in host defence is unclear. It is proposed that CD36 and SR-BI participate in host defense via endocytosis of the pathogen following ligand binding similar to that of the Class A scavenger receptors (Leelahavanichkul *et al.* 2012).

1.2.1.3 Class C scavenger receptors

Drosophila scavenger receptor CI (dSR-CI) is the only Class C scavenger receptor (Pearson *et al.* 1995). It binds to AcLDL (Pearson *et al.* 1995) and both Gram-postive (*S. aureus*) and Gram-negative bacteria (*E. coli*), and functions as a PRR in Drosophila (Ramet *et al.* 2001). It should be noted that no human homologue has been found and dSR-CI is structurally dissimilar to other scavenger receptors.

1.2.1.4 Class D scavenger receptors

CD68 in humans and its murine homologue macrosialin (Ramprasad *et al.* 1995) and lysosomal membrane glycoproteins Lamp-1, Lamp-2 and Lamp-3 (de Saint-Vis *et al.* 1998) belong to the Class D scavenger receptor family. Unlike, most other receptors, CD68 is localized intracellularly in the endosomal compartments (de Saint-Vis *et al.* 1998). It binds to OxLDL (Ramprasad *et al.* 1995) and is thought to be involved in the intracellular processing of OxLDL.

1.2.1.5 Class E scavenger receptors

Lectin-like oxidized LDL receptor-1 (LOX-1) is the sole member of the Class E scavenger receptor (Sawamura *et al.* 1997). It is a type II transmembrane protein with an extracellular C-type lectin domain that is highly conserved and mediates ligand binding (Nagase *et al.* 1997; Sawamura *et al.* 1997; Shi *et al.* 2001). LOX-1 binds to OxLDL with high affinity. Additionally, it also recognizes other endogenous ligands such apoptotic cells (Oka *et al.* 1998) and advanced glycation end-product (AGE) (Jono *et al.* 2002).

1.2.1.6 Class F scavenger receptors

Class F scavenger receptors include Scavenger Receptor Expressed by Endothelial Cells (SREC) type I (Adachi *et al.* 1997) and II (Ishii *et al.* 2002). SREC preferentially binds and internalizes AcLDL but not OxLDL. SERC is also structurally different from other scavenger receptors. It is a type I transmembrane protein that has five extracellular epidermal growth factor-like (EGF) domains (Adachi *et al.* 1997). The structurally similar SREC-II is less able to internalize modified LDL (Ishii *et al.* 2002) compared to SERC I.

1.2.1.7 Class G scavenger receptors

Scavenger receptor that binds phosphatidylserine and oxidized lipoprotein, or SR-PSOX is the only member of class G scavenger receptors (Shimaoka *et al.* 2000). In contrast to the ligand binding properties of SERC I, and similar to that of LOX-1, SR-PSOX binds to OxLDL with high affinity but not to AcLDL. It is expressed mainly on resident macrophages and dendritic cells (Shimaoka *et al.* 2000). SR-PSOX has also been shown to be the chemotactic ligand for CXC chemokine receptor 6 that is expressed on activated T cells and natural killer T cells (NKT). Therefore it is proposed that SR-PSOX draws T cells and NKT cells to macrophages and dendritic cells during inflammation (Shimaoka *et al.* 2004).

1.2.1.8 Class H scavenger receptors

Fasciclin, epidermal growth factor-like, laminin-type epidermal growth factor-like, and link domain-containing scavenger receptor, or FEEL, belongs to the class H scavenger receptors (Adachi and Tsujimoto 2002). FEEL-1 and FEEL-2 are type I transmembrane proteins of similar structure, except that FEEL-1 has one more EGF domain than FEEL-2 (Adachi and Tsujimoto 2002). They bind to AcLDL and Gram-positive and –negative bacteria, indicating that these receptors might have an important role in host defence (Adachi and Tsujimoto 2002).

1.3 MARCO

Macrophage receptor with collagenous structure, or MARCO, is a member of the Class A scavenger receptor family. It is structurally very similar to SR-A. Therefore, the ligand binding properties of MARCO are very similar to that of SR-A. It was thought that MARCO and SR-A are redundant and share a similar role in homeostasis and host defence. Recently, an increasing amount of evidence has established that SR-A and MARCO actually play non-redundant roles in host defence (Elomaa *et al.* 1998; Arredouani *et al.* 2005; Plüddemann *et al.* 2008; Dorrington *et al.* 2012).

1.3.1 Splice variants of human MARCO

There are two spice variants of MARCO, MARCO-I (Elomaa *et al.* 1995) and MARCO-II (Bowdish *et al.* 2012). Similar to SR-AII, MARCOII has a truncated cysteine-rich

domain. Little is known about the function of MARCO-II, and since it lacks the SRCR domain, it is thought that MARCO-II is deficient in ligand binding. The discussions in the following sections will be focused on MARCO-I with the comparison to SR-AI, since SR-AI is closely related to MARCO and relatively well-characterized. Thus, findings concerning the functions of SR-AI might shed light into unravelling the biochemistry of MARCO-I.

1.3.2 The structure of MARCO

MARCO is structurally very similar to that of SR-AI. As indicated in Figure 1.3, MARCO, like SR-AI, is a type II transmembrane glycoprotein. It is a 210 kDa disulphide-bonded trimer. MARCO has five domains. The SRCR domain (V), which contains 101 amino acids (a.a.) (Elomaa *et al.* 1995) is extracellular and required for ligand binding. The extracellular collagenous domain (IV) contains 270 a.a. It is much longer than its SR-AI counterpart and is involved in trimerization of the molecule. Unlike SR-AI, the collagenous domain is connected to the transmembrane domain (II) by a 75 a.a spacer domain (III) without an alpha helical coiled-coil domain. Like SR-AI, MARCO has a very short (50 a.a) cytoplasmic domain (I) (Kraal *et al.* 2000).

As a type II transmembrane glycoprotein, MARCO is presumably synthesized and modified in the ER and then transported to the cell surface via the Golgi network. Unlike type I transmembrane proteins, which have two kinds of topogenic sequences, an N-terminal cleaved signal sequence or stop-transfer membrane-anchor sequence, a type II transmembrane protein contains only the hydrophobic stop-transfer sequence that also

functions as an internal signal sequence. The orientation of the insertion of the protein into the plasma membrane is determined by the charged residues that are close to the hydrophobic sequence (Lodish *et al.* 2003).

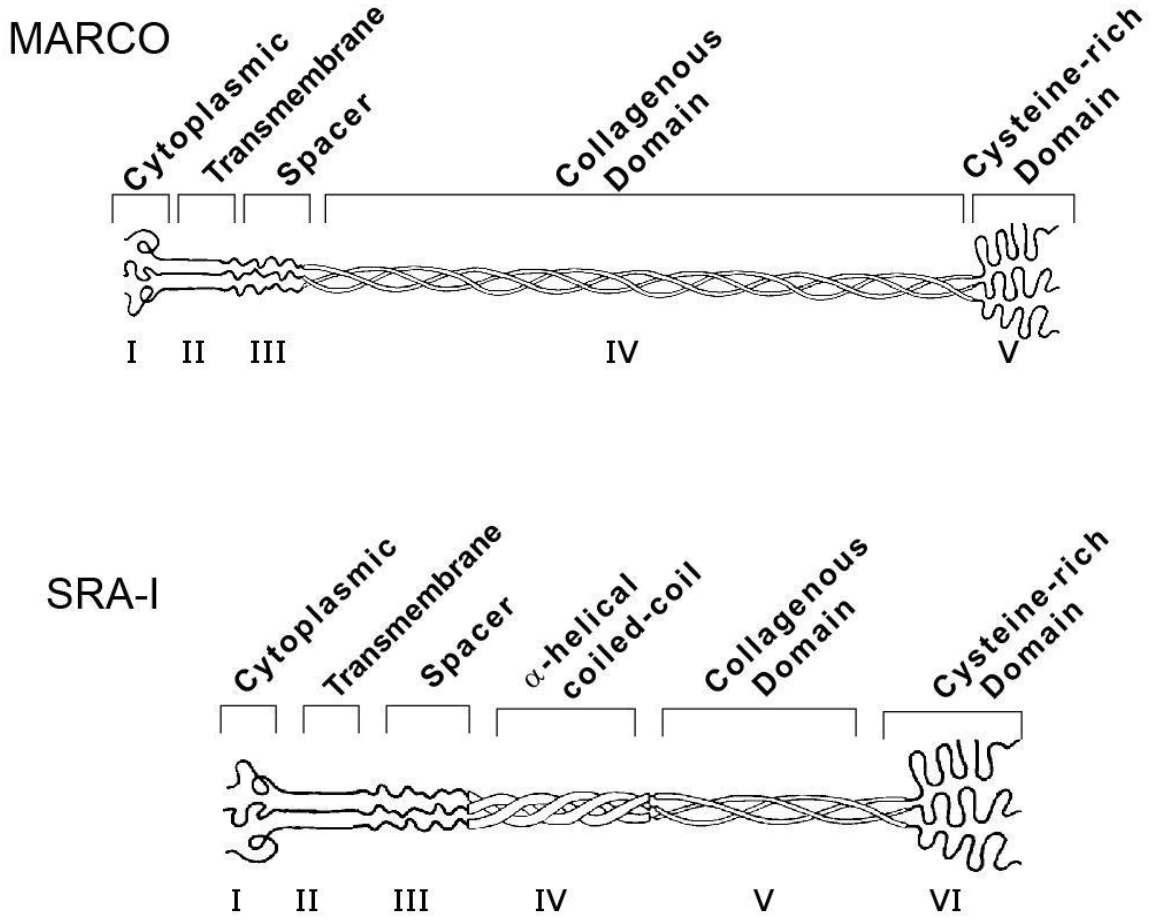


Fig 1.3 The structure of MARCO and SR-AI. MARCO consists of a I cytoplasmic tail, II transmembrane domain, III spacer region, IV collagenous domain and V C-terminal scavenger receptor cysteine-rich domain. SR-AI also has an α-helical coiled-coil region. Adapted from (Rohrer *et al.* 1990; Elomaa *et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

1.3.2.1 Domain I: the cytoplasmic domain

To date, no study has been published regarding the function of the cytoplasmic domain of MARCO.

For SR-A, it has been shown that the cytoplasmic domain of SR-A plays a very important role in its functions. For example, the six membrane-proximal amino acids are required for cell surface expression in transfected HEK293T cells (Kosswig *et al.* 2003).

In addition, the cytoplasmic domain of SR-A has also been shown to contain amino acid sequences that are important for SRA-mediated endocytosis. For example, the VXFD motif between amino acids 18 and 27 from the N-terminus is crucial for efficient ligand uptake (Morimoto *et al.* 1999). A di-leucine motif in SR-A and S20, a phosphorylation site, have also been shown to be important for SR-A mediated internalization of AcLDL (Fong 1996; Fong and Le 1999; Chen *et al.* 2006).

The function of the cytoplasmic domain of MARCO is unknown. It is likely that the cytoplasmic tail has an important role in MARCO function similar to SR-A. However, it should be noted that the primary structure of the cytoplasmic domain of MARCO is different from that of SR-A, thus suggesting that the regulation of receptor functions by the cytoplasmic tail is likely to be different. In order to predict whether there were potential sequence motifs in the cytoplasmic tail of MARCO, the ELM (Eukaryotic Linear Motif) program (Dinkel *et al.* 2012) was used. Residues 10-13 (DELL) were identified as a potential dileucine motif. Since these residues have been demonstrated to be required for SR-A-mediated endocytosis (Chen *et al*. 2006), it is reasonable to predict

that it may also be important for the endocytic function of MARCO Fig 1.4 summarizes known amino acid sequences in the cytoplasmic tail that are important for SR-A function and the predicted sequences that might be important for MARCO function.

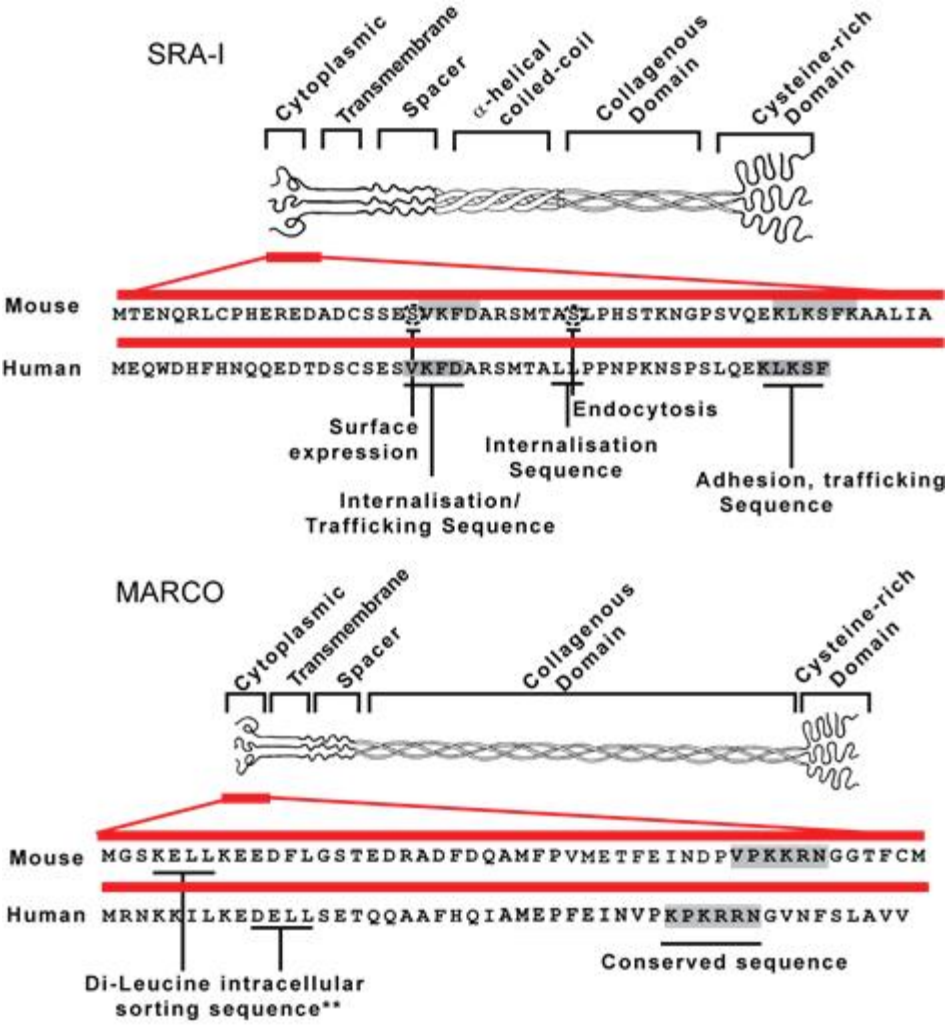


Fig 1.4 Summary of amino acid sequences of the cytoplasmic domain of human SR-A and MARCO that contribute to receptor functions.

The VXFD motif was shown to be responsible for efficient internalisation and efficient cell surface expression of SR-A. In addition, Val28 appeared to be needed for internalisation and Lys22, for cell surface expression (Morimoto *et al.* 1999). These sites have not been found in human MARCO. Ser21 and Ser49 was shown to be important for SR-A surface expression and AcLDLendocytosis, respectively (Fong and Le 1999). Kosswig and colleagues showed that the six membrane proximal amino acids are essential for cell surface expression (Kosswig *et al.* 2003). It is shown that in human SR-A the di-leucine motif contributes to SR-A-mediated endocytosis. (Chen *et al.* 2006). In the cytoplasmic tail of MARCO the amino acid sequences highlighted are putative, as their functions have not been experimentally proven.

1.3.2.2 Domain II : the transmembrane domain

Domain II of MARCO contains twenty four transmembrane hydrophobic amino acids. These amino acids are thought to be important for membrane anchoring. (Elomaa *et al.* 1995).

1.3.3.3 Domain III: the spacer domain

Domain III of MARCO is a 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 formation of disulphide bonds between monomers (Elomaa *et al.* 1995).

1.3.3.4 Domain IV: the collagenous domain

The collagenous domain of MARCO consists of 270 amino acids that form 89 G-X-Y triplets that are interrupted with A-E-K sequences. The A-E-K sequence is thought to be the hinge region in the triple helix (Elomaa *et al.* 1995). It should be noted that all collagenous transmembrane proteins are type II proteins, presumably due to the positively charged residues in the collagenous domain. In SR-A, it has been shown that collagenous domain is the ligand binding domain for a variety of ligands such as Lipid A, LPS and CpG-DNA (de Wet and Gordon 2001).

1.3.3.5 Domain V: the cysteine-rich domain

The cysteine-rich domain consists of about 101 amino acids and contains six cysteine residues that form intrachain disulfide bonds (Kraal *et al.* 2000). It is a conserved domain

and can be found in SR-AI. Unlike SR-A, it has been shown that the bacterial binding region of MARCO was located proximal to the cysteine-rich region of the COOH-terminal of the protein (Elomaa *et al.* 1998).

The crystal structure of the monomeric form of the cysteine-rich domain of the mouse receptor has been determined. In this study, it was found that the SRCR domain of MARCO contains a twisted five-stranded antiparallel β-sheet and a long loop covering a single α-helix. It has a dipolar charge distribution. The β-sheet region, which contains several arginines, forms a basic cluster. Dimerization brings the positively charged clusters together, while negative clusters point in separate directions. Therefore, it is proposed that the positively charged cluster in the SRCR domain of MARCO has enabled MARCO to bind to polyanionic ligands (Ojala *et al.* 2007).

