THE MANNOSE RECEPTOR IN MACROPHAGE BIOLOGY

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Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

DECEMBER 2010

Abstract

The Mannose receptor (MR) is a type I membrane molecule involved in both haemostasis and pathogen recognition. Its extracellular domains have broad ligand specificities: the cysteine-rich (CR) domain is involved in sulphated sugar binding, the C-type lectin-like domains (CTLDs) are responsible for the detection of sugars terminated in mannose, fucose or N-acetylglucosamine, and the fibronectin-type II (FNII) domain mediates collagen binding.

Its recently discovered collagen binding ability raised the question of MR facilitating cellular adhesion which would then influence its function as an endocytic receptor in collagen-rich mammalian tissues. For this purpose, the level of MR-mediated endocytosis, and MR expression was analyzed by using bone-marrow-derived macrophages (BM-MΦ) plated on extracellular matrix (ECM) proteins including fibronectin (not a MR ligand), collagen type I or IV (MR-ligands). The results showed no difference in the level of MR-mediated endocytosis and MR expression at both mRNA and protein levels upon MΦ adhesion to collagen. This suggests that MR interaction with collagen may simply be crucial for tissue remodelling and wound healing, rather than adhesion.

MR is also expressed in a soluble form (sMR) which is comprised of the extracellular region of intact cell-associated MR (cMR). Even though its precise role is not yet clear, enhanced sMR production was previously shown

to help Pneumocystis carinii to evade MΦ phagocytosis by forming a protective coat around the organism. In this work, the mechanism responsible for the fungi-induced MR-shedding was studied by treating MΦ with fungal particles in the presence and the absence of a wide-range of inhibitors. After treatment in serum-free conditions, the cell lysate and cell culture supernatants were analyzed by western blot, for cMR and sMR expression respectively.

It was shown that fungi species other than P. carinii can also trigger sMR production, and that this effect mainly takes place through β-glucan recognition. Using bio-active particulate β-glucan, it was also demonstrated that MR cleavage upon β-glucan recognition requires dectin-1-mediated signalling involving Syk, PI3K, and, partially, Raf-1 and that is mediated by a non-secreted metalloproteinase.

Dectin-1-mediated MR-shedding may partially explain the contradictive data on the involvement of cMR in the development of immunity against fungi, as well as other pathogens recognised by dectin-1. The ability of pathogens to evade or activate the immune response may depend on the balance between sMR and cMR expression levels.

Publications

GAZI, U. & MARTINEZ-POMARES, L. (2009) Influence of the mannose receptor in host immune responses. Immunobiology, 214**,** 554-61.

Anknowledgements

I would like to thank Dr. Luisa Martinez-Pomares for setting up such an interesting and challanging project that enabled me to develop a wide-range of research skills, as well as for providing guidance throughout the project and writing period. I also appreciate greately the Lectin Biology Group members, and other stuff and students in the School of Molecular Medical Sciences for creating such a good working atmosphere. Additionally, I would like to thank current and past Pathology Research Group members for teaching me qPCR techniques, Sigrid Heinsbroek (University of Amsterdam) for the Candida albicans particles, Philip Taylor (Cardiff University) for dectin-1-KO mice macrophages, David Williams (East Tennesse University) for β-glucan particles and β-glucan-phosphate, Gordon Brown (University of Aberdeen) for the Aspergillus fumigatus particles, and Simon Johnson (University of Nottingham) for his help in zymography. I am grateful to the MRC and Mizutani Foundation for Glycoscience for funding this work. Finally, I would like to thank my parents and sister for their love and support throughout my studies, as well as Elpida Petraki for her endless understanding and help especially throughout the writing period of this thesis.

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List of Abbreviations

AA-MΦ	Alternatively activated macrophages
ADAM	A disintegrin and metalloprotease
APC	Antigen presenting cells
BCL10	B-cell lymphoma protein 10
BCR	B-cell receptor
BM-MΦ	Bone marrow-derived macrophages
BP	Bacteriologic Plastic
BSA	Bovine serum albumin
CA-MΦ	Classically activated macrophages
CARD9	Caspase recruitment domain-containing protein 9
CR	Cysteine-rich
CRD	Carbohydrate recognition domain
CTL	C-type lectin
CTLD	C-type lectin-like domain
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
Fc	Fragment crystallizable
FcR	Fc receptors
FcRγ	FcR common γ chain
FCS	Foetal calf serum
FNII	Fibronectin type II
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
HRP	Horseradish peroxidase
Ig	Immunoglobulins
IL	Interleukin
IRF	Interferon-regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
KC	Keratinocyte chemoattractant
LCM	L929-conditioned media
LPS	Lipopolysaccharide

LRR	Leucine-rich repeat
MALT1	Mucosa-associated lymphoid tissue transformation protein 1
MBL	Mannose binding lectin
MCP	Macrophage chemoattractant protein
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
$mPLA_2R$	M-type phospholipase A_2 receptor
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor-kappa beta
PAA	Polyacrylamide
Pam_3CSK_4	Tripalmytoyl-cysteinyl-seryl-(lysyl)3-lysine
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol myristate acetate
PRR	Pathogen recognition receptor

qPCR	Real-time quantitative PCR
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SP	Surfactant protein
SR	Scavenger receptor
TC	Tissue culture plastic
TCR	T-cell receptor
TIR	Toll/IL-IR
TLR	Toll-like receptors

1. INTRODUCTION

1.1. The Immune Response: An Overview

The immune system is comprised of both innate and pathogen-specific adaptive immunity. Innate immunity provides the first line of defence, and its components are mostly present before the beginning of infection. In contrast, adaptive immunity is not triggered until there is an antigenic challenge to the host, and responds with high degree of specificity. This branch also exhibits a "memory" property which enables the host to neutralise the pathogen upon its second entry more quickly and effectively. Lymphocytes and antibodies are the main components of adaptive immunity.

1.1.1. Innate immune response

Innate immunity is comprised of at least three types of defensive barriers: an anatomical barrier, which involves mechanical (e.g. skin and mucous membranes), chemical (e.g. low pH, and chemicals released by epithelial cells) and biological factors (e.g. resident/native flora); cellular barriers (e.g. macrophages (MΦ), dendritic cells (DC), natural killer (NK) cells, and mast cells); and humoral barriers (e.g. complement system). Once the anatomical barrier is evaded, pathogens are faced with the humoral and

cellular barriers that are able to initiate an immediate response leading to inflammation.