1.4 Endocytosis

One important feature of the macrophage is its ability to bind and internalize particles such as bacterial pathogens, apoptotic cells, AcLDL and environmental particles. In fact, "phagocyte" was coined by the Russian zoologist Ilya Metchnikoff, who collected a minute, transparent starfish larva and pierced it with a thorn from a rose. In response, tiny amoeboid cells covered the thorn in an attempt to ingest the invading menace. He hypothesized that these cells with phagocytic ability were capable of ingestion and might play a key in host defence and tissue homeostasis (Tauber 2003). In the mid-1950s,

researchers made the observation that large molecules and particles are internalized in vesicles derived from the invagination and pinching-off of segments of the plasma membrane in a process they termed endocytosis. Endocytosis also controls the protein and lipid composition of the plasma membrane. Therefore, endocytosis is involved in the regulation of different cellular processes, such as antigen presentation, mitosis, cell adhesion and migration (Tauber 2003). Endocytosis is an extremely complicated process that can be subdivided into ten categories such as clathrin-mediated endocytosis, caeveolae-dependent endocytosis, phagocytosis and macropinocytosis and six other pathways summarized in Table 1.2. Phagocytosis is a process by which macrophages ingest particles with a nominal diameter of more than 0.5 μm. Bacteria, apoptotic cells, cell debris and environmental dust are all members of this category (Russell 2001). During phagocytosis, internalization is initiated by cell surface specific receptors interacting with their cognate ligand on the particles. The particles are then internalized after actin has polymerized at the site of receptor binding (Huynh *et al.* 2007). Actin is then shed from the phagosome, and the phagosome matures as the pH drops to form the phagolysosome. Consequently, pathogens are destroyed in the phagolysosome (Aderem and Underhill 1999; Groves *et al.* 2008).

Alternatively, endocytosis is characterized by internalizing particles smaller than 0.5 μm. Small particles and macromolecules are members of this category. Like phagocytosis, endocytosis is also receptor-mediated and the two most common pathways are: the clathrin-dependent and the caveolae-dependent pathways. The subsequent internalization mechanism is similar to that of phagocytosis. In contrast, pinocytosis is usually not

receptor-mediated, and is the random sampling of the extracellular matrix and occurs by random invagination and pinching-off from the highly ruffled region of the plasma membrane (Mellman 1996). Fig 1.5 shows a schematic representation of the major endocytic pathways.

Table 1.2 Summary of the molecular characteristics of endocytic pathways. (Doherty and McMahon 2009)

Endocytic mechanisms	Morphology	Implicated cargoes[b]	Small G-protein dependence	Dynamin implicated?	Other proteins implicated
Clathrin mediated	Vesicular	RTKs, GPCRs, transferrin receptor, anthrax toxin	Rab5, Arf6 implicated	Well established	Clathrin, AP2, epsin, SNX9, synaptojanin, actin amphiphysin, plus many others
Caveolae-/ caveolin1-dependent	Vesicular/ tubulovesicular	CTxB, SV40, GPI-linked proteins	Unclear (cavelolins may regulate cdc42 activity)	Some evidence	Caveolins, PTRF, src, PKC, actin (many signaling proteins localize to these sites)
CLIC/GEEC	Tubular/ring like	Fluid phase markers, CTxB, GPI-linked proteins	Cdc42, Arf1	Not as yet	ARHGAP10, actin, GRAF1, other GRAFs
IL2Rβ pathway	Vesicular?	IL2Rβ, FCεRI, Kir3.4, γc-cytokine receptor	RhoA, Rac1	Implicated	PAK1, PAK2
Arf6 dependent	Vesicular/tubular	MHC class I proteins, CD59, carboxypeptidase E	Arf6	Not as yet	Unclear as yet
Flotillin dependent	Vesicular	CTxB, CD59, proteoglycans	Unclear	Implicated but unclear	Flotillin 1 and 2
Phagocytosis	Cargo shaped	Pathogens, apoptotic remnants	Arf6/cdc42/rac1/rhoA (depending on type)	Implicated	Actin, IQGAP1, amphiphysin1, Rho kinase, adhesion proteins
Macropinocytosis	Highly ruffled	Fluid phase markers, RTKs	Rac1	Not as yet (CtBP1/BARS implicated in scission)	Actin, PAK1, PI3K, Ras, Src, HDAC6
Circular dorsal ruffles	Highly ruffled	Fluid phase markers, RTKs	Unclear	Implicated	Cortactin, actin
Entosis	Cell shaped	Matrix-deligated cells	RhoA	Not as yet	Adherens junctions

Abbreviations: CLIC, clathrin-independent carrier; GEEC, GPI-AP enriched early endosomal compartment; GPCRs, G protein–coupled receptors; GPI, glycosylphosphatidylinositol; MHC, major histocompatibility complex; RTK, receptor tyrosine kinase.

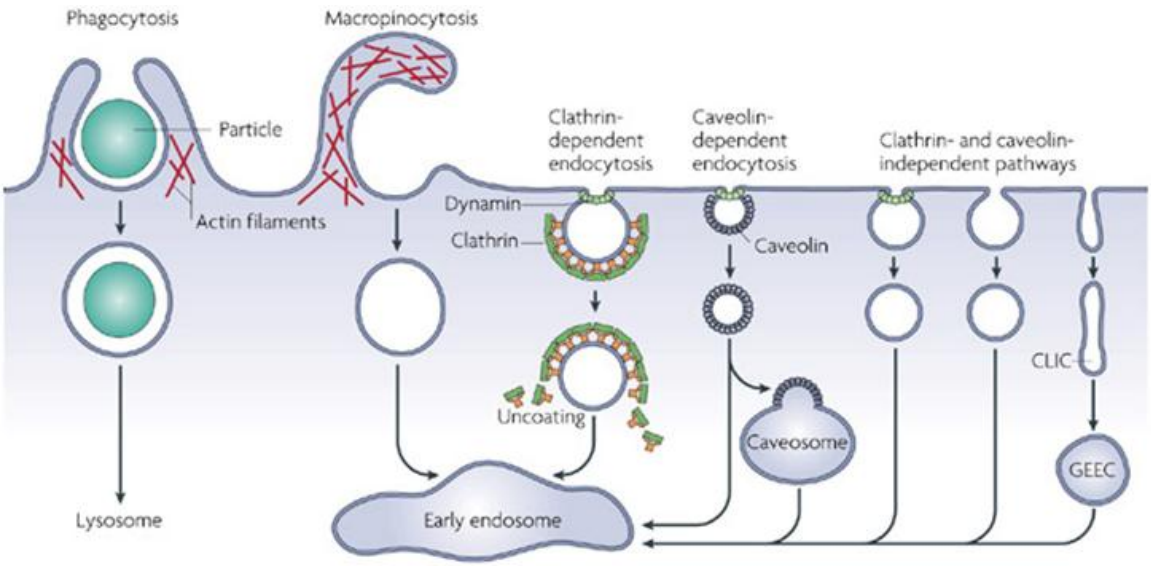


Fig 1.5 Schematic representation of the major endocytic pathways

Phagocytosis is responsible for the uptake of large particles, whereas fluid uptake occurs via macropinocytosis. Both processes are triggered by actin-dependent remodelling of the plasma membrane. Smaller particles can be taken up by both clathrin-dependent and clathrin-independent mechanisms that may or may not require the large GTPase, dynamin (Mayor and Pagano 2007)

1.4.1 Clathrin-mediated endocytosis

The clathrin-mediated endocytosis (CME) is the best-characterized endocytic pathway to date. It features the recruitment of soluble clathrin from the cytoplasm to the plasma membrane. The clathrin treskelion consistis of three clathrin heavy chains and three clathrin light chains which assemble into a polygonal lattice at the plasma membrane to form coated pits that bud and pinch off from the membrane in a dynamin-dependent manner and give rise to clathrin-coated vesicles (CCVs) (Le Roy and Wrana 2005). Clathrin-binding adaptors, such as adaptor protein-2 (AP-2), bind to clathrin as well as the cargo proteins during this process. Cargo receptors bind to AP-2 via AP-2 binding sites in their cytoplasmic domain of cargo receptors. The tyrosine- and di-leucine-based motifs are such recognition sequences by AP-2 (Nesterov *et al.* 1999). Cargo receptor monoubiquitylation is required for receptor endocytosis, as in the case of epidermal growth factor receptor (EPGR) internalization (Polo 2002), in which monobubiquitylated receptors form a complex with the adaptor proteins to promote clathrin-coated pits formation. In addition to adaptor proteins, phospholipids like phosphatidylinositol-4,5-bisphosphate are also found in the clathrin-coated pits to facilitate vesicle formation and budding by binding to clathrin adaptors such as epsins (Legendre-Guillemin *et al.* 2004). Following endocytosis of cargo proteins, clathrin-coated vesicles are uncoated to fuse with the early endosome. The early endosome is highly enriched in phosphatidylinositol-3-phosphate (PtdIns3P) and has unique protein components such as the PtdIns3P-binding 'Fab1, YOTB, Vac1, EEA1' (FYVE)-domain proteins (Doherty and McMahon 2009) that can bind to the cargo protein and determine their intracellular destinations. Therefore the

early endosome is a key control point for receptor trafficking. Receptors can be directed to Rab11-positive recycling endosomes and back to the cell surface. Alternatively, they can be directed to the intralumenal vesicle of multivesicular endosomes, and, therefore to the multivesicular body, late endosome and lysosome for degradation (Madshus 2006). Polyubiquitylation of receptors in the endosomes might serves as important signals for targeted-degradation (Katzmann *et al.* 2001). Transferrin is the best-characterized ligand for clathrin-mediated endocytosis, and it is often used as the model ligand to study or control for the clathrin-mediated endocytosis.

1.4.2 Clathrin-independent endocytosis

The majority of the clathrin-independent endocytic pathways are caveolae-dependent. They can be sub-categorized into two major pathways by their dependency for dynamin: dynamin-dependent and dynamin-independent.

Caveolae-mediated endocytosis is the best-characterized dynamin-dependent clathrin-independent pathway. It is characterized by the presence of caveolae, 50–80 nm flask-shaped plasma membrane invaginations that express caveolin family proteins (Mineo and Anderson 2001). These structures are enriched in sphingolipids and cholesterol (Simons and Gerl 2010), signalling proteins and clustered glycosyl phosphatidylinositol-anchored proteins (Sprenger 2004). Therefore, unlike clathrin-mediated endocytosis, the cargo is diverse in caveolae-mediated endocytosis. The downstream pathway is poorly understood. Evidence suggests that certain downstream pathways are dependent on the

small GTPase RhoA (Lamaze 2001). This was identified while studying the pathway that is responsible for internalizing the β-chain of the interleukin-2 receptor. It was found that neither dominant negative inhibitors of clathrin polymerization, nor clathrin assembly, nor adaptor recruitment affected endocytosis of IL-2R. In contrast, uptake was inhibited by DN dynamin and RhoA. Since RhoA plays a critical role in the regulation of actin cytoskeleton dynamics, it might be essential for the recruitment of the actin machinery to regulate endocytosis via this pathway.

The clathrin-independent and dynamin-independent endocytic pathway has been found at non-permissive temperatures in *Drosophila melanogaster* haemocytes carrying a temperature-sensitive mutation in the GTPase domain of the protein that is encoded by shibire, the dynamin gene in *D.melanogaster*. The common feature of constitutive dynamin-independent, clathrin-independent endocytosis is the use of small GTPases, either the Rho family member CDC42 or the Arf family member ARF6. (Chadda 2007)

The primary carriers that bud from the cell surface by CDC42-regulated endocytosis have long and relatively wide surface invaginations, and consequently a large volume of fluid phase is co-internalized in a single budding event compared with these other mechanisms of internalization (Fivaz 2002). In fact CDC42 is also involved in regulating macropinocytosis and phagocytosis (Garrett 2000). Additionally, it has been found that cholesterol-sensitive CDC42 activation is linked to the recruitment of an actin-polymerization machinery.

A role for the Arf family GTPase ARF6 is not fully understood. It is established that ARF6 has a potent role in actin remodelling, stimulates the formation of phosphatidylinositol 4,5-bisphosphate by activating phosphatidylinositol 4-phosphate-5-kinase (D'Souza-Schorey and Chavrier 2006).

Cholera toxin subunit B (CTxB) and Simian virus 40 (SV40) are the best-characterized ligands for caveolae-dependent endocytosis. They are often used as the control ligands to study caveolae-dependent endocytosis. Fig 1.6 depicts the caveolae-dependent endocytosis.

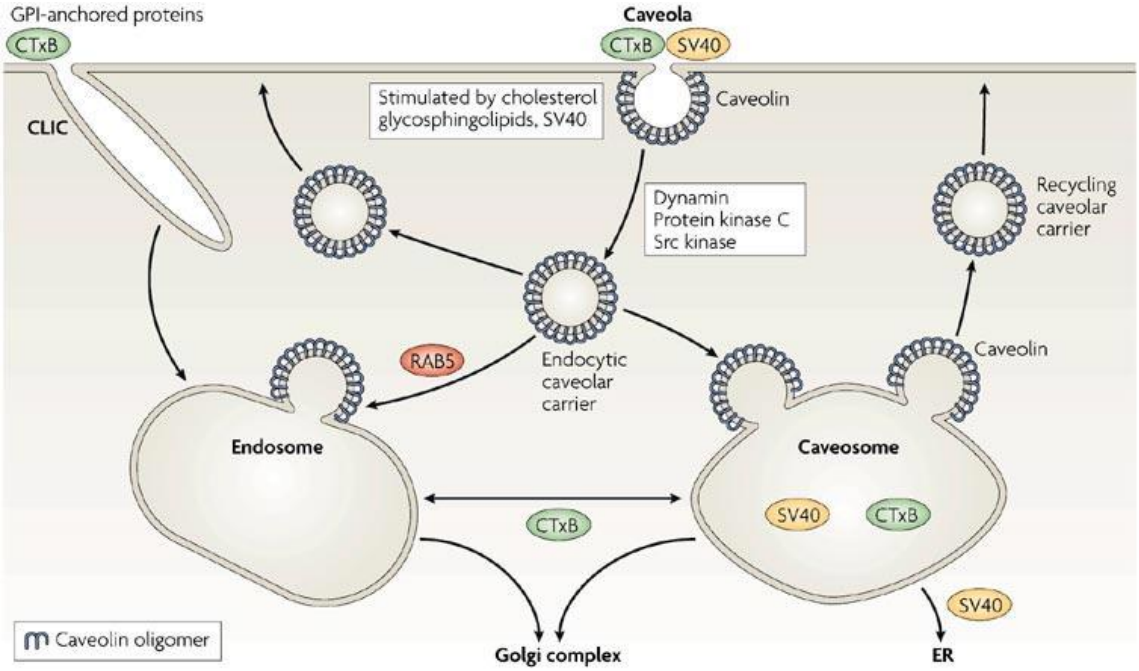


Fig 1.6 Caveolae dependent endocytosis

Caveolae is located at the cell surface. In a dynamin-dependent process, it can bud into the cell carrying CTxB and SV40. This internalization process is also regulated by protein kinase C and tyrosine kinases. Endocytic caveolar can fuse with the caveosome or with the early endosome, or can fuse back to the plasma membrane. SV40 is transported from the caveosome to the ER and caveoline is recycled back to the plasma membrane in the caveolar carrier. CTxB travels via early endosomes to the Golgi apparatus. (Parton and Simons 2007)

1.4.3 Phagocytosis

Phagocytosis is classically defined as a mechanism for internalizing and destroying particles greater than 0.5 μm in size. It is mediated by the receptors on the cell surface and is actively driven by actin. Phagocytosis can be either non-opsonic or opsonic depending on whether the binding of the receptor to the ligand requires an opsonin, proteins that enhances phagocytosis, such as complement and antibodies (Groves *et al.* 2008).

Fc receptor-mediated phagocytosis is the best-characterized opsonic phagocytic pathway. Following the engagement of particles that have been opsonized with IgG by FcRs on phagocyte membranes, a phagosome is formed by a zipper-like advance of the membrane and the cytoskeleton over particle surfaces. Binding to IgG induces and conformational changes in the cytoplasmic domains of Fc receptors such that these domains recruit or activate proteins that signal cellular movements by actin polymerization and the extension of membrane over the opsonized surface. The phagocytic cup extends over the particle by sequential local responses to the ligand-coated surface, eventually engulfing particles covered with IgG (Swanson 2008).

Alternatively, some receptors do not require an opsonin and directly bind to the particle to mediate phagocytosis. These non-opsonic receptors include the mannose receptor and the Class A scavenger receptors. The role of Class A scavenger receptor in phagocytosis and endocytosis will be discussed in Section 1.5.

1.5 Class A scavenger receptor mediated endocytosis and phagocytosis

As discussed in Section 1.3, MARCO and SR-A have very similar ligand binding properties. They can recognize and phagocytose/endocytose a variety of materials such as LPS and LTA, intact bacteria, apoptotic cells, modified LDL and environmental particles (Pluddemann *et al.* 2006; Pluddemann *et al.* 2007).

In vitro studies on the phagocytosis of apoptotic thymocytes by thymus-derived macrophages revealed that SR-A phagocytoses apoptotic cells. In this study, it was shown that the internalization of the thymocytes could be substantially inhibited with a monoclonal antibody to SR-A or by polyanionic ligands. Furthermore, phagocytosis of thymocytes was inhibited 50% in thymic macrophages derived from SR-A knockout mice (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). MARCO was also shown to recognize apoptotic B cells in the marginal zone of the spleen (Wermeling *et al.* 2007).

In addition to the clearance of apoptotic cells, SR-A- and MARCO-mediated phagocytosis also plays an important role in host defense. Both receptors have shown to mediate phagocytosis of Gram-positive and –negative bacteria. For example, SR-A has been shown to recognize the Gram-negative bacteria *Neisseria meningitides* independently of LPS (Peiser *et al.* 2002). MARCO is also a receptor for *Neisseria*. (Mukhopadhyay *et al.* 2006). In an *in vivo* mouse model, MARCO and SR-A recognize different *Neisseria* cell surface antigens (Plüddemann *et al.* 2008), which indicates that a

fine specificity in ligand recognition exists between those two class A scavenger receptors. For example, recently MARCO was shown to be the phagocytic receptor for unopsonized particles and bacteria in human aveolar macrophages (Arredouani *et al.* 2005). Thus, it was proposed that MARCO plays a very important role in aveolar macrophage defence against inhaled particles and pathogens. Furthermore, it was revealed that MARCO, and to a lesser extent SR-A, is the main receptor for mycobacterial trehalose 6,6'-dimycolate (TDM), the immunogenic lipid component of the *Mycobacterium tuberculosis* cell wall (Bowdish *et al.* 2009). These experiments demonstrated that MARCO was required for the activation of Toll-like receptor 2 and CD14 to form a functional receptor complex for TDM (Bowdish *et al.* 2009). In the same study, the authors also determined that the cytoplasmic tail of MARCO is dispensable for MARCO-induced TDM binding and subsequent signal activation. However, no actual mechanism of phagocytosis was investigated in these studies. Several attempts were made to determine the factors that are involved in the endocytosis mediated by the Class A scavenger receptors.