1.1.1.i. Cellular barriers of the innate immune system

1.1.1.i.a Macrophages

MΦ are found in almost all tissues and are derived from monocytes circulating in the blood-stream (discussed below). Since most pathogens enter the host body through the mucosa of the gut and the respiratory system, tissue MΦ constitute the first line of defence against an invading pathogen, and throughout the innate immune response they are involved in several different processes (Soehnlein and Lindbom, published online in 2010). One such activity is the removal and the killing of invading microorganisms by phagocytosis. Even though monocytes can also phagocytose, differentiation into MΦ enhances its phagocytic ability. As a first-line of defence, MΦ also play a crucial role in the coordination of the immune response through the release of inflammatory mediators such as cytokines (e.g. interleukin (IL)-1β, IL-6, IL-12, and tumor necrosis factor (TNF)-α) and chemokines (e.g. CXC chemokine ligand (CXCL)-8, CC chemokine ligand (CCL)-4, and CCL2) involved in the induction of inflammation and the activation of other immune-system cells. Activated MΦ also express high levels of class II major histocompatibility complex (MHC) molecules that enable them to function as an antigen-presenting cell (APC) involved in the initiation of adaptive immune

response (discussed below) (Soehnlein and Lindbom, published online in 2010, Taylor et al., 2005b).

1.1.1.i.b. Dendritic cells

DC acquired their name from their long membrane extensions that look like the dendrites of nerve cells. Through the blood-stream, immature DCs migrate from the bone marrow to the tissues where they are involved in the internalization of antigens by phagocytosis or endocytosis. Even though they are able to degrade antigens they take up, their main role is not the clearance of invading pathogens, rather, following the uptake of microorganism immature DCs mature into cells which are capable of activating helper T-cells (T_H-cells) and migrate to secondary lymphoid organs (Geissmann et al., published online in 2010) (Lee and Iwasaki, 2007, Melief, 2008, Satthaporn and Eremin, 2001).

1.1.1.i.c. Natural killer cells

Natural killer (NK) cells were initially identified by their ability to destroy tumour cells in the absence of any previous immunization with the tumour. Today, they are also known to facilitate the early immune response against infections with certain viruses and intracellular bacteria. NK cells are non-T, non-B lymphoid cells with well-characterised intracellular granules rich in perforin and granzymes which are able to induce apoptosis of the target cell upon release, and granulysin which has a direct antimicrobial activity. NK cells

can detect abnormal cells through two different ways. In some cases, NK cells distinguish the target cell through the detection of the reduced expression level of MHC class I molecules and the unusual profile of surface antigens by inhibitory and activating receptors respectively. NK cells cannot target the cells that express normal levels of MHC class I molecules. However, the inhibitory signal is lost during infection due to the reduced expression of MHC class I molecules. Another way of targeting cell detection by NK cells is known as antibody-dependent cell-mediated cytotoxicity and is mediated through the recognition of antibodies bound to the target cell by the Fc receptors (FcR) expressed on the NK cell surface (e.g. FcRγIII) (Caligiuri, 2008) (Shibuya, 2003).

1.1.1.i.d. Mast cells

Even though mast cells are best known for their role in allergic responses, they are also believed to facilitate the protection of internal surfaces of the host against pathogens, and are involved in the immune response against parasitic worms. During their development, mast cells migrate to peripheral tissues, such as skin, mucosa and airways where they differentiate into their mature forms. Their intracellular granules contain a mixture of chemical mediators (e.g. histamine, leukotrienes) that increase the vascular permeability required for the induction of inflammation (discussed below). Degranulation is mainly triggered by the signalling through FcεRI (specific for IgE) expressed on the mast cell surface (Gilfillan and Tkaczyk, 2006).

1.1.2. Inflammation

The cellular barrier of innate immunity can discriminate self from non-self through the recognition of repetitive structures unique to microorganisms, called pathogen-associated molecular patterns (PAMPs), directly by the cell-associated pattern recognition receptors (PRRs) (e.g. Toll-like receptors) or indirectly by soluble PRR (e.g. mannose binding lectin, MBL) or complement fragments (e.g. C3b) (Lee and Kim, 2007). Apart from inducing phagocytosis, pathogen recognition also results in the secretion of cytokines (e.g. IL-1β, IL-6, IL-12, TNF-α), chemokines (e.g. CXCL8, CCL4, CCL2) and lipid mediators (e.g. prostaglandins, leukotrienes, and platelet-activating factor) that are quickly produced through an enzymatic degradation of membrane phospholipids (Soehnlein and Lindbom, published online in 2010).

The secreted products initiate the process known as inflammation which involves the recruitment of immune cells and molecules of innate immunity into the sites of infection. The recruitment is promoted by the dilation and increased permeability of blood vessels, as well as by the enhanced expression of cell-adhesive molecules by endothelial cells (e.g. P-selectin and E-selectin) (Soehnlein and Lindbom, published online in 2010, Huang and Vita, 2006). The resultant increased local blood flow and leakage of fluid into tissues causes heat, redness, swelling and pain. Additionally, inflammation also helps the activation of adaptive immunity by increasing the flow of lymph with microbes and antigen-bearing cells to nearby lymphoid

tissues where the adaptive immune response is activated (Lee and Iwasaki, 2007).

Neutrophils are the predominant cell type initially recruited to the site of infection as a result of the CXCL8 chemokine released by the MΦ in response to an invading pathogen. In turn, neutrophils are also involved in the recruitment of MΦ to the site of inflammation through the production of chemokines such as macrophage inflammatory proteins (MIP-1α and -1β) (Soehnlein and Lindbom, published online in 2010, Arndt et al., 2002). Therefore, the initial phase of an inflammatory response is mostly dependent on MΦ and neutrophils, and as the response progresses, other cell types such as monocytes and immature DC are also recruited to contribute to the destruction of an invading microorganism. The recruited monocytes can differentiate into MΦ as well as DCs depending on the signals received from the surrounding environment (Soehnlein and Lindbom, published online in 2010, Gordon and Taylor, 2005).