Zhu and colleagues demonstrated that in a human macrophage cell line, THP-1, SR-A-mediated endocytosis of AcLDL was primarily clathrin-mediated and elicited ERK signalling. SR-A-mediated uptake of fucoidan requires caveolae-dependent endocytosis and p38 and JNK pathways are subsequently activated (Zhu *et al.* 2011). In a previous study by this group, they found that the di-leucine motif in the cytoplasmic domain of SR-A was required for AcLDL endocytosis (Chen *et al.* 2006). Additionally, with the use of pharmacological inhibitors, DeWitte-Orr and colleagues revealed that SR-A mediated

viral double-stranded RNA entry into the cell is clathrin-dependent and might involve actin (DeWitte-Orr *et al.* 2010).

To date, almost nothing is published regarding the mechanism of MARCO-mediated endocytosis.

Hirano and Kanno showed that, by transiently expressing mouse MARCO in CHO-K1 cells, MARCO-expressing cells are capable of internalizing polystyrene particles that are 20 nm and 200 nm in size. Actin does not seem to be actively involved in the internalization process, as cytochalasin D, a potent actin depolymerizing agent, did not perturb ligand uptake (Kanno *et al.* 2007). They also showed that MARCO is capable of binding multi-walled carbon nanotubes, a promising nanomaterial, via membrane ruffling, a mechanism similar to macropinocytosis (Hirano *et al.* 2012). However, they did not investigate the potential importance of the cytoplasmic domain of MARCO in ligand uptake.

1.6 Hypothesis and the aim of this thesis

Although MARCO and SR-A have a similar ligand binding capacity, they differ in ligand specificities and their regulation (Plüddemann *et al.* 2008). SR-A has received much more attention. The functions of the domains of SR-A are relatively well-characterized. Little is known about the function of these domains in MARCO, especially the cytoplasmic domain. In SR-A, it was shown that the cytoplasmic domain is important for receptor

endocytosis, trafficking and surface expression. Yet no research is published regarding the same aspects in MARCO. As discussed in Section 1.5, another outstanding question is how MARCO mediates ligand uptake and if any regions of MARCO are important for this process.

I hypothesised that the cytoplasmic domain of MARCO is important for cell surface expression, trafficking and endocytosis. I also sought to determine the molecular components of the MARCO-mediated endocytic pathway.

Chapter Three investigates the role of the cytoplasmic tail of MARCO in MARCO surface expression and trafficking. In Chapter Four, we describe the mechanism of MARCO-mediated endocytosis. The aim of Chapter Five was to characterize the role of the cytoplasmic tail of MARCO in endocytosis. Chapter Six summarizes the results and the implications of this study are further discussed.

2 Chapter Two: Materials and methods

2.1 Materials

2.1.1 Antibodies

Rabbit polyclonal anti-human MARCO antibodies called 9805 were generated at McMaster Central Animal Facility by immunizing female New Zealand White (NZW) rabbits purchased from Charles River (Wilmington, MA) with a synthetic MARCO peptide ordered from Genscript (Piscataway, NJ) described in Section 2.2.1. 9805 is the ID of the rabbit that was immunized with human MARCO peptide. Secondary detection was performed with R-phycoerythrin (RPE) and allophycocyanin (APC)-AffiniPure $F(ab')_2$ Fragment goat anti rabbit secondary antibodies that were purchased from Jackson Immunoresearch (West Grove, PA). Alexa488 goat anti-rabbit $F(ab')_2$ secondary antibody was purchased from Invitrogen (Burlington, ON). Normal rabbit IgG, an isotype control was obtained from Calbiochem (San Diego, CA).

2.1.2 Ligands and related reagents

Alexa488 conjugated human transferrin and Alexa 488-conjugated cholera toxin subunit B were acquired from Invitrogen. Fluoresbrite Plain YG 0.5 and 0.2 micron microspheres were obtained from Polysciences (Warrington, PA). Bovine Serum Albumin (BSA) Fraction V was purchased from Calbiochem. Maleic anhydride was purchased from Sigma.

2.1.3 Molecular cloning reagents

MARCO constructs that contained truncated cytoplasmic regions were created by PCR amplification followed by restriction enzyme digest and ligation with vectors followed by transformation. Briefly, primers that amplify hMARCO and mutants were ordered from IDT (Toronto, ON). Table 2.1 summarizes the primers used in this study. KOD Hot Start Polymerase was purchased from Novagen (San Diego, CA). DNA was amplified with an Eppendorf Pro S Mastercycler (Mississauga, ON). Restriction enzymes HindIII and XbaI were obtained from Fermentas (Burlington, ON), FspI that is used to linearize DNA before stable transfection was purchased from NEB (Pickering, ON). T4 Ligase was purchased from NEB. Chemically competent DH5α *E. coli* cells were obtained from Invitrogen. DNA mini-prep kit was purchased from Bio Basics. DNA midi-prep kit was purchased from Invitrogen. The empty vectors pCDNA3.1/Hygro(+)was a kind gift from Dr. Peter Pelka (University of Manitoba). The plasmid containing full-length human MARCO was a kind gift from Dr. Timo Pikkarainen (Karolinska Institutet). Full-length hMARCO- pCDNA3.1/Hygro(+)and the mutant Δ1-34hMARCO pCDNA3.1/Hygro(+) were generated as described in Section 2.2.2.

Table 2.1 Summary of the primers used to contruct MARCO mutants

Sequence Name	Sequence (5'→3')	Restriction Enzymes
hMARCO-Forward	ACT GTT AAG CTT ACC ATG AGA AAT AAG AAA ATT CTC	HindIII
hMARCO-Reverse	ACT GTT TCT AGA TCA GAC GCT GCA CTC CAC GC	XbaI
hMARCOΔ1-34-Forward	ACT GTT AAG CTT ACC ATG AAG CCC AAG AGG AGA AAT G	HindIII

2.1.4 Pharmacological inhibitors

Table 2.1 shows the inhibitors tested. Pitstop2 was obtained from Abcam (Toronto, ON), and all the rest on the list were obtained from Sigma (Oakville, ON). Optimal dose concentrations of drugs were determined by titration.

Table 2.2: Summary of Inhibitors Tested.

Inhibitors	Target / Mechanism	Final Concentration	Vehicle
Chlorpromazine	Reversibly translocates clathrin and adaptor proteins to intracellular vesicles; inhibit clathrin-mediated endocytosis	28.2 μM	H_2O (Orlandi and Fishman 1998)
Valinomycin	Promotes K+ efflux from cells, thus inhibit clathrin-mediated endocytosis	10 μM	DMSO (Lee *et al.* 2009)
Pitstop2	Binds terminal domain of clathrin to inhibit clathrin-mediated endocytosis	15 μM	DMSO (von Kleist *et al.* 2011)
Dynasore	Act on the GTPase domain of dynamin to inhibit GTP hydrolysis	80 μM	DMSO (Macia 2006)
Cytochalasin D	Depolymerize Filamentous actin	5 μg/mL	PBS (Sulahian *et al.* 2008)

2.1.5 Other reagents

Sterile phosphate-buffered saline (PBS), Dulbecco's Modified Eagle Medium (DMEM), L-glutmine, penicillin/ streptomycin (pen/strep) and fetal bovine serum (FBS) were supplied by McMaster Immunology Research Centre (MIRC). Opti-MEM was obtained from Invitrogen. HEK293T cells were a generous gift from Dr. Brian Lichty.

2.2 Methods

2.2.1 General culture conditions for mammalian cells

The human embryonic kidney 293T (HEK293T) cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Human MARCO-expressing stable HEK 293T cells were additionally supplemented with 350μg/ml hygromycin B. Cells were incubated at 37 ℃ in a 5% CO2, 95% humidity incubator. Cells were detached by incubation with 4 mg/ml lidocaine in cold PBS for 5 minutes. The cells were routinely sub-cultured 1:10 twice weekly and only cells with low passage numbers ($p<10$) were used in all the experiments.

2.2.2 Cloning of hMARCO and related constructs

Human MARCO mutants were cloned into pCDNA3.1/Hygro(+) vector. Briefly, primers were designed with sequences such as restriction enzymes recognition sites, HindIII and XbaI, and start codon including a Kozak sequence, stop codon and protective

sequences. HMARCO gene was PCR-amplified from plasmids containing full-length human MARCO. PCR products (inserts) and empty vectors were digested with HindIII and XbaI followed by gel purification. The concentration of both inserts and vectors was determined by a Nanodrop spectrophotometer from Thermo Scientific (Mississauga, ON). Purified inserts and vectors were mixed at a molar ratio of 3:1 with NEB T4 ligase and buffer at 16 ℃ overnight. The ligation reaction was transformed into chemically competent DH5α *E. coli* cells, and the transformed cells were plated on to LB agar purchased from Invitrogen with the addition of antibiotics. DNA was extracted from the cultures of each colony. The sequences of all constructs were validated by sequencing performed by MOBIX at McMaster University.

2.2.3 Transient and stable transfection of HEK293T cells

HEK293T cells were transfected with GeneJuice from Novagen as *per* manufactuer's protocol. Briefly, HEK293T cells were seeded into 6-well tissue culture plates at a density of 3 x 10^5 cells/well in 3 mL of DMEM overnight. The following day cells were transfected with 3 μL GenJuice per 1 μg of DNA. Cells were incubated with fresh media 5 hours after transfection and were used for analysis 24 hours after transfection. In the case of stable transfection with hMARCO-pCDNA3.1/Hygro(+), plasmids were linearized with FspI before transfection. Cells were transferred to a T175 flask from BD Biosciences (Mississauga, ON) and incubated with 350 μg/mL Hygromycin B 48 hours after transfection. The incubation of transfected cells with Hygromycin B was allowed to continue for approximately two weeks until MARCO expression was stable.

2.2.4 Rabbit anti hMARCO polyclonal antibody production and purification

A peptide, GHHDCSHEEDAGVECSV, from the C-terminus of human MARCO was selected and synthesized with KY residues added to the N-terminus to facilitate conjugation to the carrier proteins. The peptide was then cross-linked to carrier protein keyhole limpet hemocyanin (KLH) via the side chains of their tyrosine residues with bisdiazonium-benzidine (BDB) generously provided by Dr. Peter Whyte (McMaster University). Basic local alignment search tool (BLAST) searches were performed to ensure the peptide sequence is not present in any other human proteins to prevent cross-reactivity. Briefly, the coupling reaction was carried out by mixing 5 mg of synthetic peptide in 0.5 ml of 0.2 M sodium borate buffer and with 0.5 ml of KLH (8 mg/ml) in borate buffer at 4℃ with 100 µL BDB for two hours with gentle rocking. Two NZW rabbits were injected subcutaneously with 400 micrograms of peptide-KLH emulsified with complete Freund's adjuvant followed by 6 subsequent immunizations subcutaneously with 400 micrograms of peptide-KLH emulsified with incomplete Freund's adjuvant. Serum from the rabbit was diluted in PBS 1:1 and purified by affinity chromatography with MARCO peptide-conjugated Affigel 10 (Bio-Rad, Mississauga, ON) column. Dialysis was performed to eliminate impurities. Concentrations of the purified antibody were determined by Nanodrop. Antibody titers were tested by flow cytometry. All animal work was approved by the McMaster Animal Research Ethics Board.

2.2.5 Maleylation of bovine serum albumin (BSA)

Maleylation of BSA was achieved by adding small amounts of maleic anhydride (final concentration 0.1 M) to BSA (10 mg/ml) to the reaction buffer (0. 2M Na2CO3, pH=8.5). The pH of the reaction was readjusted by adding 5M NaOH to maintain the reaction pH above 7.5. Protein aliquots were stored at -20 ℃. To determine whether maleylation was successful, the proteins were visualised by silver staining detailed in Section 2.2.10 as shown in Fig 2.1. Briefly, 500 µg of proteins were loaded on a 12% SDS-PAGE gel and ran for 40 minutes at 150 Volts followed by silver staining.

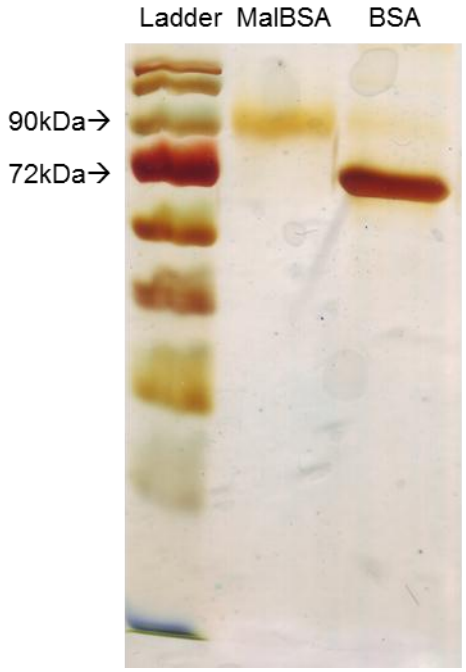


Figure 2.1 Silver staining of MalBSA and BSA 500 µg of proteins were loaded on a 12% SDS-PAGE gel and ran for 40 minutes at 150 Volts and the proteins were visualized by silver staining. The molecular weight of MalBSA is higher than BSA due to covalent modification.

2.2.6 Alexa488 labeling of MalBSA and BSA and coating of beads

MalBSA or BSA was dialyzed against 20mM PBS with three buffer changes (pH 7.4) and quantified by the Bradford assay. Proteins were reduced by a 10-fold molar excess of dithiothreitol (DTT). Reduced proteins were immediately purified via size exclusion chromatography by a desalting column PD-10 from GE healthcare (Mississauga, ON) and quantitated by Bradford assay. AlexaFluor®488 C5 maleimide were added to reduced protein solutions at a molar ratio of 10:1 and the reaction was allowed to proceed in the dark for two hours with gentle rocking. Upon completion of the reaction, protein conjugates and unincorporated dyes were separated by size exclusion chromatography. Successfully labelled proteins were aliquoted and stored in -20 ℃.

Additionally, fluorescent polystyrene latex microspheres from Polysciences (Warrington, PA) were passively coated with either MalBSA or normal BSA as a control, by incubating overnight at room temperature in 5 mg of BSA or MalBSA in 0.1 M borate buffer, pH 8.5 with gentle rocking. In both cases, the beads were centrifuged and re-suspended in 1 ml of PBS. The number of beads in the suspension was calculated as per manufacturer's protocol.

2.2.7 Endocytosis assay

Stable MARCO-expressing HEK293T cells were washed with PBS and re-suspended in serum-free media at a density of 1 x 10^6 cells/ml. To determine the cell association of ligand and ligand binding, cells were subsequently incubated with AF488-MalBSA,

MalBSA-coated 200 nm beads or MalBSA-coated 500 nm beads with gentle rocking on a nutating mixer from VWR (Mississauga, ON) at 37 ℃ or 4 ℃ for 90 minutes, after which time interval they were washed and fixed for 20 min in 4% (w/v) paraformaldehyde (PFA) at 37 ℃. The cells were analyzed on a fluorescence plate reader from Bio-Tek (Winooski, VT) or Canto from BD Biosciences (Mississauga, ON). For analysis, ligand uptake was expressed as the difference between cell association and ligand binding. The values were obtained from the plate reader by reading the fluorescence with Ex_{441nm}/Em_{486nm}. On the Canto, the fluorescence values were obtained with readings from the fluorescein isothiocyanate (FITC) channel, which is similar to Ex_{441nm}/Em_{486nm}.

2.2.8 Inhibition studies

For inhibition and specificity studies, 10^6 cell/mL HEK293T cells were incubated with the indicated concentration of inhibitors in serum-free media for the appropriate time with or without specific ligands. The data were quantitatively evaluated by flow cytometry and qualitatively by immunofluorescence microscopy.

2.2.9 Cytotoxicity Analysis

The cytotoxicity of different inhibitors was assessed with the CytoTox96 cytotoxicity assay kit purchased from Promega (San Luis Obispo, CA). This assay is a (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based test where tetrazolium salts are reduced into intensely coloured formazan derivatives by

mitochondrial enzymes, which are rapidly inactivated within a few minutes after cell death. The assay was performed per manufactuer's protocol. In brief, cells were seeded at 10^4 cells/well in a 96-well plate overnight. They were incubated in serum-free media with various concentrations of the inhibitors described above for 90 minutes. Subsequently, the cells were immediately incubated with the substrate containing the tetrazolium salts. After 3 hours at 37℃, the absorbance of the coloured formazan was measured on a BioTek plate reader (Winooski, VT).

2.2.10 SDS-Polyacryamide Gel Electrophoresis (SDS-PAGE)

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 5x SDS-PAGE loading buffer (312.5 mM Tris base, 10% glycerol, 11.5% SDS, with or without a reducing agent 500 mM DTT, bromophenol blue 0.1%, pH 6.8). Gels were cast and run using a Mini-PROTEAN III gel system from Bio-Rad (Mississauga, ON). Gels were electrophoresed at 150 mV per 1.0 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 10 to 250 kD (Precision plus protein kaleidoscope, Bio-Rad, Mississauga, ON).

Visualization of the proteins on the gel was performed by silver staining. The gel was soaked in the fixing solution containing 50% methanol, 40% double-distilled water (ddH_2O) and 10% acetic acid for 1 h. Next, it was washed 2x 10 min in 50% ethanol. The

gel was then pre-treated 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 3x 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, and 0.4 mg sodium thiosulfate) was added and the gel was agitated gently. When the bands seemed sufficiently bright, the fixing solution was added to stop the reaction.

2.2.11 Western Blotting

The proteins were transferred from SDS-PAGE gels to Immun-Blot PVDF membrane (Bio-Rad, Mississauga, ON) at 100 V for 60 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 at 4 ^oC in blocking buffer (4% fat-free milk powder, 0.1% Tween in TBS, Brampton, ON) for one hour and then incubated overnight in blocking buffer containing 1 μg/ml polyclonal rabbit anti human MARCO antibody 9805. The membrane was washed three times in 0.1% Tween in TBS, before incubation with horse radish peroxidise (HRP)-conjugated anti-rabbit secondary antibody (dilution 1:100,000) 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 purchased from GE Healthcare (Mississauga, ON) onto Kodak film (Etobicoke, ON).