1.1.3. Adaptive immunity

1.1.3.i. T-cell activation

The adaptive immune response is triggered in the secondary lymphoid organs as a result of the activation of naïve T_H-cells by APCs (e.g. DC, which are specialised in the activation of naïve T-cells). Following antigen internalization via phagocytosis or endocytosis, APCs are able to process the

antigen into smaller peptides and present the processed peptides in the context of MHC II molecules on the cell surface. This antigen processing is mediated by the endocytic pathway, which is comprised of three increasingly acidic compartments: early endosome (pH ~6.0-6.5), late endosome (pH ~5.0-6.0), and lysosome (pH ~4.5-5.0) (Male, 2006, Leyva-Cobian and Carrasco-Marin, 1994).

Especially during the immune response against intracellular pathogens (e.g. virus) not infecting APC directly, or against not endogenously expressed tumour antigens, APC can also present the internalized exogenous antigens in the context of MHC class I molecules through the process called 'cross-presentation' (Burgdorf et al., 2008). The underlying mechanism responsible is not yet clear. However, it is suggested that the internalized material can exit the endocytic pathway and enter the cytosolic pathway, which facilitates the processing of endogenous antigens (e.g. viral proteins), and involves the same route followed during the degradation of misfolded intracellular proteins (Burgdorf et al., 2007, Heath and Carbone, 2001).

In contrast to the cells expressing antigenic peptides in the context with MHC class II molecules, the cells displaying antigen-MHC class type I complex are destroyed by $CD8^+$ T_C-cells, and therefore are named target cells. Since almost all nucleated cells express MHC class I, they can all be designated as target cells.

The detection of antigen:MHC complex by T-cells is mediated through the T-cell receptors (TCRs). However, the signal transduced by the recognition

of the antigen-MHC complex is not enough for T-lymphocyte activation on its own. It requires an additional, antigen-nonspecific, co-stimulatory signal induced by the engagement of T-cell CD28 with B7 family member molecules on APC (signal two). In the absence of this second signal, antigen-MHC complex interaction with TCR leads to the state of non-responsiveness, called clonal anergy, which is characterized by the inability of cells to proliferate and by minimal cytokine production (especially IL-2) (Fathman and Lineberry, 2007, Krammer et al., 2007, Dure and Macian, 2009).

1.1.3.ii. Effector T-cell subsets

There are two distinct large groups of naïve T-cells ($CD4^+$ and $CD8^+$ T-cells) which detect different types of MHC molecules. $CD4^+$ T-cells are involved in the detection of antigens in association with class II MHC molecules, which are expressed only by APCs, while $CD8^+$ T-cells are responsible for the recognition of cells displaying antigens bound to class I MHC molecules.

Upon activation, $CD8^+$ T-cells differentiate into $CD8^+$ cytotoxic T-cells (also known as cytotoxic lymphocytes or CTL), which aim to destroy target cells. Most $CD8^+$ T-cells cannot become activated solely by the APCs, and therefore require help from $CD4^+$ helper T-cells. Helper T-cells both release IL-2 and induce an increased level of co-stimulatory activity on the same APC which helps to drive $CD8^+$ T-cell proliferation (Zhang et al., 2009). Like NK

cells, activated CTLs mediate cell killing mainly through the release of perforin and granzyme that trigger apoptosis in the target cell. They also express the membrane-bound effector molecule Fas ligand (CD178) which, upon interacting with Fas on the target cell, signals apoptosis (Choy, published online in 2010).

In contrast to $CD8^+$ T-cells, $CD4^+$ T-cells differentiate into a number of effector T-cells (T_H1, T_H2, T_H17, and regulatory T cells) which are determined by the cytokines secreted by the APC. Therefore, the receptors involved in microbial detection and the intracellular signalling triggered are of crucial importance for the determination of the appropriate type of immune response (Gutcher and Becher, 2007). For instance, among the PRRs involved in the detection of fungi, dectin-1 and dectin-2 were previously shown to promote the differentiation of T_H17 cells (LeibundGut-Landmann et al., 2007) (Robinson et al., 2009).

T_H17 cells are induced by cytokines IL-6, IL-21, and IL-23, and mediate immune responses against extracellular pathogens. T_H17 cells derived their name from the ability to release IL-17 which has a key role in the induction of inflammatory responses by triggering the release of many inflammatory cytokines (e.g. IL-6) and chemokines (e.g. IL-8). T_H17 cells can also recruit and activate neutrophils during immune response, and were shown to promote many organ-specific autoimmune diseases (Zhu and Paul, 2008).

Both T_H1 and T_H2 cells are induced by the cytokines (interferon (IFN)-γ and IL-12, and IL-4, respectively) that suppress T_H17 cell-mediated immune

responses. Both of these T-cell subsets can be distinguished from each other by the cytokines they produce: T_H1 cells mainly produce IFN-γ and IL-2, while T_H2 cells secrete predominantly IL-4 and IL-5 (McGhee, 2005).

T_H1 cells mediate the immune response against intracellular pathogens (Zhu and Paul, 2008). They increase the microbicidal activity of MΦ by both producing pro-inflammatory cytokines (e.g. TNF-α, granulocyte-MΦ colony-stimulating factor (GM-CSF) and IFN-γ) and interacting with the specific antigen:MHC type II complex, and CD40 on the MΦ surface (Suttles and Stout, 2009) (Murphy, 2008). They are also involved in the induction of opsonising antibody production (mainly IgG) by B-lymphocytes.

T_H2 cells are associated with humoral immunity. They mediate the immune response against extracellular parasites (e.g. helminths) and are also involved in the induction and persistence of allergic diseases (Zhu and Paul, 2008). T_H2 cells produce cytokines (e.g. IL-4, IL-5, and IL-13) that induce B-cell activation, differentiation, proliferation, and production of alternative immunologlobulin types, especially IgE. IgE is the antibody responsible for allergies as it degranulates both mast cells and basophils, leading to the release of active mediators such as histamine, and serotonin (Zhu and Paul, 2008). T_H2 cells are also involved in the activation of naïve B-cells, in order to proliferate and release IgM.