2.2.12 Immunofluorescence Microscopy

Stable MARCO-expressing HEK293T were seeded into Lak-Tek II chamber slides (Thermo Fisher Scientific, Mississauga, ON) at 1x 10^5 cells per well in DMEM overnight. 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 the permeabilizing agent, 0.1% Triton-X100. After blocking, primary antibodies were 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, tetramethylrhodamine B isothiocyanate (TRITC)-labelled phalloidin were added to cells for 45 min. The chamber slides were washed with PBS and mounted in ProLong Gold antifade reagent with DAPI (Invitrogen, Burlington, ON). The images were captured by Zeiss fluorescence light microscope or Zeiss confocal laser scanning microscope model LSM510 (Carl Zeiss Microimaging, New York, NY). Z-stacks (two μm between scans) were taken of each field for five random fields.

2.2.13 Flow cytometry

Cells were harvested and fixed with 4% PFA for 20 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 1 μg/ml in blocking mixture. After 1 h the cells were washed twice in FACS wash solution, and appropriate

secondary antibodies were added (dilutions: 1:200 for PE/APC-conjugated goat anti-rabbit IgG and 1:1000 for Alexa488-conjugated goat anti-rabbit IgG). Finally, the cells were washed 4 times with FACS Wash solution and analysed on a FACSCanto (BD Biosciences, Burlington, ON). Data were analysed using FlowJo software (Treestar Inc, Ashland, OR).

2.2.14 Bioinformatic analysis of the putative motifs in the cytoplasmic tail of MARCO

The sequence of the cytoplasmic domain of MARCO was acquired from the National Center for Biotechnology Information. The accession number is NP_006761.1. The sequence of the cytoplasmic domain of MARCO (residues 1-49) was analyzed for potential signalling motifs using Eukaryotic Linear Motif (Dinkel *et al.* 2012), an online database with a comprehensive database of known experimentally validated motifs.

2.2.15 Statistical analysis

Experiments were performed in triplicate, unless noted otherwise. The Student's T-test was used to determine the differences between the means of samples. Differences were considered significant when $p \leq 0.05$. The statistical analysis was performed using Graphpad Prism (Graphpad Software, San Diego, CA).

3 Chapter 3: The role of the cytoplasmic tail in MARCO trafficking

3.1 Introduction

The studies in this chapter investigated which regions of MARCO are important for MARCO trafficking. It is evident that trafficking of scavenger receptors is crucial for their functions. For example, the long-tailed variant of CD163, which differs from the short-tailed variant only in the cytoplasmic domain, is primarily abundant in the Golgi apparatus and endosome whereas the short-tailed variants are mostly expressed on the cell surface. Consequently, the long-tailed variant display low endocytic activities (Nielsen *et al.* 2006). To date, it is poorly understood which regions of MARCO other than the transmembrane domain are important for cell surface expression and trafficking within the cell.

MARCO has a short N-terminal cytoplasmic tail consisting of 49 amino acids that is similar to SR-A (Elomaa *et al.* 1995). SR-A has been shown to require the cytoplasmic six membrane-proximal amino acids for cell surface expression (Kosswig *et al.* 2003). It is suggested that the conserved motif, VKFD, in the cytoplasmic tail of SRA is responsible for SRA internalization (Morimoto *et al.* 1999). I speculated that the cytoplasmic tail of MARCO might contain similar sequences that can affect trafficking of the protein and cell surface expression. Bioinformatic analysis revealed a putative di-leucine motif and conserved sequences in MARCO that might contribute to MARCO internalization and trafficking as shown in Fig 3.1. To investigate the role of the

cytoplasmic tail in MARCO trafficking, a MARCO cytoplasmic mutant was created as shown in Fig 3.1.

3.2 The aims of this chapter

The aims of this chapter are to determine whether the cytoplasmic tail of MARCO is required for MARCO trafficking, such as surface expression, internalization and recycling during natural receptor turnover without the presence of ligand and during endocytosis, in which the ligand is present. I hypothesized that the cytoplasmic tail of MARCO is important for MARCO sub-cellular localization. To test this hypothesis, I created the cytoplasmic mutant of hMARCO by truncating amino acids 1-34 in the cytoplasmic domain and stably expressing this construct in HEK293T cells. I then conducted specific assays on hMARCO and Δ1-34hMARCO cells to test the role of the cytoplasmic domain in MARCO trafficking.

3.3 Results

3.3.1 Creation of stable hMARCO and Δ1-34hMARCO HEK293T cells

To study whether the cytoplasmic domain of MARCO plays a role in MARCO trafficking and endocytosis, cytoplasmic mutants of MARCO were created for subsequent stable transfection into HEK293T cells. Stable transfection was preferred over transient transfection to reduce variability in MARCO expression. The HEK293T cell line was chosen due to its highly transfectable, non-phagocytic nature. RT-PCR and western

blotting were performed to eliminate the possibility that there might be endogenous MARCO expression in HEK293T cells.

Human MARCO mutants were cloned into the pCDNA3.1/Hygro(+) vector as shown in Figure 2.1. This vector was chosen because it contains a cytomegalovirus (CMV) promoter that can lead to sufficient expression of the protein of interest.

Figure 3.1 summarizes all the constructs made that were used in the subsequent studies. These included hMARCO and Δ1-34hMARCO- pCDNA3.1/Hygro(+). Δ1-34hMARCO lacked the putative di-leucine motif but retained the conserved sequence and other amino acids that might be important for MARCO trafficking or endocytosis. The entire cytoplasmic tail (Δ1-50hMARCO) was not truncated, because it is very likely that a Δ1-50hMARCO might be deficient in cell surface expression (Kroos 2011) ,and this expression deficit between hMARCO and Δ1-50hMARCO could introduce difficulties in data interpretation.

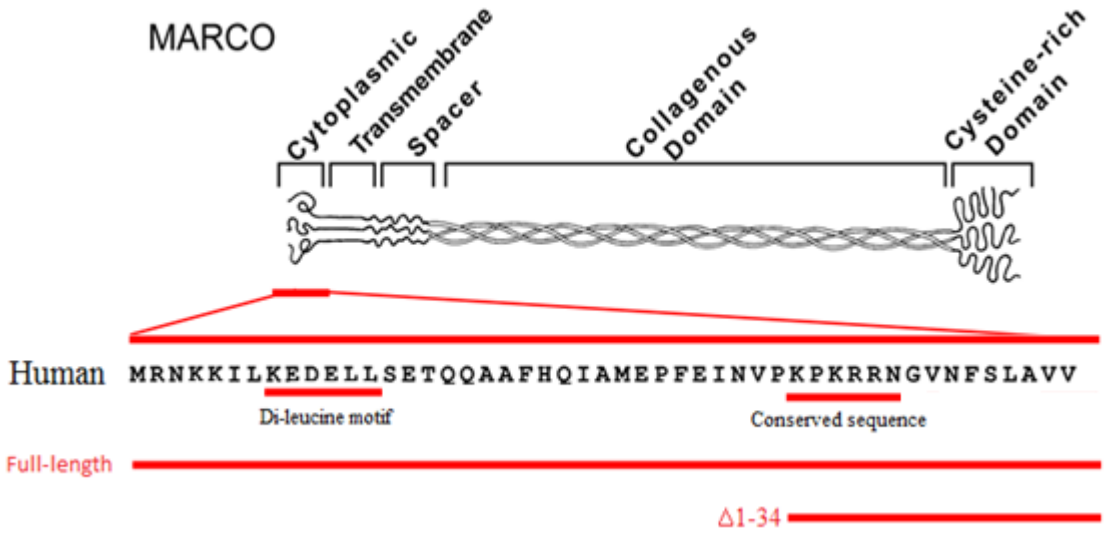
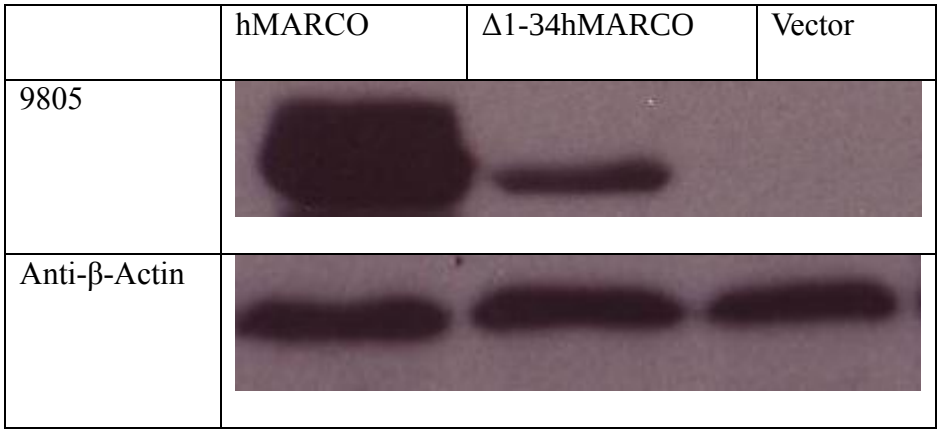


Fig 3.1 Summary of hMARCO constructs. Full-length hMARCO and Δ1-34hMARCO were constructed for this study. Δ1-34hMARCO lacked the conserved sequence but retained the putative di-leucine motif and other amino acids that might be important for MARCO trafficking or endocytosis.

3.3.2 The first thirty-four amino acids within the cytoplasmic tail of hMARCO are not necessary for MARCO surface expression

To test the hypothesis that the cytoplasmic domain of MARCO is necessary for cell surface expression, stably transfected HEK 293T cells that expressed either the full-length hMARCO or Δ1-34hMARCO were used. Western blotting on whole cell lysate showed that the total protein expression of hMARCO is greater than Δ1-34hMARCO (Fig 5.2 A). Flow cytometry analysis (Fig 5.2B) using the rabbit anti human MARCO antibodies 9805 revealed that both hMARCO and Δ1-34hMARCO showed a similar level of surface expression. These results indicated that at least the first thirty-four amino acids within the cytoplasmic tail of hMARCO were not necessary for MARCO surface expression. However, truncation of the first thirty-four amino acids seemed to reduce the intracellular pool of MARCO in Δ1-34hMARCO-HEK293T cells. The results also suggested that there seems to be more intracellular hMARCO than Δ1-34hMARCO.

(A)



(B)

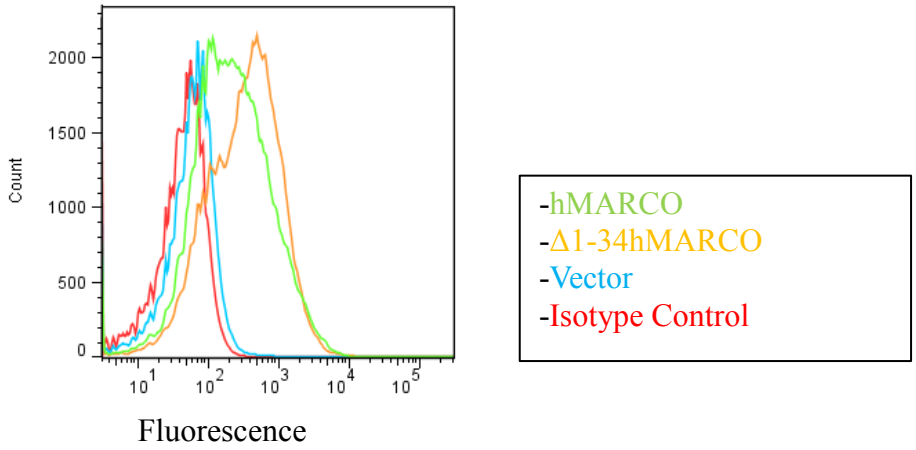


Fig 3.2 Expression of hMARCO and the cytoplasmic domain mutants in HEK 293T cells. **A.** Equal amount of hMARCO and mutant whole cell lysates were resolved on a non-reducing 12% SDS-PAGE gel and MARCO or β-actin, the loading control, expression was detected with primary rabbit anti-human MARCO antibodies 9805 or anti-β-actin antibody followed by the secondary anti-rabbit-HRP by western blotting. Lanes: 1. hMARCO; 2. Δ1-34hMARCO; 3. Vector control; hMARCO expression is higher than Δ1-34hMARCO and no band was detected in the vector control. **B.** Measurement of cell surface expression of MARCO in non-permeabilized transfected HEK293T cells by flow cytometry. MARCO expression was detected with rabbit anti human MARCO antibodies 9805 and an anti-rabbit APC secondary, normal rabbit IgG was used as an isotype control. The graph shown is representative of 3 experiments with cells that have different low passage numbers (P= 5, 7 and 8). The cytoplasmic domain mutants of MARCO and the full-length MARCO display a similar level of cell surface expression.

3.3.3 The first thirty-four amino acids within the cytoplasmic tail of hMARCO are not necessary for MARCO receptor internalization

To determine whether the first thirty-four amino acids are necessary for MARCO receptor internalization, I utilized monensin to block MARCO recycling in the absence of MARCO ligands, the fluorescently-labelled MalBSA-coated beads, first. Monensin is an ionphore that has a very high affinity to Na^+, therefore it mediates Na^+/H^+ exchange and consequently collapses the pH gradient of the membrane. It exerts its most profound effects on the trans cisternae of the Golgi apparatus stacks, which are important for the secretion of proteins (Mollenhauer *et al.* 1990). Therefore monensin blocks exocytosis and receptor recycling via the Golgi network. After monensin treatment, cells were immediately fixed on ice with 4% PFA and stained with rabbit anti-hMARCO antibody 9805 followed by a secondary APC-conjugated anti-rabbit antibody. The results were evaluated by flow cytometry. The data are expressed as the relative fluorescence intensity, with values at time 0 set to 1. No surface expression change was observed when monensin is absent. Monensin-induced MARCO internalization was observed with both the full-length MARCO and the cytoplasmic mutant Δ1-34hMARCO. However, there was no significant difference of surface MARCO expression at each time point between hMARCO and Δ1-34hMARCO HEK293T cells. Taken together, the first thirty-four amino acids are not required for MARCO internalization when the ligand is not present.

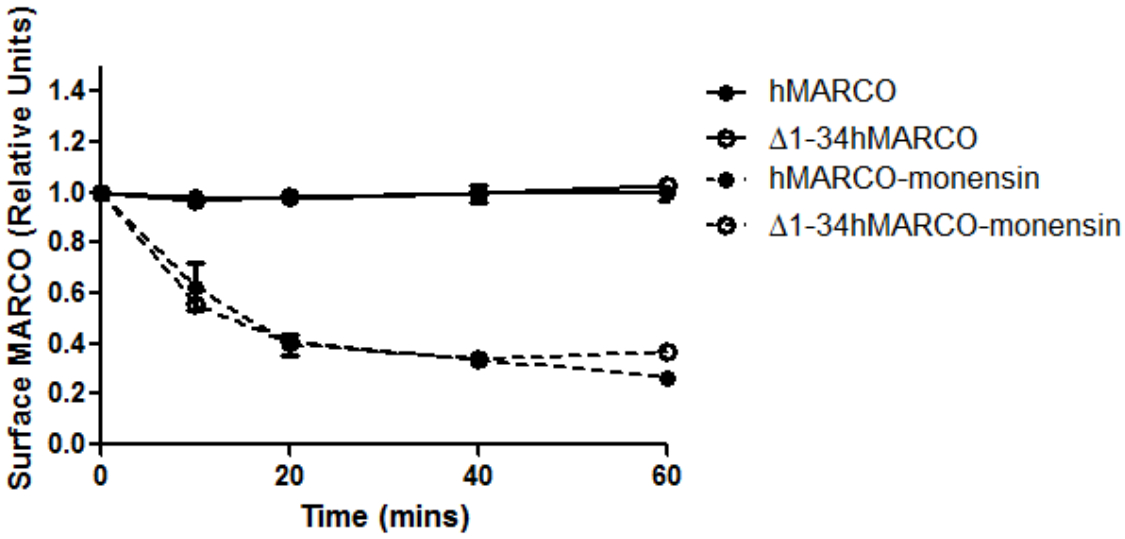


Fig 3.3 Characterization of MARCO receptor internalization without ligands. HMARCO (solid circles) and Δ1-34hMARCO (open circles) HEK293T cells were incubated with (dotted line) or without (solid line) 25μM monensin for the time indicated. After incubation with monensin, cells were immediately fixed on ice with 4% PFA and stained with primary rabbit anti-hMARCO antibodies 9805 followed by a secondary APC-conjugated anti-rabbit antibody. The results were evaluated by flow cytometry. The data are expressed as the relative fluorescence intensity, with values at time 0 set to 1. Data were plotted as mean ±SEM, n=3

3.3.4 Amino acids 1-34 of the cytoplasmic tail of hMARCO might be important for hMARCO recycling to the cell surface

To test if the first thirty-four amino acids are necessary for MARCO internalization, I utilized monensin to halt MARCO recycling in the presence of MARCO ligands first. After monensin and fluorescent MalBSA bead treatments, the cells were immediately fixed on ice with 4% PFA and stained with rabbit anti-hMARCO 9805 antibodies followed by a secondary APC-conjugated anti-rabbit antibody. The results were evaluated by flow cytometry. The data were expressed as the relative fluorescence intensity, with values at time 0 set to 1. With ligands, monensin-induced MARCO internalization was kinetically similar between the full-length MARCO and the cytoplasmic mutant Δ1-34hMARCO (Fig 3.4). In contrast, when monensin was absent, hMARCO surface expression was elevated drastically within the first twenty minutes of incubation with MalBSA-coated beads while Δ1-34hMARCO expression remained unchanged during that period. Eventually, hMARCO surface expression returned to a level that was comparable to Δ1-34hMARCO (Fig 3.4). Monensin primarily halts the recycling of MARCO, so when this blockade was removed, hMARCO, but not Δ1-34hMARCO surface expression increased significantly in the presence of beads (Fig 3.4), therefore the first thirty-four amino acids might be required for hMARCO recycling to the cell surface. Since I demonstrated that the full-length MARCO has an larger intracellular pool of protein than Δ1-34hMARCO, it is possible that the intracellular pool of MARCO contributed to the acute ligand-induced up-regulation of MARCO surface expression.