Among the $CD4^+$ T-cell subsets, regulatory T cells (T_{reg}) function to suppress T-cell responses by limiting the immune response and preventing autoimmune responses. They develop in conditions free of pathogen-related

danger signals, which are characterised by a high abundance of transforming growth factor (TGF)-β and lack of IL-6, IFN-γ, and IL-12 (Zhu and Paul, 2008, Sakaguchi et al., 2008).

1.1.3.iii. B-cell activation

In many infections, pathogens multiply in the extracellular spaces of the body and spread through extracellular fluids. The protection of extracellular spaces is mediated by the humoral immune response in which antibodies produced by B-lymphocytes have a key role.

As with T-lymphocytes, B-cell activation requires two different signalling mechanisms. One signalling comes from the B-cell receptor (BCR) whose antigen-specificity is mediated by membrane-anchored antibodies (mIg). Besides MΦ and DC, B-cells are also involved in antigen presentation to T-cells. Upon mIg engagement, the bound antigen is internalized and then processed within the endocytic pathway into short peptides to be presented with class II MHC molecules on the cell surface. B-cells require BCR-mediated signalling to function as an APC in T_H-cell activation. The increased expression of both co-stimulatory and MHC class II molecules enable the interaction between T- and B-cells forming a T-B conjugate (Murphy, 2008).

The T-B conjugate formation supports the activation and proliferation of the B-cells through both cytokine production and the up-regulation of a T_H-cell membrane protein, CD40L (aka CD154). The CD40-CD40L interaction

delivers the required signal two which acts in concert with signal one to drive the resting B-cell into the cell cycle (Haxhinasto et al., 2002, Harwood and Batista, published online in 2010).

Once activated, B-cells start to express receptors for various cytokines including IL-2, IL-4, and IL-5. The engagement of these receptors by the T_H-cell-derived cytokines further support B-cell proliferation as well as enabling B-cell differentiation into antibody secreting plasma cells and memory B-cells, class-switching, and affinity maturation (Mallat et al., 2009, Harwood and Batista, published online in 2010).

1.2. Macrophages

Since the later chapters are on MΦ interaction with fungal pathogens, the following section will focus on the MΦ and membrane-anchored receptors involved in triggering an anti-fungal immune response.

MΦ belong to the mononuclear phagocyte system which is composed of cells that share the same common haematopoietic precursors that differentiate into blood monocytes (Hume et al., 2002). Following their migration into tissues, monocytes undergo maturation, and differentiate into various cell types according to the signals in the tissue microenvironment. Apart from MΦ, other differentiated cell types include osteoclasts and myeloid-derived DCs (Gordon and Taylor, 2005).

The name 'macrophage' is derived from the Greek words macros and phagein, which means "big" and "to eat", respectively. They constitute an effective branch of the phagocytic barrier against the invading pathogens, and like DCs, facilitate the cross-talk between innate and adaptive immunity by acting as APCs. However, their APC function is mostly restricted to the secondary immune responses during which MΦ facilitate the activation of already primed (memory) T-cells. Upon pathogen recognition, they release a wide-range of secretory products that influence the migration and activation of other immune cells (e.g. neutrophils during the initial phases of inflammation) (Gordon and Taylor, 2005).

Nevertheless, the importance of MΦ is not restricted solely to microbial detection, and they are also involved in the recognition of self-molecules required for maintaining heamostasis. This contributes to the resolution of inflammation which is activated once the pathogen is neutralised. As mentioned above, inflammation involves the release of a wide-range of secretory mediators which, apart from facilitating the entry of leukocytes to the sites of injury, and the communication between the immune cells, trigger deleterious effects for the tissue environment. During the resolution of inflammation, the tissue debris and secreted inflammatory enzymes are removed to allow tissue remodelling.

MΦ are mostly positioned at strategic points within tissues, while some remain mobile travelling throughout the body. The tissues in which MΦ can reside include lymphoid organs, as well as non-lymphoid organs like the liver (kupffer cells), lung (alveolar MΦ), nervous system (microglia), epidermis (langerhans cells), reproductive organs and serosal cavities. They are also abundant within the lamina propria of the gut and the interstitium of organs such as the heart, pancreas, and the kidney (Taylor et al., 2005b).

1.2.1. Monocyte heterogeneity

Like other mononuclear phagocytes, MΦ are differentiated from circulating monocytes which descend from hematopoietic stem cells (HSCs) that undergo myeloid differentiation and produce multipotent precursors

(Figure 1.1). These precursors give rise to common lymphoid progenitor cells (CLPs), and lineage-restricted common myeloid progenitor cells (CMPs) which, in response to the macrophage colony-stimulating factor (M-CSF), differentiate into monoblasts, pro-monocytes and eventually monocytes (Serbina et al., 2008) (Mosser and Edwards, 2008). After their release into the peripheral blood, monocytes circulate for several days before migrating to the tissues. Depending on the signals received from the microenvironment, the recruited monocytes then become tissue-MΦ, DCs or osteoclasts (multinucleate cells that resorb bone) (Gordon and Taylor, 2005).

The circulating monocytes constitute approximately 5-10% of the peripheral-blood leukocytes in humans and show morphological heterogeneity such as variability of size, granularity and nuclear morphology. In humans, monocytes are divided into two subsets according to the differential expression of CD14 (part of the receptor involved in lipopolysaccharide, LPS, recognition) and CD16 (also known as FcγRIII): $CD14^{hi}CD16^{-}$, and $CD14^{+}CD16^{+}$ cells (Passlick et al., 1989, Gordon and Taylor, 2005). Among those, $CD14^{+}CD16^{+}$ cells resemble mature tissue MΦ, and express higher levels of MHC class II molecules and CD32 (alternatively known as FcγRII). They are smaller in size and less granular than $CD14^{hi}CD16^{-}$ monocytes (Strauss-Ayali et al., 2007).

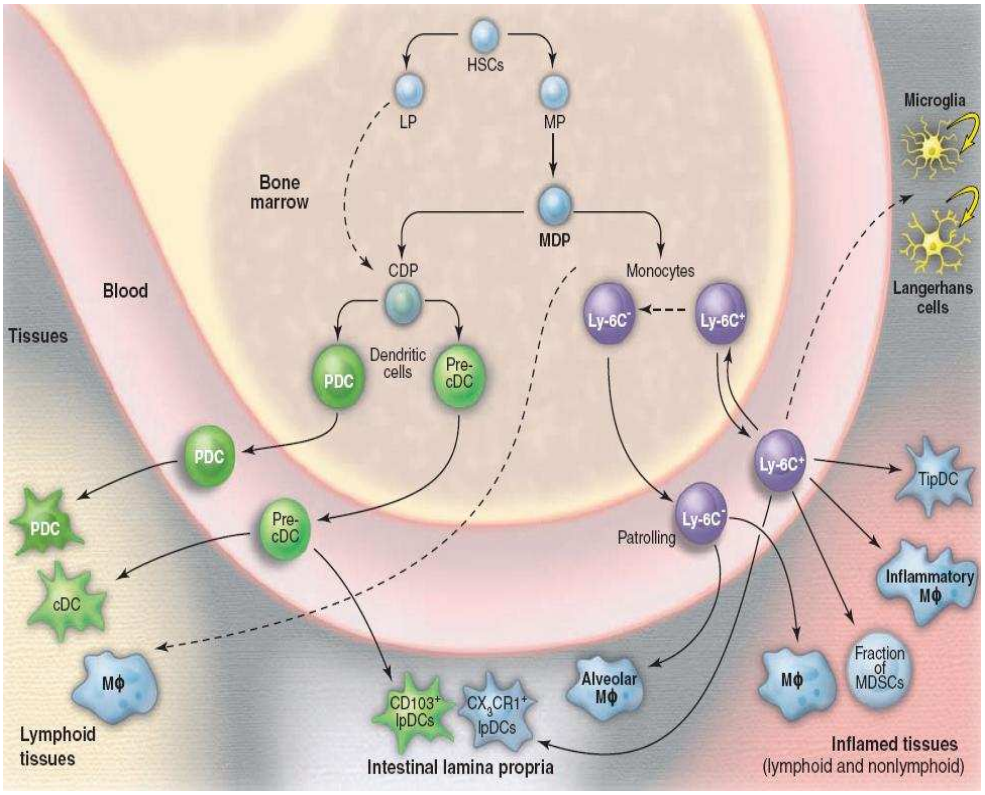

Figure 1.1: MΦ and DC differentiation in mice. HSCs differentiate into myeloid (MP) and lymphoid (LP) committed precursors in bone marrow. MPs produce monocyte, MΦ and DC precursors (MDPs), which give rise to monocytes, some populations of MΦ, and common DC precursors (CDPs). The two monocyte subsets Ly6C$^+$ and Ly6C$^-$, are released from the bone marrow to enter the blood circulation. Some studies suggested that Ly6C$^+$ monocytes can shuttle between the blood and bone marrow and lose Ly6C expression. CDPs differentiate into preclassical dendritic cells (pre-DCs), and plasmacytoid dendritic cells (PDCs). Among those pre-DCs give rise to CD8α$^+$ and CD8α$^-$ cDCs in lymphoid tissues, and CD103$^+$ lamina propria DCs (lpDCs) in nonlymphoid tissues. Under steady-state conditions, Ly6C$^-$ and Ly6C$^+$ monocytes differentiate into alveolar MΦ, and CX_3CR1^+ lpDCs respectively. During inflammation Ly6C$^+$ monocytes can differentiate into monocyte-derived DCs, for example, TNF and inducible nitric oxide synthase (iNOS)-producing DCs (TipDCs), inflammatory MΦ, and myeloid-derived suppressor cells (MDSCs) associated with tumours. They are also thought to give rise to microglia and Langerhans cells (dashed arrow), which can renew independently from the bone marrow (curved arrow). Additionally during inflammation HSCs can also enter the peripheral tissues (dahes arrow) to differentiate into myeloid cells. Today it is still not clear whether LPs contribute to PDCs and cDCs (dashed arrow) (Geissmann et al., published online in 2010).

In mice, monocytes can be divided into two main subsets according to their expression of CCR2 (CC-chemokine receptor-2), CD62L (L-selectin), CX_3CR1 (CX_3C-chemokine receptor 1), and Ly6C (part of the GR1 epitope): $CCR2^{+}CD62L^{+}CX_3CR1^{low}Ly6C^{+}$, and $CCR2^{-}CD62L^{-}CX_3CR1^{hi}Ly6C^{-}$. Among those $CCR2^{+}CD62L^{+}CX_3CR1^{low}Ly6C^{+}$ subset corresponds to human $CD14^{hi}CD16^{-}$ monocytes which are also $CCR2^{+}CX_3CR1^{low}$, while $CCR2^{-}CD62L^{-}CX_3CR1^{hi}Ly6C^{-}$ subset corresponds to human $CD14^{+}CD16^{+}$ which also express high levels of CX_3CR1 (Table 1.1) (Geissmann et al., published online in 2010) (Gordon and Taylor, 2005).

The two subsets also differ in the expression of adhesion molecules and chemokine receptors. That results in both monocyte subsets displaying differences in their susceptibility to infections, as well as migration patterns (Geissmann et al., published online in 2010, Strauss-Ayali et al., 2007). Due to the expression of CCR2 and CD62L, which are involved in inflammatory cell recruitment, $CCR2^{+}CD62L^{+}CX_3CR1^{low}Ly6C^{+}$ are rapidly recruited to the areas of damage, or inflamed tissue, and therefore are alternatively known as inflammatory monocytes. In contrast, $CCR2^{-}CD62L^{-}CX_3CR1^{hi}Ly6C^{-}$ are called resident monocytes, since they persist longer in the blood and are recruited to non-inflamed tissues (Strauss-Ayali et al., 2007) (Geissmann et al., published online in 2010) (Geissmann et al., 2003).