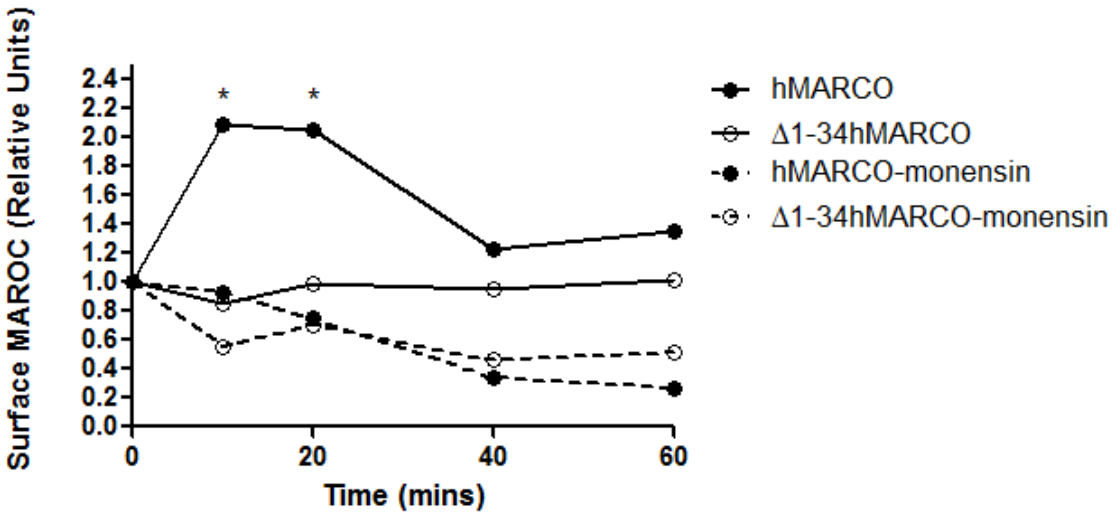


Fig 3.4 Characterization of MARCO receptor internalization in response to MalBSA-coated beads. HMARCO (solid circles) and Δ1-34hMARCO (open circles) HEK293T cells were incubated with (dotted line) or without (solid line) 25μM monensin for the time indicated with the presence of MalBSA-coated beads as the MARCO ligand. After incubation with monensin, cells were immediately fixed on ice with 4% PFA and stained with primary rabbit anti-hMARCO 9805 antibodies followed by a secondary APC-conjugated anti-rabbit antibody. The results were evaluated by flow cytometry. The data are expressed as the relative fluorescence intensity, with values at time 0 set to 1. Data were plotted as mean ±SEM, n=6, *$p<0.05$.

3.4 Discussion

In this study, I showed that the first thirty-four amino acids were not necessary for MARCO surface expression or internalization. I concluded that the first thirty-four amino acids might be necessary for MARCO surface recycling, as revealed by the bead endocytosis assay with monensin perturbation.

Previously, it was reported that the cytoplasmic tail of hMARCO is important for MARCO surface expression (Kroos 2011). This seems to contradict my findings shown in Section 3.3.1. In that study, full-length hMARCO and Δ1-40hMARCO were transiently transfected into HEK293T cells and it was found that 58.2±10.1% of hMARCO and 36.9±4.1% of Δ1-40hMARCO transfected cells expressed the protein on the cell surface. Combined with my findings in Section 3.3.1, it is possible that KPKRRN, i.e. amino acids 35-40 are required for sufficient MARCO surface expression. Interestingly, bioinformatics analysis of the putative functional sites of the cytoplasmic domain of MARCO by the program ELM revealed that the RRN sequence in the amino acids 35-40 is a potentially useful motif for the secretion of the membrane-bound protein from the ER. Further experiments need to be performed to validate this finding. Additionally, it should be noted that membrane-proximal amino acids of MARCO might alter cell surface localization, but are not obligatory for trafficking to the membrane, since hMARCO with the entire cytoplasmic tail truncated can still be expressed at the cell membrane (Kroos 2011), an observation similar to that finding was made by me with mouse MARCO (data not shown).

Interestingly, our findings on the role of the cytoplasmic tail of MARCO in receptor surface expression was strikingly different than the role of the cytoplasmic tail of SR-A on its surface expression. It was shown that SR-A required the membrane proximal region consisting of six amino acids for cell surface expression. In addition, it has been shown 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 my cytoplasmic mutants showed no change in surface expression compared to full-length MARCO. This suggested that there is a remarkable difference in regulation of cell surface expression between MARCO and SRA. It is likely that the ER processing and the secretory pathway of the two receptors from the Golgi apparatus to the cell membrane requires very different amino acid sequences that might or might not be present in the cytoplasmic domain of the receptors.

I also showed that amino acids 1-34 are not required for MARCO internalization. It is possible that the internalization sequence was not present within amino acids 1-34. Experiments with further truncated MARCO might help to reveal which, if any, sequence in the cytoplasmic tail of MARCO is important for MARCO internalization.

I showed that the full-length but not the truncated MARCO surface expression is drastically increased when recycling is not impaired by monensin. A possible explanation for this observation is that hMARCO is more readily available in the cytoplasm than Δ1-34hMARCO as shown in Figure 3.2A, so when MARCO expressing cells were stimulated with ligands, there would be more hMARCO that can be targeted on to the cell surface. To investigate this possibility, I could conduct a “pulse-chase” analysis on these

cells to determine whether the MARCO targeted to the cell surface is directly from the cytoplasm or nascently synthesized and processed through the ER. It is likely that MARCO recycling and initial membrane targeting via the secretory pathway required different amino acid sequences in the cytoplasmic tail of MARCO, since Δ1-34hMARCO surface expression was not impaired, which indicated that amino acids 1-34 were not involved in the secretion of MARCO to the cell surface.

In summary, this study showed that amino acids 1-34 of hMARCO are not necessary for MARCO surface expression and internalization but might be important for hMARCO recycling.

4 Chapter 4: Characterization of MARCO-mediated endocytosis

4.1 Introduction

Scavenger receptors recognize a variety of ligands of different sizes with a diverse degree of specificity. MARCO has been reported to recognize and mediate the uptake of AcLDL, OxLDL (Platt and Gordon 2001), polystyrene particles (Kanno *et al.* 2007), *S. pneumonia* and environmental particles (Arredouani *et al.* 2005). However, the mechanism of MARCO-mediated endocytosis is not understood and has never been investigated.

As discussed in Section 1.4, clathrin-mediated endocytosis and caveolae-dependent endocytosis are the two main ways that small particles, such as AcLDL and viruses, are bound and taken up via a receptor. For example, it has been shown that SR-A mediates AcLDL uptake via the clathrin-mediated pathway and also fucoidan uptake via the caveolae-depedent pathway in a human macrophage cell line, THP-1 (Zhu *et al.* 2011), and this AcLDL uptake requires a di-leucine motif within the cytoplasmic domain of SR-A (Chen *et al.* 2006). Additionally, it has been shown that SR-A mediates viral double-stranded RNA uptake via the clathrin-mediated pathway (DeWitte-Orr *et al.* 2010). To date, no study regarding the mechanism of MARCO-mediated endocytosis of the similar kind is published.

4.2 The aims of this chapter

The aims of this chapter are to characterize what endocytic pathways involve MARCO. I hypothesised that MARCO mediates ligand uptake of small particles via clathrin-mediated endocytosis or caveolae-dependent endocytosis. To test this hypothesis, I sought to identify suitable ligands to measure MARCO-mediated endocytosis first. Second, to identify the components of MARCO-mediated endocytic pathway in an logical order, I chose to follow the classification system for endocytic mechanisms proposed by Mayor and Pagano shown in Fig 4.1 (Mayor and Pagano 2007) I employed a variety of pharmacological inhibitors that are known to block specific steps of endocytosis. The results indicated that MARCO-mediated endocytosis was clathrin-dependent. Additionally, dynamin was required for MARCO-mediated endocytosis. Lastly, actin involvement was also critical for MARCO-mediated endocytosis.

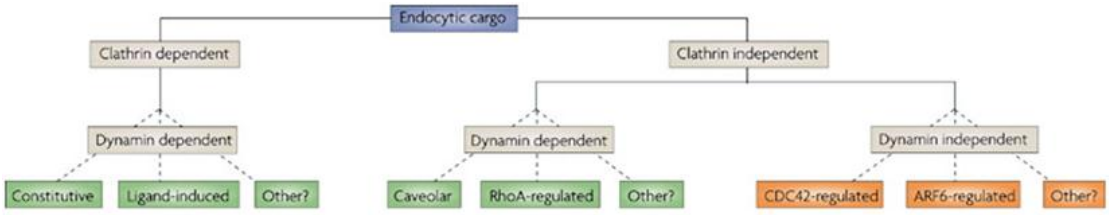


Fig 4.1 Proposed classification system for endocytic mechanisms. A cargo protein can be endocytosed by either clathrin-dependent or clathrin-independent mechanisms. Clathrin-independent pathways can be further categorized first by their dependence on the large GTPase dynamin, and then by other mechanistic components of the internalization pathway. (Adapted from Mayor and Pagano 2007).

4.3 Results

4.3.1 MalBSA-coated 500 nm beads are MARCO ligands

MalBSA has been reported to be a ligand for scavenger receptors (Acton *et al.* 1994). To test whether MalBSA is a ligand for MARCO, MalBSA was coated onto 500 nm fluorescent beads, as described in Section 2.2.6. The binding and uptake of MalBSA coated beads by WT and $MARCO^{-/-}$ resident peritoneal macrophages (RPMϕs) was evaluated by flow cytometry (Fig. 4.2). The results indicated that WT RPMϕs bind and internalize significantly more MalBSA-coated beads than $MARCO^{-/-}$ RPMϕs. This showed that MARCO was a receptor for MalBSA-coated beads. Since there are other scavenger receptors, such as SR-BI and CD36, expressed on the RPMϕs which can bind and internalize MalBSA-coated beads (Acton *et al.*), it will be difficult to study MARCO-mediated endocytosis in a specific manner in primary macrophages. Therefore, I decided to stably overexpress MARCO in HEK293T cell lines, a non-phagocytic cell line, to study MARCO-mediated endocytosis. Additionally, the use of this cell line had enabled study of the contribution of the cytoplasmic tail to MARCO-mediated endocytosis by expressing a MARCO mutant with a truncated cytoplasmic tail described in Chapters 3 and 5.

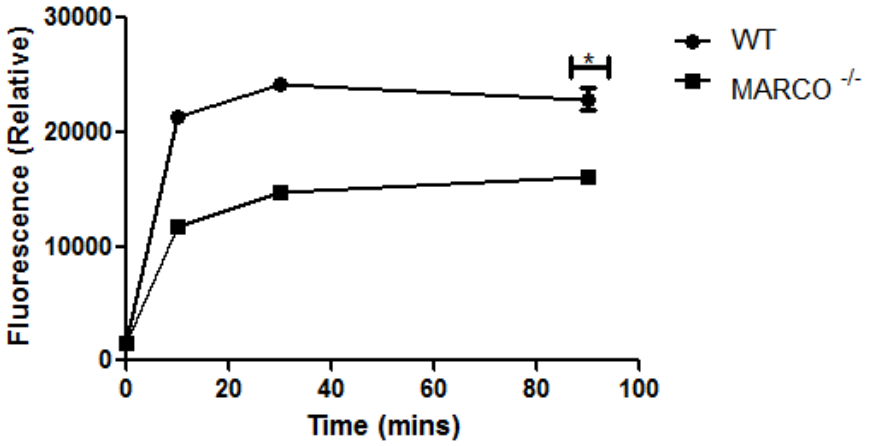


Fig 4.2 MalBSA-coated 500nm beads are MARCO ligands. Mouse resident peritoneal macrophages were isolated from WT and MARCO $^{-/-}$ mice and incubated with fluorescent, MalBSA-coated polystyrene beads at a ratio of 300 beads: 1 cell for the time indicated at 37℃. Bead binding and uptake were quantitated by flow cytometry. The values are relative mean fluorescence intensity. Data were plotted as mean±SEM, n= 3 mice per group, *$p<0.05$.

4.3.2 MalBSA-coated 500nm beads are the highly specific ligands to study MARCO-mediated endocytosis with HEK293T cells

To study MARCO-mediated endocytosis, it is of paramount importance to choose a ligand that is specific for MARCO in HEK293T cells that stably overexpress MARCO. Dose response studies and kinetics studies were conducted on stable hMARCO-expressing HEK293T cells and plasmid vector-expressing controls with AlexaFluor488-labled MalBSA (AF488-MalBSA), 200 nmMalBSA-coated beads and 500 nm MalBSA-coated beads. The binding and uptake of ligands was assessed by fluorescence spectroscopy or flow cytometry as described in Section 2.2. As can be seen from Figs 4.3(A), (C) and (E), hMARCO-expressing HEK293T cells showed a dose-dependent response with 200 nm MalBSA beads and 500 nm MalBSA beads, and to a lesser extent AF488MalBSA. As can be seen from Figs 4.3 (B), (D) and (F), hMARCO-expressing cells showed time-dependent response towards all ligands with different degrees. Overall, judging by the difference of cell association of ligands between hMARCO- and vector-expressing cells, hMARCO-expressing cells exhibited poor specificity towards AF488MalBSA, mediocre specificity towards 200 nm MalBSA-coated beads and great specificity towards 500 nm MalBSA-coated beads. I concluded that 500 nm MalBSA-coated beads were the most specific ligand for hMARCO-expressing cells, and this ligand was used for subsequent studies unless otherwise stated. It is worth noting that the specificity of ligands towards MARCO-expressing cells seemed to be inversely-related to the size of the ligands. Whether MARCO truly favours ligands of large size is yet to be determined.

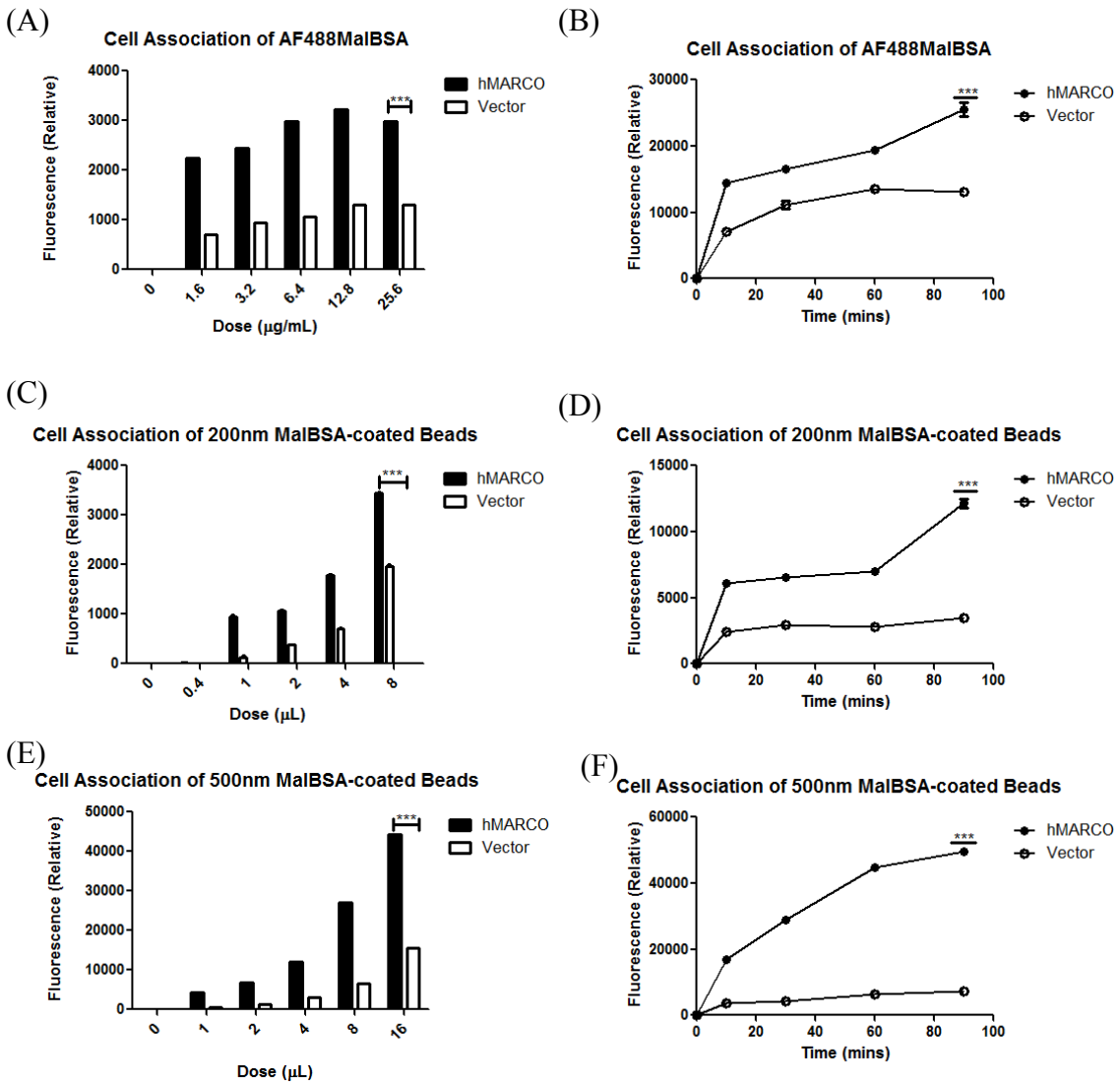


Fig 4.3 MalBSA-coated 500nm beads are highly specific ligands to study MARCO-mediated endocytosis with HEK293T cells. Stable hMARCO-expressing HEK293T and vector control were incubated with AF488MalBSA (A) and (B), 200 nm MalBSA-coated beads (C) and (D) and 500 nm MalBSA-coated beads (E) and (F) for various doses (A), (C) and (E) and various time (B), (D) and (F) at 37 ℃ with gentle rocking. Data were plotted as mean±SEM, n=3, ***p<0.001. ns p>0.05

4.3.3 Optimization of inhibition of endocytosis with pharmacological inhibitors

In order to use pharmacological inhibitors of endocytic signalling components, I concluded that it was necessary to perform optimization studies in the HEK293T cell line. In order to determine whether MARCO uptake was mediated by clathrin or not, I used human transferrin as a positive control, as it is well established that transferrin is almost exclusively taken up by clathrin-mediated endocytosis. Clathrin-mediated endocytosis can be inhibited at various stages by 1) Chlorpromazine (CPZ), which reversibly translocates clathrin into an intracellular vesicle, 2) by dynasore, which bind and inhibit the activity of dynamin, 3) by valinomycin, which promotes K+ efflux and by Pitstops, which binds to the C-terminal domain of clathrin to inhibit coat assembly. The optimal doses of these pharmacological inhibitors were determined by titration as shown in Fig 4.4. Chlorpromazine had a dose-dependent inhibitive effect on fluorescently labelled AF488-hTf uptake by HEK293T cells. The optimal inhibitive dose for CPZ was determined to be 28.8 μM, at which 98% of AF488-hTf uptake was inhibited. Similarly, dynasore (Macia 2006), showed a dose-dependent inhibitive effect on AF488-hTf uptake by HEK293T cells. The optimal dose for dynasore was determined to be 80 μM, at which 91% of AF488-hTf uptake was inhibited. In contrast, valinomycin and Pitstop2 showed dose-independent responses of AF488-hTf uptake by HEK293T cells. In conclusion, I determined that CPZ is an ideal inhibitor to test clathrin-mediated endocytosis in HEK293T cells. Additionally, dynasore is also useful to test whether dynamin is involved in MARCO-mediated endocytosis in HEK293T cells.

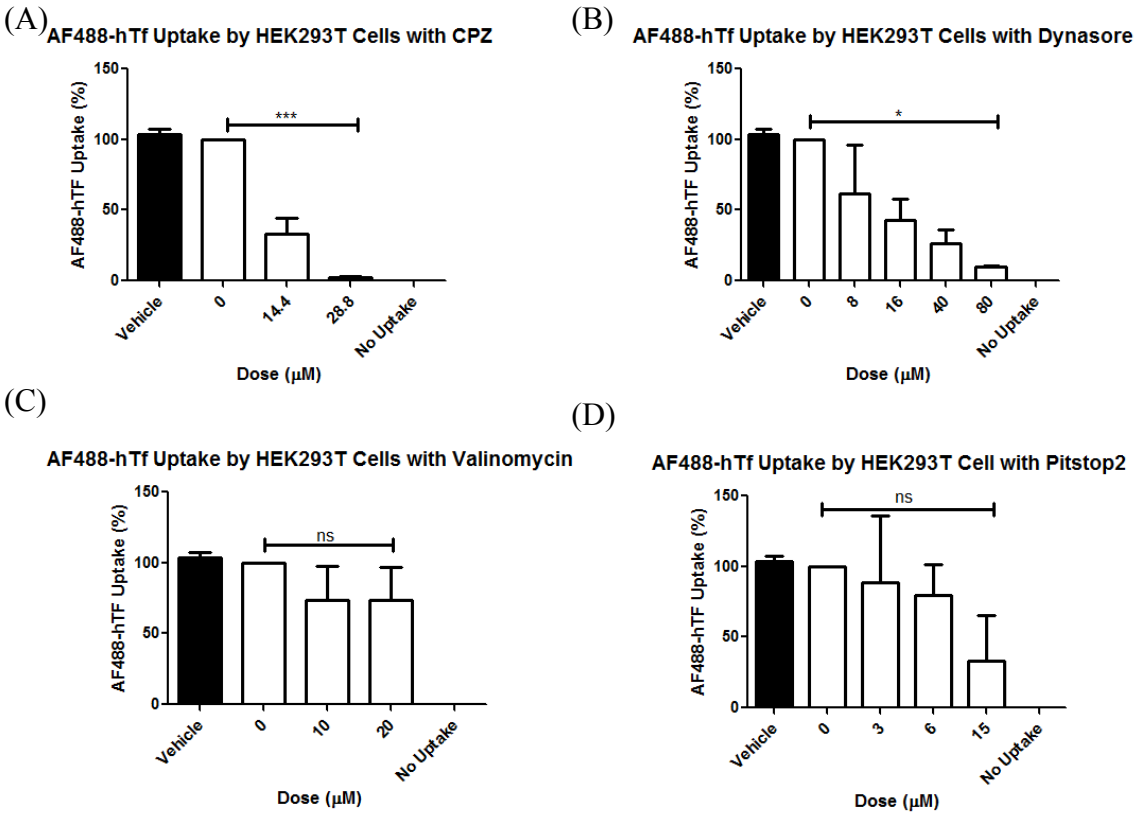
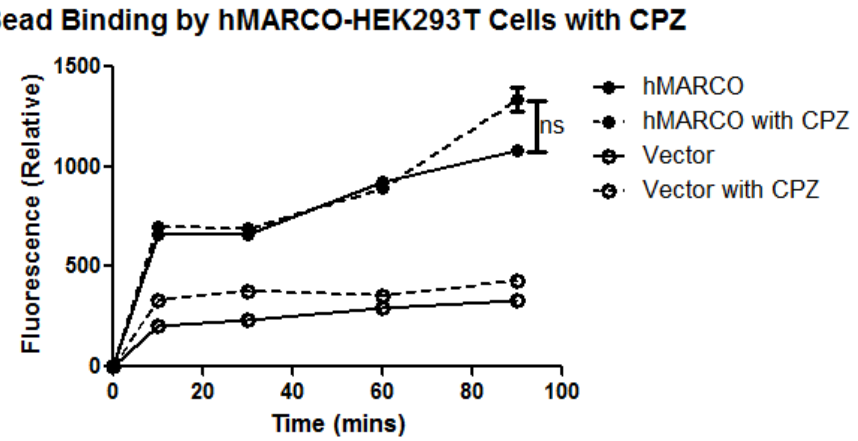


Fig 4.4 Characterization of AF488-hTf uptake by HEK293T cells with pharmacological inhibitors for endocytosis. HEK293T cells that were incubated with 10 μg/mL AF488-hTf for 90 minutes were co-treated with chlorpromazin (A), dynasore (B), valinomycin (C) and Pitstop2 (D) for the various doses indicated. AF488-hTf uptake was calculated as the difference between the fluorescence signals from the cells that were incubated at 37 ℃ and 4 ℃ with the same inhibition conditions. Data were plotted as mean ±SEM, n=3, * $p<0.05$, ***$p<0.001$. ns $p>0.05$

4.3.4 MARCO-mediated endocytosis is clathrin-dependent

In order to measure bead binding in the absence of uptake, experiments were performed at 4 ℃ . MARCO-expressing and non-expressing HEK293T cells were incubated with the inhibitor CPZ to block clathrin coat formation at the vesicle site and, thus, uptake of fluorescently-labelled MalBSA-coated beads. At 4 ℃, MARCO-expressing cells bound significantly more beads and this binding was not inhibited by CPZ, thus demonstrating that clathrin is not required for cell association. In contrast, when MARCO-expressing and non-expressing cells were incubated at 37 ℃, the temperature at which uptake occurs, there was a robust inhibition (98%) of bead uptake (Fig 4.5B). These results demonstrated that MARCO-mediated bead uptake is CPZ-sensitve, thus clathrin is required for MARCO-mediated bead endocytosis.

(A)



(B)

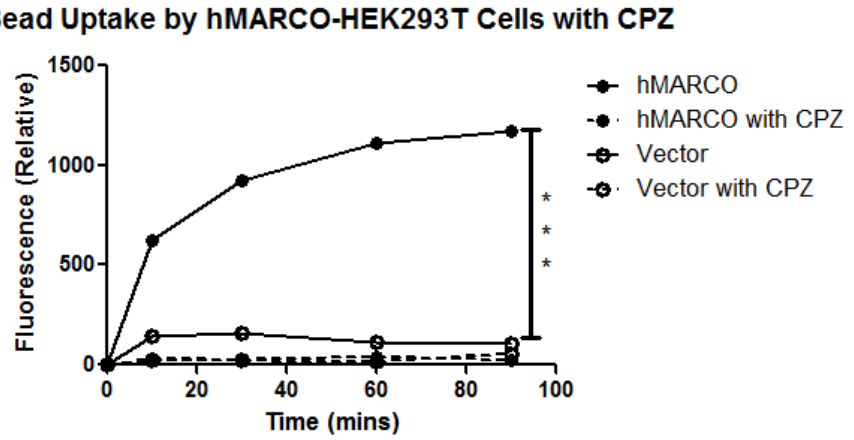


Fig 4.5 Characterization of MalBSA-coated beads binding and uptake by hMARCO-HEK293T cells with CPZ. HMARCO-HEK293T cells were incubated with MAlBSA-coated beads with the presence of 28.8 μM CPZ at 4 ℃ and 37 ℃ for the time indicated to determine beads binding (A) and uptake (B). Data were plotted as mean ±SEM, n=3, ***p<0.001. ns p>0.05

4.3.5 MARCO-mediated endocytosis is dynamin-dependent

To determine whether dynamin, a GTPase that is involved in the fission of clathrin-coated vesicles (Doherty and McMahon 2009), is required for MARCO-mediated, clathrin-dependent endocytosis, dynasore was used to block dynamin-dependent endocytosis. HMARCO-HEK293T cells were pre-treated with 80 μM dynasore before incubating with MalBSA-coated beads at 37 ℃ for 90 minutes. The cells were fixed with 4% PFA and permeabilized with 0.1% Triton-X100. The presence of MARCO was determined by primary rabbit anti-human MARCO 9805 antibodies followed by the secondary goat anti rabbit IgG conjugated with AlexaFlur647 staining and F-actin were determined by phalloidin-TRITC staining. Cells were imaged on a Zeiss confocal microscope model LSM510. Z-stacks (2 μm between scans) were constructed of twelve slices each field. Compared to the non-treated cells (Fig. 4.6A), hMARCO-expressing HEK293T cells, dynasore-treated cells (Fig. 4.6B) exhibited significantly less bead uptake. This result indicates that MARCO-mediated endocytosis is dynamin-dependent.

(A) (B)

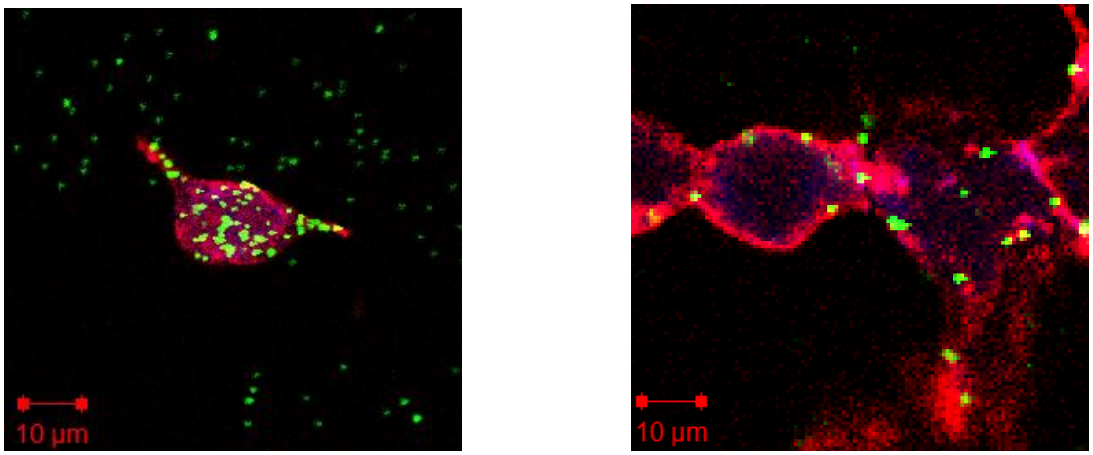


Fig 4.6 Characterization of MalBSA-coated beads uptake by hMARCO-HEK293T cells with dynasore by fluorescence microscopy. HMARCO-HEK293T cells were incubated with MalBSA-coated beads without (A) or with (B) the presence of 80 µM dynasore at 37 ℃ for 90 minutes. One representative experiment is shown.

4.3.6 Actin involvement is required for MARCO-mediated endocytosis

To determine whether actin involvement was required for MARCO-mediated endocytosis, cytochalasin D (CytoD), an F-actin depolymerizing drug (Dharmawardhane *et al.* 2000) was used to block any actin-dependent process. HMARCO-HEK293T cells and vector controls were incubated with MalBSA-coated beads with or without the presence of 5 μg/mL CytoD at 37 ℃ and 4 ℃ for the time indicated. At 90 minutes, 87% of bead uptake by hMARCO-HEK293T cells was inhibited with CytoD treatment. These results suggested that an intact actin skeleton was required for MARCO-mediated endocytosis.

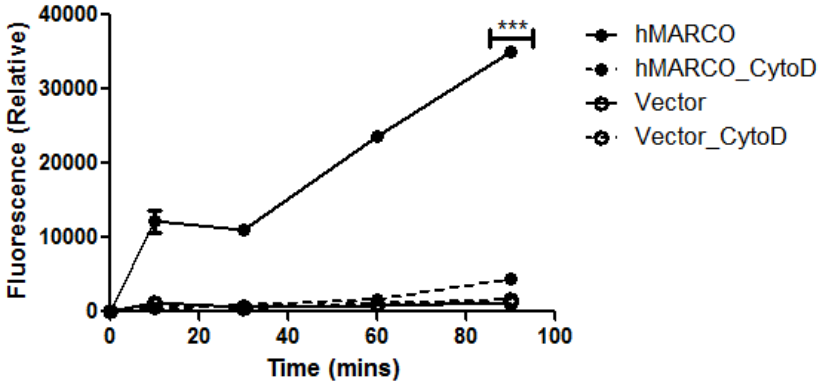


Fig 4.7 Characterization of MalBSA-coated beads uptake by hMARCO-HEK293T cells with CytoD. HMARCO-HEK293T cells and vector controls were incubated with MalBSA-coated beads or with or without the presence of 5 μg/mL CytoD at 37 ℃ or 4 ℃ for the time indicated. Fluorescently-labelled MalBSA-coated beads uptake was calculated as the difference between the fluorescence signals from the cells that were incubated at 37 ℃ and 4 ℃ under the same inhibition conditions. One representative experiment is shown. Data were plotted as mean ±SEM, n=3, ***P<0.001.

4.4 Discussion

MalBSA was previously shown to be a ligand for scavenger receptors. Here, it was demonstrated that 500nm MalBSA-coated polystyrene beads are adequate MARCO ligands. Moreover, it was demonstrated that MalBSA-coated 500nm beads are the highly specific ligands to study MARCO-mediated endocytosis with HEK293T cells. More non-MARCO specific ligand binding and uptake was observed with smaller ligands. The non-specific ligand binding and uptake is likely contributed by SR-BI and CD36 that are endogenously expressed in HEK293T cells. As discussed in Section 3.3.2, MARCO ligand specificity seems to increase with particle size. This result can also be interpreted as a MARCO ligand non-specificity (e.g. SR-BI and CD36 specificity) decrease with particle size. To test these two hypotheses, inhibiting non-specific binding with blocking antibodies against SR-BI and CD36 can be used to determine if ligand binding and uptake increase with particle size. If ligand binding and uptake increased with particle size when non-specific binding is blocked, it can be concluded that MARCO does favour binding and taking up large ligands.

Titration experiments performed with the pharmacological inhibitors with AF488-hTf as the positive control ligands indicated that CPZ and dynasore nearly abolish clathrin-mediated endocytosis at optimal doses. Valinomycin and Pitstop2 could not significantly halt clathrin-mediated endocytosis. It is likely that different cell lines have different sensitivities for different inhibitors and in the present case, CPZ and dynasore functioned well in HEK293T cells while valinomycin and Pitstop2 appeared to be non-inhibitory in

this system. I noted that CPZ, dynasore, and CytoD treatment on HEK293T cells with the optimal dose did not alter MARCO surface expression or induce significant cell death.

The route by which MARCO mediates ligand entry is not clear, nor is the endocytic molecular machinery that helps facilitate MARCO-mediated ligand entry. Pharmacological agents that block specific molecular components of endocytosis were used to determine the molecular components involved in MARCO-mediated endocytosis. My results demonstrated that MARCO mediates fluorescently-labelled MalBSA-coated beads uptake via a clathrin-mediated, dynamin-dependent pathway that requires actin involvement. To further validate these findings, I could perform co-immunoprecipitation of MARCO with clathrin to determine whether MARCO and clathrin are physically present within the same complex upon ligand stimulation. A dominant-negative mutant of dynamin K44A, is often utilized to block dynamin-dependent functions. Transiently-expressed K44A could help determine whether the ligand uptake is blocked. My findings that MARCO-mediated bead uptake is sensitive to CytoD are consistent with those of a previous study in which internalization of latex beads were shown to be mediated by scavenger receptors in an actin-dependent manner (Sulahian *et al.* 2008).

The potential involvement of MARCO in caveolae-dependent endocytosis was not investigated here. It is possible that MARCO mediates other ligand uptake via this pathway. For the 500 nm MalBSA-coated beads, it is highly unlikely that MARCO also mediates bead uptake via both clathrin-dependent and caveolae-dependent pathways. My preliminary results (methods and data not shown) suggest that caveolae inhibitors nystatin and filipin seem to suggest that MARCO-mediated bead uptake is not sensitive to these

drugs thus indicating that in HEK293T cells, MalBSA-coated beads are probably taken up by MARCO via the clathrin-depedent pathway.

In summary, this study showed that 500 nm MalBSA-coated beads are highly specific ligand for MARCO in hMARCO-expressing HEK293T cells. Based on the studies with pharmacological inhibitors, MARCO-mediated bead uptake is clathrin-dependent, dynamin-dependent and requires actin involvement.

5 Chapter 5: The role of the cytoplasmic tail in MARCO-mediated endocytosis

5.1 Introduction

In Chapter 4, I characterized the endocytic pathway utilized by MARCO with perturbations caused by an array of pharmacological inhibitors, including CPZ, dynasore and CytoD. I concluded from these studies that MARCO-mediated endocytosis is clathrin-dependent, dynamin dependent and requires the involvement of actin. However, it is still not clear how MARCO mediates endocytosis and which amino acid sequences of MARCO are important for MARCO-mediated endocytosis.

As discussed in Section 1.3, the cytoplasmic tail of scavenger receptors might contain important amino acid sequences that contribute to endocytosis. The cytoplasmic domain of SR-A contains sequences for internalization 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 internalization (Morimoto *et al.* 1999). In addition, the cytoplasmic domain of SR-A contains a di-leucine motif, which has been demonstrated to be required for AcLDL endocytosis (Chen *et al.* 2006). The potential signalling sequences in the cytoplasmic domain of MARCO have not been investigated.