	Monocytes Subsets		Cellular Activities		
	Human	Mouse	Phagocytic activity	Cytokine production	Co-stimulatory activity
Inflammatory monocytes	$CD14^{hi}$ $CD16^{-}$	$CCR2^{+}$ $CD62L^{+}$ CX_3CR1^{low} $Ly6C^{+}$	Higher	Higher	Lower
Resident monocytes	$CD14^{+}$ $CD16^{+}$	$CCR2^{-}$ $CD62L^{-}$ CX_3CR1^{hi} $Ly6C^{-}$	Lower	Lower	Higher

Table 1.1: Monocyte subsets in mice and humans. $CD14^{hi}CD16^{-}$ and $CCR2^{+}CD62L^{+}CX_3CR1^{low}Ly6C^{+}$ subsets in humans and in mice are regarded as inflammatory monocytes, and are associated with a higher phagocytic and cytokine production ability than the resident monocytes ($CD14^{+}CD16^{+}$ in humans and $CCR2^{-}CD62L^{-}CX_3CR1^{hi}Ly6C^{-}$ in mice) which have a higher co-stimulatory activity (Strauss-Ayali et al., 2007, Grage-Griebenow et al., 2001, Gordon and Taylor, 2005).

Additionally, adaptive transfer experiments showed that the $Ly6C^{+}$ monocytes can shuttle between the blood and the bone marrow, and loose Ly6C expression (Figure 1.1). Furthrmore, it was suggested that

$CCR2^{+}CD62L^{+}CX_3CR1^{low}Ly6C^{+}$ monocytes pass through an intermediate phenotype of $CCR2^{+}CCR7^{+}CCR8^{+}Ly6C^{mid}$, before acquiring the $CCR2^{-}CD62L^{-}CX_3CR1^{hi}Ly6C^{-}$ phenotype (Qu et al., 2004).

The intermediate phenotype was proposed to have similar allogeneic cell stimulating capacity to the $Ly6C^{-}$ monocytes, and to express higher CCR7 and CCR8 mRNA levels than the other two monocyte subsets (Gordon and Taylor, 2005). In case of infection, both $CCR2^{+}CCR7^{+}CCR8^{+}Ly6C^{mid}$ and $CCR2^{+}CD62L^{+}CX_3CR1^{low}Ly6C^{+}$ monocytes can respond to pro-inflammatory signals, and migrate to inflamed tissues. Following their recruitment to the site of infection, they start to express higher levels of CD11c, and MHC class II molecules, and differentiate into MΦ or DCs. Because of the enhanced expression of CCR7 and CCR8, the intermediate phenotype is suggested to be particularly more prone to migrate to draining lymph nodes and differentiate into DCs (Gordon and Taylor, 2005).

Nevertheless, in most recent studies, the generation of $Ly6C^{-}$ monocytes were not affected by genetic defect in or antibody-mediated depletion of $Ly6C^{+}$ monocytes (Feinberg et al., 2007) (Mildner et al., 2007, Geissmann et al., published online in 2010).

1.2.2. MΦ heterogeneity

The migration from blood, through the endothelia, the interstitium, and epithelia, requires adhesion molecules including integrins (such as β_1, β_2),

immunoglobulin-superfamily molecules (e.g. CD31), and selectins, as well as matrix metalloproteinase activity that breaks down the extracellular matrix (ECM) proteins and enables the passage between the cells (Gordon, 2003). Within the tissues, monocytes migrate along a concentration gradient formed by the interaction of proteoglycans, present in the ECM and on endothelial cells, with chemokines, cytokines and growth factors (Gordon, 2003).

Depending on the signalling received from the microenvironment (e.g. surface and secretory products of neighbouring cells, and ECM (Gordon, 2003)) tissue resident MΦ display a high degree of heterogeneity (differing in life span, morphology, and phenotype) that enables them to perform their tissue-specific functions, such as bone remodelling by osteoclasts, pathogen killing by alveolar MΦ, and apoptotic cell removal by thymic MΦ (in thymus). Nevertheless, MΦ heterogeneity can also be observed within a single organ. For instance in the spleen, marginal zone MΦ express an array of PRRs and function in clearance of blood-borne pathogens, while metallophilic MΦ that surround the splenic white pulp adjacent to the marginal sinus, express high levels of sialoadhesin. Even though the function of metallophilic MΦ is still not yet clear, they are thought to have a role in the initial response to systemic infection. The spleen also contains tingible body MΦ which facilitate the removal of apoptotic lymphocytes during germinal centre formation (Taylor et al., 2005b).

More heterogeneity is derived from the MΦ activation states that further allow MΦ to adopt their wide-range actions (Taylor et al., 2005b,

Gordon, 2003, Mosser, 2003) (Figure 1.2). These include the classical activation pathway that results in activated MΦ (CA-MΦ) induced by the combined effect of IFN-γ and LPS (Nathan, 1991). CA-MΦ have high microbicidal activity, and are associated with pro-inflammatory cytokine release and cellular immunity. This is in contrast to the alternative activation which facilitates tissue repair, and humoral activity, and is induced by IL-4 or IL-13 (Gordon and Taylor, 2005).