5.2 The aims of this chapter

I hypothesised that the cytoplasmic domain of MARCO contains sequences required for efficient uptake of MalBSA-coated beads. To test this hypothesis, two constructs of MARCO were used: hMARCO and Δ1-34hMARCO which is missing a putative di-leucine motif and a region of conserved sequences (Fig 3.1). I chose to delete amino acids 1-34 instead of the entire cytoplasmic domain because preliminary data suggested that the surface expression of MARCO was significantly decreased when the entire cytoplasmic tail was truncated; this would introduce difficulty for data interpretation as the level of surface expression of this hypothetical construct might be very low. I also hypothesized that the cytoplasmic tail of MARCO is important for the clathrin-mediated, dynamin-regulated and actin-dependent endocytosis by MARCO. To study the role of the cytoplasmic domain in the endocytic pathway characterized in Chapter 4, I used the same panel of inhibitors on hMARCO and Δ1-34hMARCO-HEK293T cells.

5.3 Results

5.3.1 The cytoplasmic tail of MARCO is crucial for MARCO-mediated bead uptake

Surface expression of MARCO constructs is characterized in Section 3.3.2. To test whether the cytoplasmic domain is important for MARCO-mediated endocytosis, hMARCO-expressing HEK293T cells, Δ1-34hMARCO-HEK293T cells and vector-expressing control cells were incubated with MalBSA-coated beads at 37 ℃ for 90 minutes. Bead binding and uptake were evaluated by immunofluorescence microscopy

and flow cytometry (Fig. 5.1). The results showed that bead binding was not altered when the cytoplasmic tail was truncated. In contrast, bead uptake was reduced approximately 50% when the cytoplasmic tail is truncated. Taken together, the cytoplasmic tail was required for complete ligand uptake but not binding by MARCO.

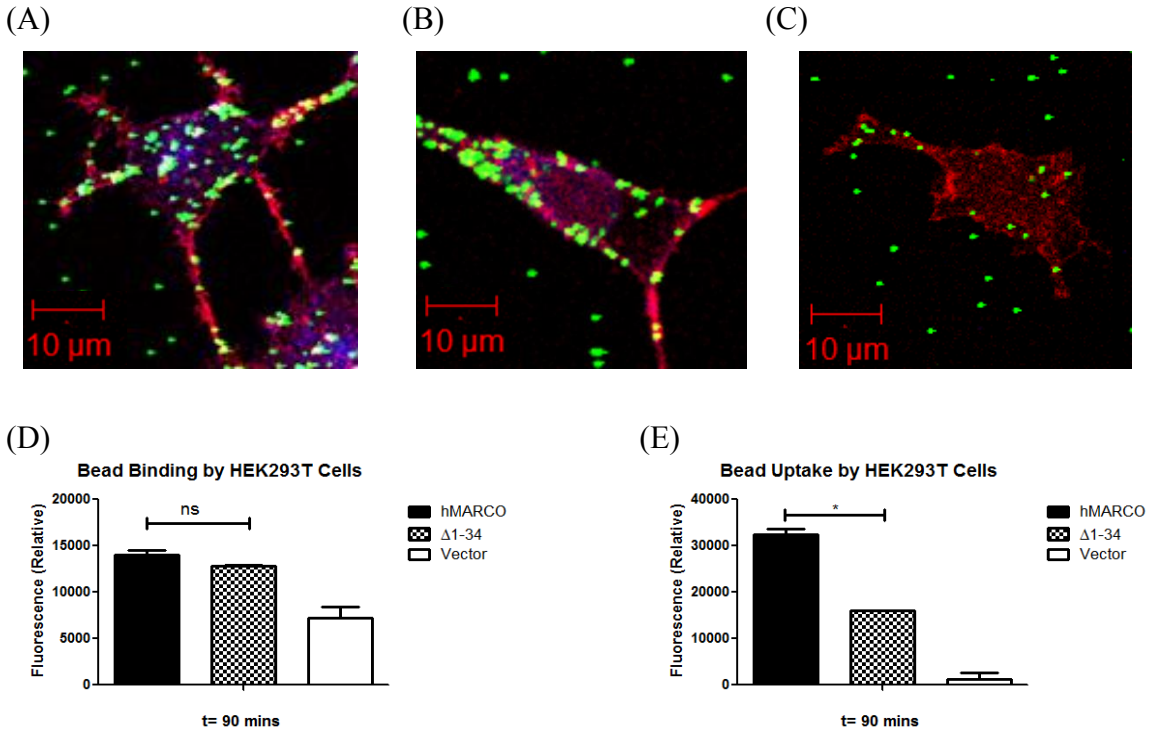


Fig 5.1 The cytoplasmic tail of MARCO is crucial for MARCO-mediated bead uptake HMARCO-HEK293T cells (A), Δ1-34hMARCO-HEK293T cells (B) and vector controls (C) were incubated with MalBSA-coated beads at 37 ℃ for 90 minutes. One representative experiment is shown. Cells were fixed with 4% PFA and permeabilized with 0.1% Triton-X100. MARCO was stained in blue (9805 and AlexaFluor647) and F-actin were stained in red (phalloidin-TRITC). Cells were imaged on Zeiss confocal microscope model LSM510. Z-stacks (2 µm between scans) were taken of each field for 5 random fields. In addition to the qualitative evaluation by immunofluorescence microscopy, we also performed the endocytosis assay by the incubation of fluorescently-labelled beads at a ratio of 300 beads to one cell with hMARCO-, Δ1-34hMARCO- and vector-expressing HEK293T cells at 37 ℃ and 4 ℃ to quantitatively evaluate the ligand binding (D) and uptake (E) properties with flow cytometry. Ligand binding (D) is presented as the mean cell-associated fluorescence intensity at 4 ℃ and ligand uptake (E) is presented as the difference of the mean cell-associated fluorescence intensity at 37 ℃ and 4 ℃. Data were plotted as mean±SEM, n=3, * $P<0.05$. ns $P>0.05$

5.3.2 The cytoplasmic tail of MARCO is required for clathrin-dependent, MARCO-mediated bead uptake

To determine whether the cytoplasmic tail of MARCO is required for ligand uptake via the clathrin-mediated endocytic pathway shown in Section 3.3.4, I treated hMARCO-HEK293T and Δ1-34hMARCO-HEK293T cells with CPZ alongside MalBSA-coated beads at 4 ℃ or 37 ℃ for 90 minutes. The results were assessed by flow cytometry (Fig 5.2). The outcome of the endocytosis assay showed that inhibition by CPZ does not alter the bead binding capacity of either hMARCO or Δ1-34hMARCO. Importantly, bead uptake by Δ1-34hMARCO-HEK293T cells was insensitive to CPZ inhibition, while hMARCO-HEK293T cells exhibited a 98% reduction. Overall, the data suggested that the clathrin-mediated, MARCO-specific bead uptake required the cytoplasmic tail of MARCO.

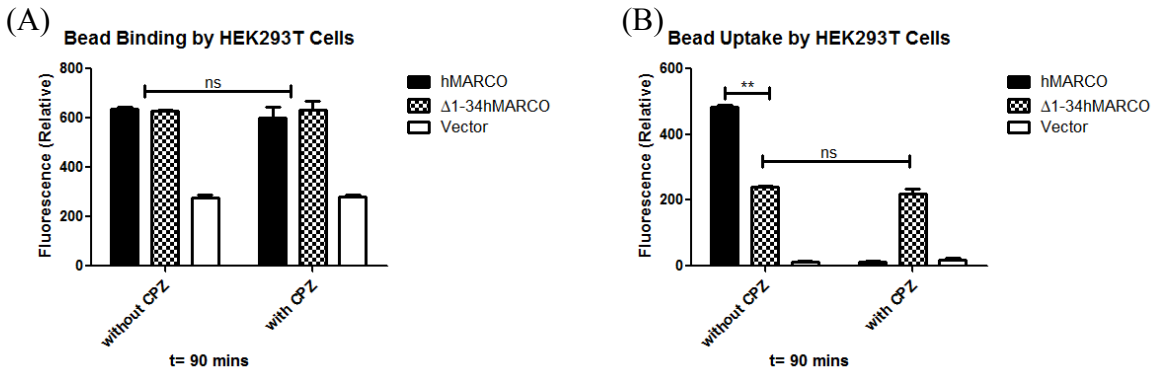


Fig 5.2 Characterization of MalBSA-coated beads binding and uptake by hMARCO-HEK293T cells and Δ1-34hMARCO-HEK293T cells with CPZ. Cells were incubated with MAlBSA-coated beads at a ratio of 300 beads to one cell with the presence of 28.8 μM CPZ at 4 ℃ and 37 ℃ for 90 minutes to determine beads binding (A) and uptake (B). No statistically significant inhibition of beads binding by hMARCO-HEK293T or Δ1-34hMARCO-HEK293T cell with CPZ was observed. On the other hand, a 98% reduction of bead uptake by hMARCO-HEK293T cells were observed with CPZ, while no reduction of bead uptake by Δ1-34hMARCO-HEK293T was observed with CPZ. Data were plotted as mean ±SEM, n=3, ** $P<0.01$. ns $P>0.05$

5.3.3 The cytoplasmic tail of MARCO is required for dynasore-dependent, MARCO-mediated bead uptake

To determine whether the cytoplasmic tail of MARCO is required for ligand uptake via the dynamin-dependent endocytic pathway shown in Section 3.3.5, we treated hMARCO-HEK293T and Δ1-34hMARCO-HEK293T cells with dynasore plus MalBSA-coated beads at 4 ℃ and 37 ℃ for 90 minutes. The results were evaluated by flow cytometry (Fig 5.3). Similar to CPZ inhibition shown in Section 4.3.2, the inhibition by dynasore did not alter the bead binding capacity of either hMARCO or Δ1-34hMARCO. However, bead uptake by Δ1-34hMARCO-HEK293T cells was insensitive to dynasore inhibition, while hMARCO-HEK293T cells exhibited a nearly complete reduction. In conclusion, the data indicated that the dynamin-dependent, MARCO-specific bead uptake required the cytoplasmic tail of MARCO.

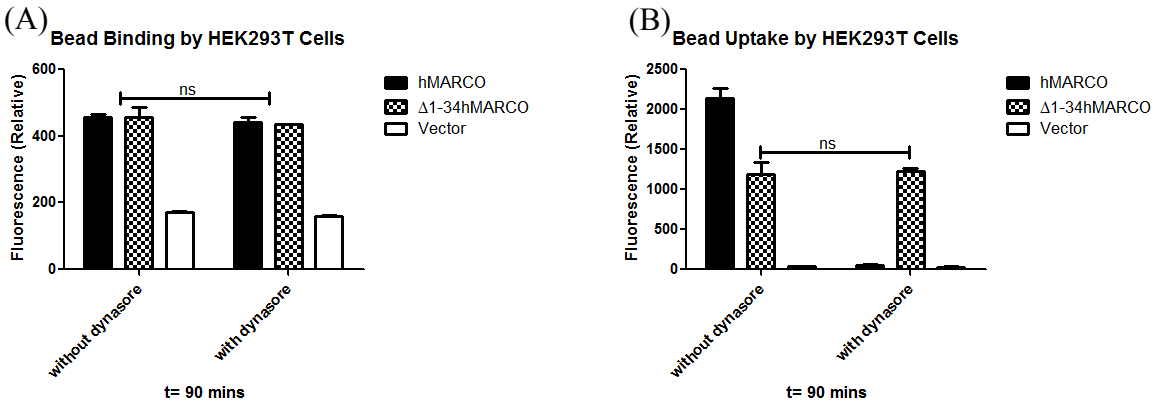


Fig 5.3 Characterization of MalBSA-coated beads binding and uptake by hMARCO-HEK293T cells and Δ1-34hMARCO-HEK293T cells with dynasore. Cells were incubated with MAlBSA-coated beads at a ratio of 300 beads to one cell with the presence of 80 μM dynasore at 4 ℃ and 37 ℃ for 90 minutes to determine beads binding (A) and uptake (B). Data were plotted as mean ±SEM, n=3, ns P>0.05

5.3.4 Actin involvement of MARCO-mediated endocytosis does not require the cytoplasmic tail

To determine whether the actin involvement of MARCO-mediated endocytosis shown in Section 3.3.6 requires the cytoplasmic tail of MARCO, I inhibited MARCO-mediated uptake with the actin depolymerizing agent, CytoD. The results were evaluated by flow cytometry. Similar to CPZ and dynasore inhibition, Cyto D inhibition did not alter the bead binding capacity of hMARCO-HEK293T and Δ1-34hMARCO-HEK293T cells. Approximately 90% of bead uptake by both hMARCO-HEK293T and Δ1-34hMARCO-HEK293T cells was inhibited when treated with CytoD, unlike CPZ and dynasore inhibition showed in Sections 5.3.2 and 5.3.3. The results suggested that the actin involvement in MARCO-mediated endocytosis is universal and does not require the cytoplasmic tail of MARCO.

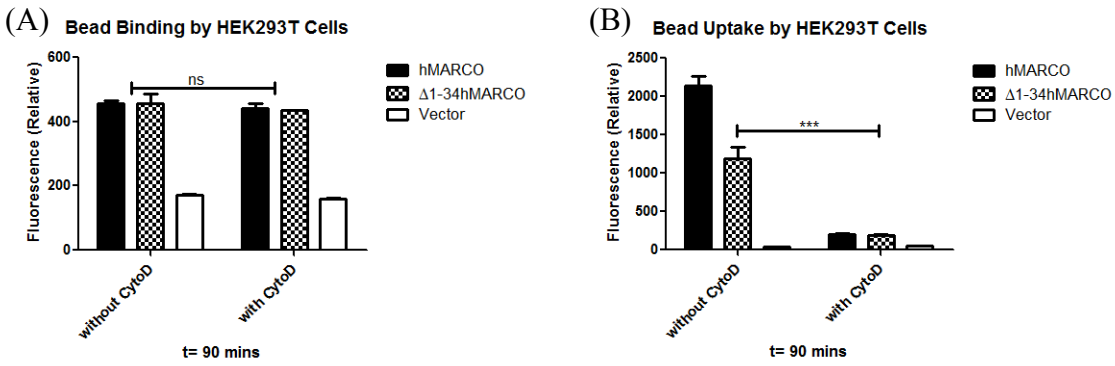


Fig 5.4 Characterization of MalBSA-coated beads binding and uptake by hMARCO-HEK293T cells and Δ1-34hMARCO-HEK293T cells with CytoD. Cells were incubated with MAlBSA-coated beads at a ratio of 300 beads to one cell with 5μg/mL of CytoD at 4 ℃ and 37 ℃ for 90 minutes to determine beads binding (A) and uptake (B). Data were plotted as mean±SEM, n=3, *** $P<0.001$ ns $P>0.05$

5.4 Discussion

In Chapter 4, I characterized the endocytic pathway utilized by MARCO with perturbations by an array of pharmacological inhibitors, including CPZ, dynasore and CytoD. I concluded from these inhibition studies that MARCO-mediated endocytosis was clathrin-dependent, dynamin dependent and requires the involvement of actin. However, it is still not clear how MARCO mediates endocytosis and which amino acid sequences are important for MARCO-mediated endocytosis. As discussed in Section 1.6, the cytoplasmic tail of MARCO might contain important amino acid sequences that contribute to MARCO-mediated endocytosis. I created a stable cell line that expressed Δ1-34hMARCO, which lacks the first thirty-four amino acids including the putative di-leucine motif, to study whether the cytoplasmic domain of MARCO is required for endocytosis. In this chapter, I used the same inhibitors on hMARCO-HEK293T cells and Δ1-34hMARCO-HEK293T cells to determine whether the cytoplasmic tail of MARCO was required for all the elements in the MARCO-dependent endocytic pathway characterized previously.

The endocytosis assay with hMARCO-HEK293T cells and Δ1-34hMARCO-HEK293T cells revealed that the cytoplasmic tail of MARCO is important for ligand uptake but not ligand binding. This was understood since the SRCR domain of MARCO is hypothesized to be the ligand recognition and binding domain (Elomaa *et al.* 1998). It should also be noted that ligand binding was not reduced completely; this is indicative of other cytoplasmic sequences within the thirty-fourth to fiftieth amino acid sequence of the

cytoplasmic domains that also contributes to MARCO-mediated endocytosis. Thus, a further truncation on the cytoplasmic domain will be helpful to determine the additional sequences that are required for MARCO-mediated endocytosis.

Inhibition with CPZ, dynasore and CytoD all suggested that the capacity of MARCO-mediated ligand binding was not changed and was independent of the cytoplasmic domain. Interestingly, bead uptake by Δ1-34hMARCO-HEK293T cells was insensitive to CPZ and dynasore inhibition whereas hMARCO-HEK293T cells was sensitive to CPZ and dynasore inhibiton indicating that the first thirty-four amino acids are important for clathrin and dynamin to mediate MARCO-mediated endocytosis. Ligand uptake by both hMARCO-HEK293T and Δ1-34hMARCO-HEK293T cells was inhibited to the basal level when treated with CytoD. This suggested that actin involvement was independent of the cytoplasmic tail of MARCO. It is important to note here that treatment of cells with all the inhibitors did not alter the surface expression of hMARCO nor Δ1-34hMARCO, and that the levels of expression of both constructs on the surface were similar. So the change in ligand uptake upon inhibitor treatment is not due to the drug-induced alteration of surface protein expression nor uneven cell surface expression between constructs.

To explore whether the first thirty-four amino acids of the cytoplasmic tail were truly involved in clathrin binding, the lysates of hMARCO and Δ1-34hMARCO-HEK293T cells can be immunoprecipitated by an anti-clathrin antibody and analyzed by western blotting with anti-hMARCO antibody 9805. If it is the case that the cytoplasmic tail is required for MARCO-mediated endocytosis via clathrin-mediated endocytosis, I would expect to find full-length hMARCO but not Δ1-34hMARCO co-immunoprecipitaed by an

anti-clathrin antibodies. If this is validated, one can further investigate whether AP-2 is the adaptor protein that links MARCO to clathrin and whether the di-leucine motif of MARCO is the binding sequence for AP-2.

The large GTPase dynamin regulates clathrin-mediated endocytosis (Doherty and McMahon). Here, I showed that the cytoplasmic tail of MARCO was required for dynamin-regulated, MARCO-mediated uptake of beads. It is currently unknown how the regulation of endocytosis by dynamin is linked to the cargo protein. It is postulated that dynamin regulates endocytosis by regulating the maturation of clathrin-coated pits and subsequent budding of the clathrin-coated vesicles (Loerke *et al.* 2011). Since Δ1-34hMARCO might be incapable of binding to the adaptor proteins and clathrin, CCP will not be mature enough for dynamin to act upon. This might explain why ligand uptake by Δ1-34hMARCO-HEK293T cells was insensitive to dynasore perturbation.

I also showed that the cytoplasmic tail is not required for the actin involvement in MARCO-mediated endocytosis. This suggested that actin was actively involved in probably the early stages of this endocytic pathway. Actin engagement is required for the deformation of the clathrin-coated vesicles after CCVs are completely internalized (Boulant *et al.* 2011) so it is expected that no uptake can occur if actin is depolymerized, and this should be independent of the cytoplasmic tail of MARCO. Additionally, the observation that CytoD, but not dynasore, can almost completely inhibit bead uptake by Δ1-34hMARCO-HEK293T cells indicated that actin was involved in early endocytic steps before dynamin regulation takes place.

In summary, I showed that the cytoplasmic tail was required for complete bead uptake by MARCO. In addition, the cytoplasmic tail of MARCO was also required for clathrin and dynamin regulation of MARCO-mediated endocytosis. Lastly, the cytoplasmic tail of MARCO was not required for the involvement of actin in MARCO-mediated endocytosis.

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

The aim of the studies presented in this thesis has been to characterize the role of the cytoplasmic domain of MARCO and to characterize the molecular pathway of MARCO-mediated endocytosis. By studying the cell surface expression, receptor internalization, recycling, binding and endocytosis of the stably transfected HEK293T cells expressing the cytoplasmic domain mutants of MARCO, I was able to answer various questions regarding the MARCO-mediated endocytosis and the role of the cytoplasmic tail in MARCO function. However, there are still many areas that need further clarification and investigation, namely the molecular mechanism of MARCO-mediated endocytosis and the specific signalling regions within the cytoplasmic tail for MARCO-mediated endocytosis and trafficking.

Little is known about the function of the cytoplasmic tail of MARCO. The cytoplasmic domain of the other major Class A scavenger receptor, SR-A, has been characterized more extensively. In Section 1.3.2.1, I discussed the differences and similarities between the cytoplasmic tail of MARCO and SR-A. Therefore, the studies on the role of the cytoplasmic tail of SR-A guides the experiments performed in the thesis.

Since the receptor-mediated endocytosis is well characterized, I chose to follow the classification system for endocytic mechanisms proposed by Mayor and Pagano (Mayor and Pagano 2007) (Fig 4.1) to logically elucidate the MARCO-mediated endocytic pathway.

6.1 Summary of main findings

First, I hypothesized that the cytoplasmic domain of MARCO is necessary for cell surface expression and trafficking. To investigate this hypothesis, the cytoplasmic mutant, Δ1-34hMARCO, which includes the conserved sequences, but lacks the remainder of the cytoplasmic domain was stably expressed in HEK293T cells. The results showed that the cell surface expression level of the cytoplasmic domain mutant was similar to that of the full-length MARCO. These results indicated that amino acids 1-34 are not required for MARCO surface expression. Combining these results with a previous study (Kroos 2011) which showed that Δ1-40hMARCO displayed a reduced level of surface expression, I further concluded that amino acids 35-40, KPKRRN, are necessary for complete MARCO surface expression. I also examined the role of the cytoplasmic tail of MARCO in trafficking and I showed that both Δ1-34hMARCO and the full-length hMARCO are internalized at the same rate from the cell membrane with or without MARCO ligands. This suggested that amino acids 1-34 were not required for MARCO receptor internalization. Additionally, I found that hMARCO surface expression increases significantly while Δ1-34hMARCO expression remains unchanged with ligand stimulation but not monensin inhibition. Thus, I concluded that amino acids 1-34 are required for ligand-induced receptor recycling.

Second, I sought we elucidate the molecular pathway of MARCO-mediated endocytosis. It was previously shown that MalBSA is a good ligand for scavenger receptors. Here, I confirmed that 500 nm MalBSA-coated beads were MARCO ligands for MARCO

expressing macrophages. Furthermore, I showed that MAlBSA-coated beads were highly specific ligands for stable hMARCO-HEK293T cells. I used this ligand for the subsequent studies. Next, I examined what elements of the endocytic machinery are involved in MARCO-mediated endocytosis by employing specific pharmacological inhibitors that act at various stages of endocytosis. I found that MARCO-mediated ligand uptake was almost complete inhibited by chlorpromazine, a specific inhibitor of clathrin. Thus I concluded that MARCO-mediated endocytosis required clathrin. Since it is well established that dynamin, a large GTPase that is responsible for the scission of clathrin-coated vesicles, is required for clathrin-mediated endocytosis (Doherty and McMahon 2009), I determined whether dynamin is required for MARCO-mediated endocytosis using a dynamin-specific inhibitor, dynasore. As a result, I showed that ligand uptake by MARCO was reduced significantly in the presence of dynasore. Thus dynamin was required for MARCO-mediated endocytosis. Lastly, since actin is also implicated in clathrin-mediated endocytosis, I tested whether MARCO-mediated endocytosis requires the involvement of actin by inhibiting endocytosis with CytoD, an actin polymerization inhibitor. My results indicated that MARCO-mediated ligand uptake was abrogated by CytoD, thus suggesting that actin was required in MARCO-mediated endocytosis. Taken together, I determined that MARCO mediates endocytosis via a clathrin-mediated, dynmin-dependent pathway that required intact actin network.

Third, I hypothesized that the cytoplasmic tail plays a critical role in MARCO-mediated endocytosis described above. The endocytosis assay comparing full-length hMARCO and the cytoplasmic domain mutant Δ1-34hMARCO-HEK293T cells showed that the ligand

uptake was significantly reduced when the cytoplasmic tail is missing. There was no significant difference in the capacity of ligand binding between hMARCO and Δ1-34hMARCO-HEK293T cells. In conjunction with the observation that there was no difference in surface expression between hMARCO and Δ1-34hMARCO, I thus concluded that amino acids 1-34 of MARCO were necessary for complete ligand uptake by MARCO. To determine at what stage the cytoplasmic tail of MARCO is involved in MARCO-mediated endocytosis of MalBSA-coated beads, I treated hMARCO and Δ1-34hMARCO-HEK293T cells with specific inhibitors CPZ, dynasore and cytoD. My results indicated that bead uptake by Δ1-34hMARCO-HEK293T cells were not inhibited by CPZ or dynasore. CytoD induced a 90% inhibition of bead uptake by Δ1-34hMARCO-HEK293T cells. Taken together, I determined that amino acids 1-34 of MARCO were required for the clathrin-dependent and dynamin-regulated ligand uptake by MARCO but were not required for the actin involvement of MARCO-mediated endocytosis.

In summary, the studies presented in this thesis determined that MARCO mediates endocytosis via a clathrin-mediated, dynamin-dependent pathway that involves actin. The cytoplasmic domain, more specifically, amino acids 1-34, are required for clathrin-dependent and dynamin-regulated ligand uptake but not required for the actin involvement of MARCO-mediated endocytosis. Additionally, the cytoplasmic tail might also be important for MARCO cell surface expression and recycling but not receptor internalization.

6.2 Proposed models of MARCO trafficking and receptor mediated endocytosis

I demonstrated in Chapter Four that MARCO-mediated endocytosis was clathrin-mediated, dynamin-regulated and required actin. I further showed in Chapter Five that the cytoplasmic domain of MARCO was essential for clathrin dependency and dynamin regulation in MARCO-mediated endocytosis while actin involvement did not require amino acids 1-34. Fig 6.1 presents a model of MARCO trafficking and endocytosis based on the findings described above. Briefly, MARCO is inserted into the ER via a hydrophobic sequence that is presumably present in the transmembrane domain of MARCO (Lodish *et al.* 2003). The PKRRN sequence in the cytoplasmic tail of MARCO might provide additional signals for trafficking to the cell surface. At the cell surface, when MARCO is engaged with its cognate ligand via its SRCR domain, a conformational change is presumbly induced in MARCO and this leads to the binding of MARCO-ligand complex to an adaptor protein, possibly AP-2 via the recognition of the putative di-leucine motif in the cytoplasmic domain of MARCO. Actin might be acting at the sites of the invagination of the plasma membrane. Clathrin subsequently binds to AP-2 to form a clathrin coat around the budding vesicles. As the clathrin-coated vesicles continue to mature, dynamin, a large GTPase, forms a spiral around the neck of the budding vesicles, extends lengthwise and constricts through GTP hydrolysis. When the vesicle is fully mature, it pinches off the vesicle from the parent membrane due to the lengthening and tightening of the coil around the vesicle neck. MARCO is then transported to the endosome, and, depending on the signals received, MARCO will be targeted back to the

cell membrane or to the late endosome for degradation. It is possible that the MARCO molecules that are targeted for lysosomal degradation are monoubiquitinated while the MARCO mlecules that are recycled back to the membrane are polyubiquinated (Thoms and Erdmann 2006). K4, K5, K35 and K37 residues might be involved in the ubiquitination of MARCO. Fig 6.2 summarizes the potential regions of the cytoplasmic tail of MARCO involved in MARCO trafficking and receptor-mediated endocytosis discussed throughout this thesis.

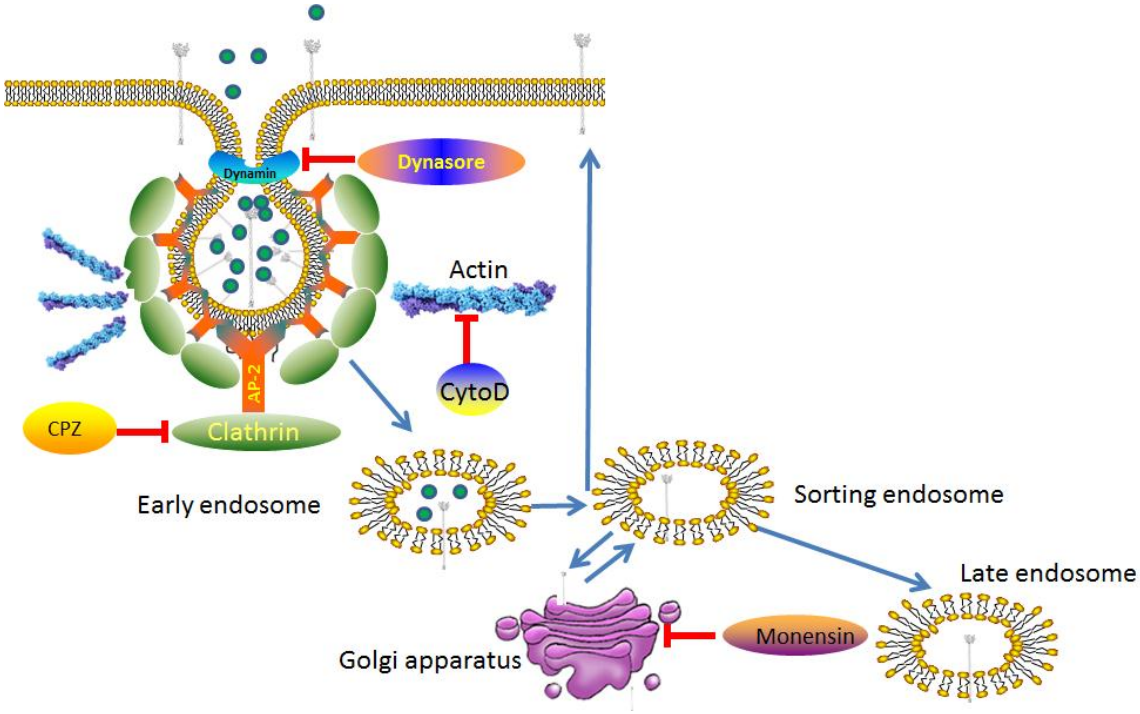


Fig 6.1 A proposed model of MARCO tranfficking and MARCO-mediated endocytosis. MARCO is inserted into the ER via a hydrophobic sequence that is present in the trasmembrane domain of MARCO (Lodish *et al.* 2003). The PKRRN sequence in the cytoplasmic tail of MARCO might provide additional signals for trafficking to the cell surface. At the cell surface, when MARCO is engaged with its cognate ligand via its SRCR domain, a conformational change is presumably induced in MARCO and this leads to the binding of MARCO-ligand complex to an adaptor protein, possibly AP-2 via the recognition of the putative di-leucine motif in the cytoplasmic domain of MARCO. Meanwhile, actin might be acting at the sites of the invagination of the plasma membrane. Clathrin subsequently binds to AP-2 to form a clathrin coat around the budding vesicles. As the clathrin-coated vesicles continues to mature, dynamin, a large GTPase, forms a spiral around the neck of the budding vesicles and extends lengthwise and constricts through GTP hydrolysis. When the vesicle is fully mature, it pinches off of the vesicle from the parent membrane due to the lengthening and tightening of the coil around the vesicle neck. MARCO is then transported to the endosome, depending on the signals received, MARCO will be targeted back to the cell membrane or to the late endosome for degradation. It is possible that the MARCO molecules that are targeted for lysosomal degradation are monoubiquitinated while the MARCO molecules that are recycled back to the membrane are polyunbiquinated. K4, K5, K35 and K37 residues might be involved in the ubiquitination of MARCO.

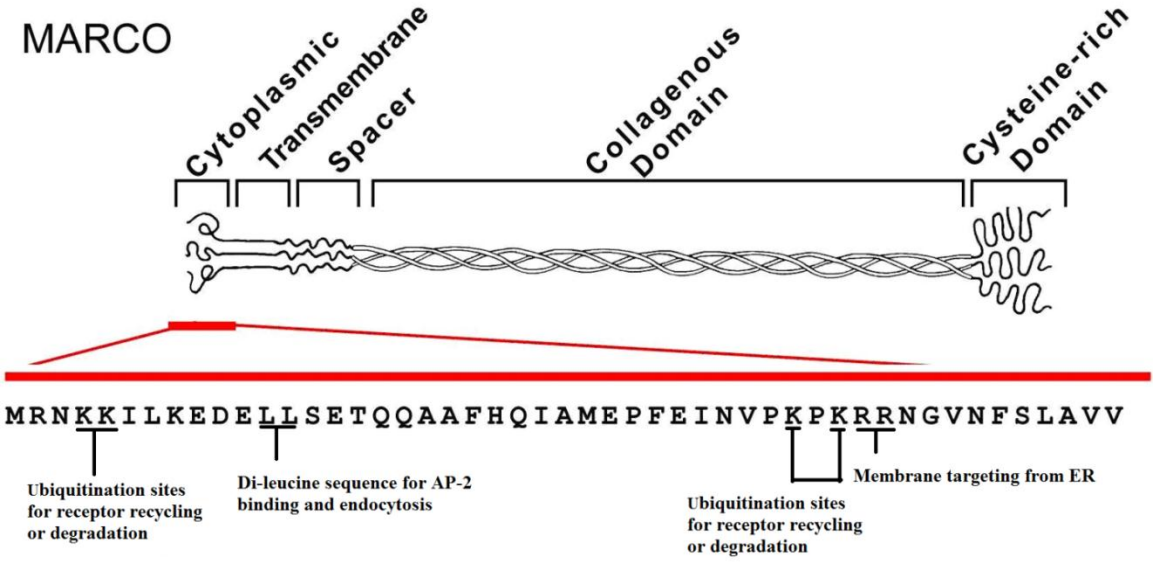


Fig 6.2 The potential regions in the cytoplasmic domain of human MARCO that are important for MARCO trafficking and endocytosis. The potential Lysine sequence might be involved in recycling and degradation of MARCO via ubiquitinnation, as was shown in Chapter Three. Other possible sequences that might affect membrane trafficking include the di-leucine motif, the R38 and R39. It is important to note that the precise function of these amino acid sequence remain to be experimentally proven.

6.3 General discussion and future directions

Although I have successfully identified that MARCO mediated ligand uptake occurs via clathrin-mediated endocytosis, we did not show that MARCO is physically associated with clathrin. For future studies, I can perform co-immunoprecipitation of MARCO with anti-clathrin antibodies and probe MARCO with an anti-human MARCO 9805 antibodies. Alternatively, high resolution microscopy can be performed to identify co-localization of MARCO and clathrin with the presence of a MARCO ligand, such as the fluorescently-labelled MalBSA-coated bead. If the di-leucine sequence is truly the binding sites for AP-2, then I would expect that the cytoplasmic tail mutant Δ1-34hMARCO not to be co-localized with clathrin.

One caveat to these studies is that I used pharmacological inhibitors. Although I noted that treatment of HEK293T cells with the inhibitors did not significantly affect cell viability or MARCO surface expression, I could design alternative experiments to confirm my findings. First, we can design siRNA that specifically targets components of the endocytic pathway to knock down the gene expression of the molecule of interest. Second, if available, I can transiently express a dominant negative mutant of a molecule to inhibit its function. Dynamin K44A is one such mutant. It has been demonstrated that over expression of this mutant impairs endocytosis of transferrin (Hinshaw, 2000).

I concluded that amino acids 1-34 were essential for MARCO-mediated and clathrin-mediated endocytosis. I note that a significant amount of MalBSA-coated beads were specifically taken up by Δ1-34hMARCO. This could suggest that amino acids 35-50

within in the cytoplasmic tail of MARCO might be important for MARCO ligand uptake via a clathrin-independent mechanism. Further experiments are needed to explore this possibility.

I also note that caveolin-1 expression in HEK293T cells is very low compared to other cell lines and primary macrophages. I only used a very specific ligand to study MARCO-mediated endocytosis. I cannot rule out the possibility that MARCO might mediate caveolae-dependent endocytosis with a different ligand in a cell that abundantly expresses caveolin.

I hypothesized which residues have endocytic signalling capacity in MARCO described in Fig 6.2. To confirm if the lysine residues are truly involved in MARCO trafficking, I can perform site-directed mutagenesis targeting K4A, K5A, K35A and K37A, individually or combined, and determine if MARCO internalization, surface expression and recycling is impaired. Alternativley, I can directly probe for ubiquitinylation of the cytoplasmic tail of MARCO with anti mono/polyubiquitin antibodies. Additionally, a Δ1-40hMARCO-HEK293T stable cell would be helpful in determining if R38 and R39 are important for MARCO surface expression.

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