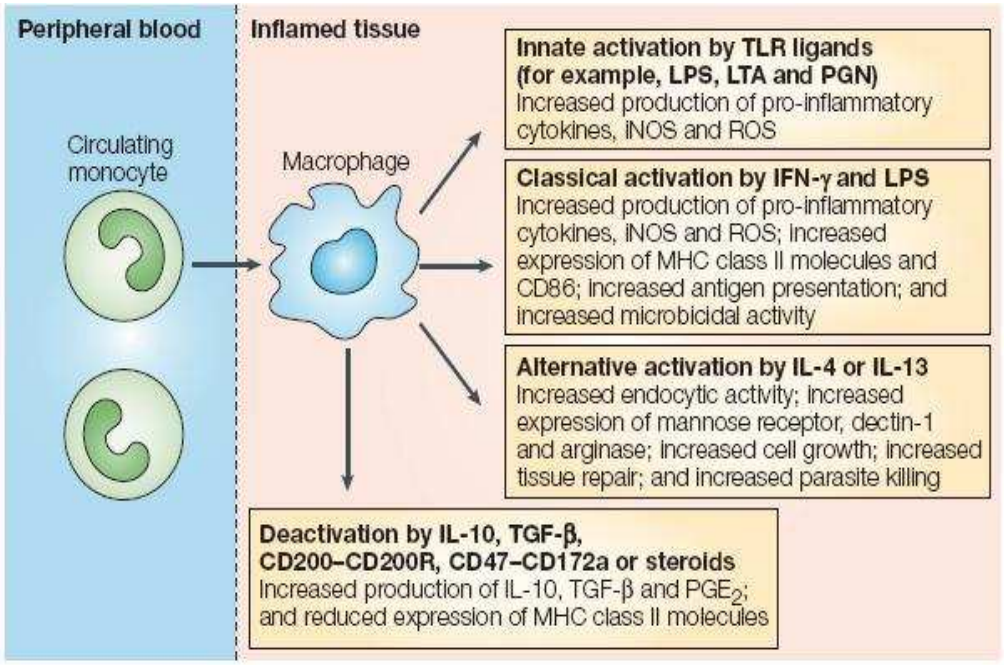

Figure 1.2: Inflammation-induced MΦ heterogeneity. During inflammation, the inflammatory peripheral-blood monocytes differentiate into different subsets with distinct phenotypes and physiological activities according to the signals available in the microenvironment (Gordon and Taylor, 2005).

MΦ can also be activated as a result of the engagement of PRRs such as Toll-like receptors (TLRs), a process which is known as innate activation (Takeda et al., 2003). Like CA-MΦ, the innate activated MΦ (IA-MΦ) possess microbicidal activity and release pro-inflammatory cytokines. However, in contrast to IA-MΦ, CA-MΦ have a higher antigen presentation and intracellular pathogen killing ability (Gordon and Taylor, 2005).

In the absence of any activating signals, such as in conditions following the neutralisation of an invading pathogen by the immune response involving both IA-MΦ and CA-MΦ, and the resolution of the inflammation by activated cells such as alternatively-activated MΦ (AA-MΦ), MΦ are found in a de-activated state. Today, it is still not clear if the fate of the MΦ is determined once, or whether it depends on a change in the microenvironment. The de-activation is mediated by cytokines such as IL-10 and TGF-β, or by signalling through inhibitory receptors, such as the CD200 receptor (CD200R). The de-activated MΦ mainly produce anti-inflammatory cytokines and have reduced MHC class II expression level (Gordon and Taylor, 2005).

1.2.3. Pathogen recognition

Microorganisms are detected by immune cells either indirectly by soluble PRR (e.g. MBL) or by complement fragments (e.g. C3b) that coat the non-self molecules, or directly by PRRs which discriminate the potentially hazardous pathogens and their associated secreted products from harmless host

cells, by recognizing pathogen-associated molecular patterns (PAMP) (Lee and Kim, 2007).

PRRs can be expressed on the cell membrane as well as in the cytoplasm. Cytoplasmic PRRs are evolved to trigger the immune response against intracellular organisms such as viral pathogens that can enter the host cell to use host machinery to replicate, and intracellular bacteria resistant to the phagocytic killing after uptake and which can gain access to the cytosol. They are also involved in the modulation of the signalling triggered by PRRs on the cell membrane to establish an efficient co-ordination of innate immune responses (Creagh and O'Neill, 2006).

In contrast to cytoplasmic PRRs, cell membrane-anchored PRRs are involved in the recognition of extracellular pathogens, and have diverse functions such as facilitating the presentation of PAMPs to other PRRs and the initiation of major signalling pathways involved in antigen uptake and the release of various inflammatory mediators. Among those, C-type lectins (CTL) are responsible for the recognition of carbohydrate structures, and together with TLRs, they have a central role in triggering the immune response against fungi whose cell wall structure is 90% carbohydrate (Geijtenbeek and Gringhuis, 2009). As the later chapters are based on the MΦ interaction with fungal pathogens in serum-free conditions, the next section will focus on the cell membrane-anchored members of these two receptor families involved in triggering anti-fungal immune responses.

1.2.3.i.Toll-like receptors

TLRs are the best characterized signal-generating PRRs, and are major cell-surface initiators of the inflammatory response to pathogens. They were first detected as a group of Drosophila proteins involved in the dorsoventral patterning in the development of embryos (Hashimoto et al., 1988). However, their effect was later found not to be development-restricted and they also have been shown to facilitate immune responses against fungal infections (Lemaitre et al., 1996). Mammalian Toll receptor homologs were detected by subsequent studies, and were therefore named as Toll-like receptors (TLRs) (Takeda and Akira, 2004).

Mammalian TLRs are type I integral membrane glycoproteins with an extracellular solenoid shaped recognition domain composed of leucine-rich repeats (LRR), and a cytoplasmic Toll/IL-IR (TIR) domain responsible for triggering an intracellular signalling cascade (Werling et al., 2009). Individual TLRs are differentially distributed within the cell and, depending on their sub-cellular location, TLRs can be categorised into two groups: TLRs that are predominantly expressed on the cell surface (e.g. TLR 1, 2, 4, 5, and 6) and TLRs that are mostly expressed in endocytic compartments (e.g. TLR 3, 7, 8, 9) (Lee and Kim, 2007).

TLRs function as homo- or hetero-dimers (e.g. TLR2-TLR6 and TLR9-TLR9 complex) or with other PRRs (e.g. TLR4-CD14) (Lee and Kim, 2007). The dimerisation is thought to be mediated by TLR-ligand engagement which results in a conformation change facilitating the two TIR domains into closer

proximity to associate symmetrically. This enforces further structural reorganization necessary for adaptor recruitment. The adaptor proteins couple to downstream protein kinases and ubiquitin ligases that eventually lead to the activation of transcription factors, such as nuclear factor-kappa beta (NF-κB), and members of the interferon-regulatory factor (IRF) family (O'Neill and Bowie, 2007).

The NF-κB transcription factors play a crucial role in establishing an effective immune response against the invading pathogen. As well as regulating the expression of a wide-variety of mediators involved in inflammation, the pathway also provides a link between innate and adaptive immune responses by inducing the release of mediators such as IL-2 that promotes T-cell proliferation and differentiation into effector cells, and TNF-α that enables the migration of APC to a nearby lymph for the activation of naïve T-cells. They also induce the cell-surface expression of co-stimulatory factors by APC (Beinke and Ley, 2004, Li and Verma, 2002) (Gerondakis and Siebenlist, published online in 2010).

IRFs are mainly involved in anti-viral immune responses by activating the transcription of type I IFNs; IFN-α and IFN-β. Both IFNs mediate their intracellular effects through binding to IFN α/β receptor that stimulates the expression of several proteins. Among those 2'-5'-oligo-adenylate synthase is responsible for the activation of ribonuclease (RNAse L) that degrades viral RNA, whereas double stranded RNA (dsRNA)-dependent protein kinase (PKR) inactivates protein synthesis and thereby blocks viral replication in

infected cells (Boo and Yang, published online in 2010). IFNs are also involved in the activation of the adaptive immune response through the induction of co-stimulatory signal expression in MΦ and DCs, and in the MHC-class type I expression in all cell types, in order to facilitate the activation of naïve and cytotoxic T-cells, respectively (Takaoka et al., 2008) (Saha et al., published online in 2010) (Tailor et al., 2006).

TLRs can detect an extremely wide-variety of PAMPs that range from intracellular structures, such as double (or single)-stranded RNA, and viral DNA to extracellular ligands such as LPS, and flagellin (Figure 1.3). Among the family members, TLR2, TLR4, and TLR9 have been reported to trigger anti-fungal immune response (Netea et al., 2008).

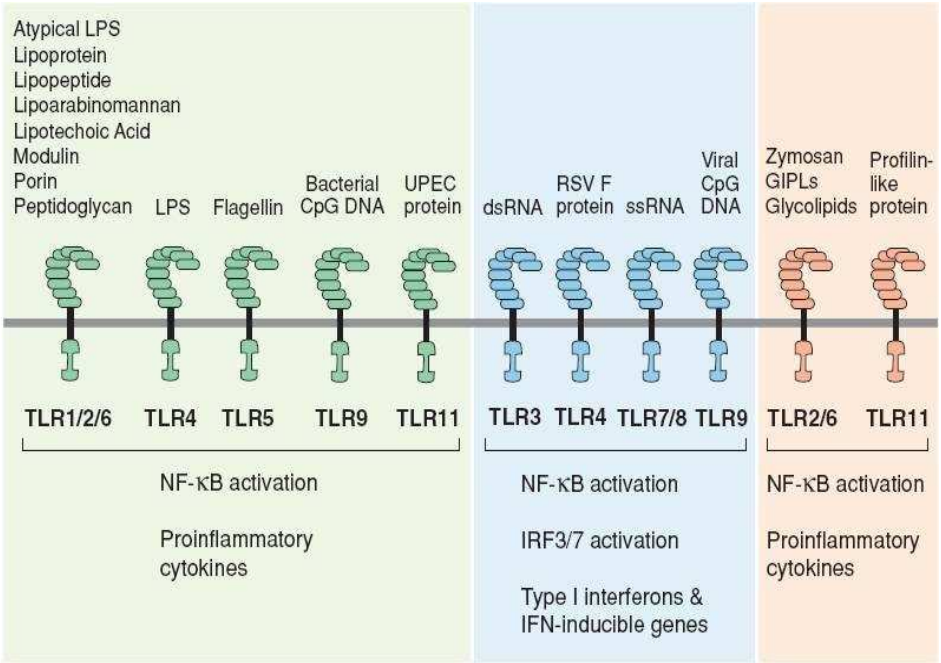

Figure 1.3: TLR ligands. TLRs are involved in the recognition of wide variety of PAMPs from bacteria, viruses, protozoa, and fungi (West et al., 2006).

TLR signalling involves a family of five adaptor proteins which are: MyD88, MyD88-adaptor-like (MAL, or TIRAP), TIR-domain-containing adaptor protein inducing IFN-β (TRIF or TICAM1), TRIF-related adaptor molecule (TRAM, TICAM2), and sterile α- and armadillo-motif-containing protein (SARM) (O'Neill and Bowie, 2007). Among these, MyD88 is a central adapter shared by almost all TLRs, and each TLR has distinct signalling pathways.

1.2.3.i.a. MyD88-dependent signalling

MyD88 has an essential role in TLR signalling and innate immunity. MyD88 deficient mice were extremely unresponsive to ligands for TLR2, TLR4, TLR5, TLR7 and TLR9, and displayed higher resistance to LPS-mediated toxic effects, as well as impaired immune responses (O'Neill and Bowie, 2007).

Following ligand binding by TLR, MyD88 is recruited to the TLR cytoplasmic domain (Akira and Takeda, 2004). This in turn brings other members of the interleukin-1 receptor associated kinase (IRAK) family, which associates with MyD88 through DD-DD (death domain) interactions (Barton and Medzhitov, 2003). So far, four IRAKs hasve been identified: IRAK1, IRAK2, IRAK4 and IRAK-M which are all ubiquitously expressed, except for IRAK-M, whose expression is monocyte/MΦ restricted (Wesche et al., 1999). Among IRAKs, only IRAK1 and IRAK4 have intrinsic serine/threonine

protein kinase activity, and the lack of kinase activity suggests a possible negative-regulatory role for both IRAK2 and IRAK-M in TLR-mediated signalling (Kawai and Akira, 2006). This was supported by Kobayashi et al, who detected an over-production of TLR-induced cytokines by IRAK-M deficient cells (Kobayashi et al., 2002).

MyD88 first activates IRAK4 that is involved in the phosphorylation and activation of IRAK-1. IRAK-1 is now able to activate the TNF receptor-associated factor-6 (TRAF-6), after which the IRAK-1/TRAF-6 complex dissociates from the receptor and interacts with additional molecules, resulting in the stimulation of the upstream kinases for NF-κB, p38 and JNK (Figure 1.4) (Akira and Takeda, 2004) (Lee and Kim, 2007) (Takeda and Akira, 2004).

MyD88 has also been reported to facilitate the induction of IRF7 by TLR7, TLR8 and TLR9 (Honda et al., 2004, Honda et al., 2005, Kawai et al., 2004). It forms a complex with IRAK1, IRAK4, TRAF6 and IRF7 such that IRF7 becomes phosphorylated by IRAK-1 (Hochrein et al., 2004). Additionally, MyD88 was suggested to promote the activation of IRF5 and IRF1 among which the latter appears to interact with MyD88 before being translocated to the nucleus (Negishi et al., 2006).

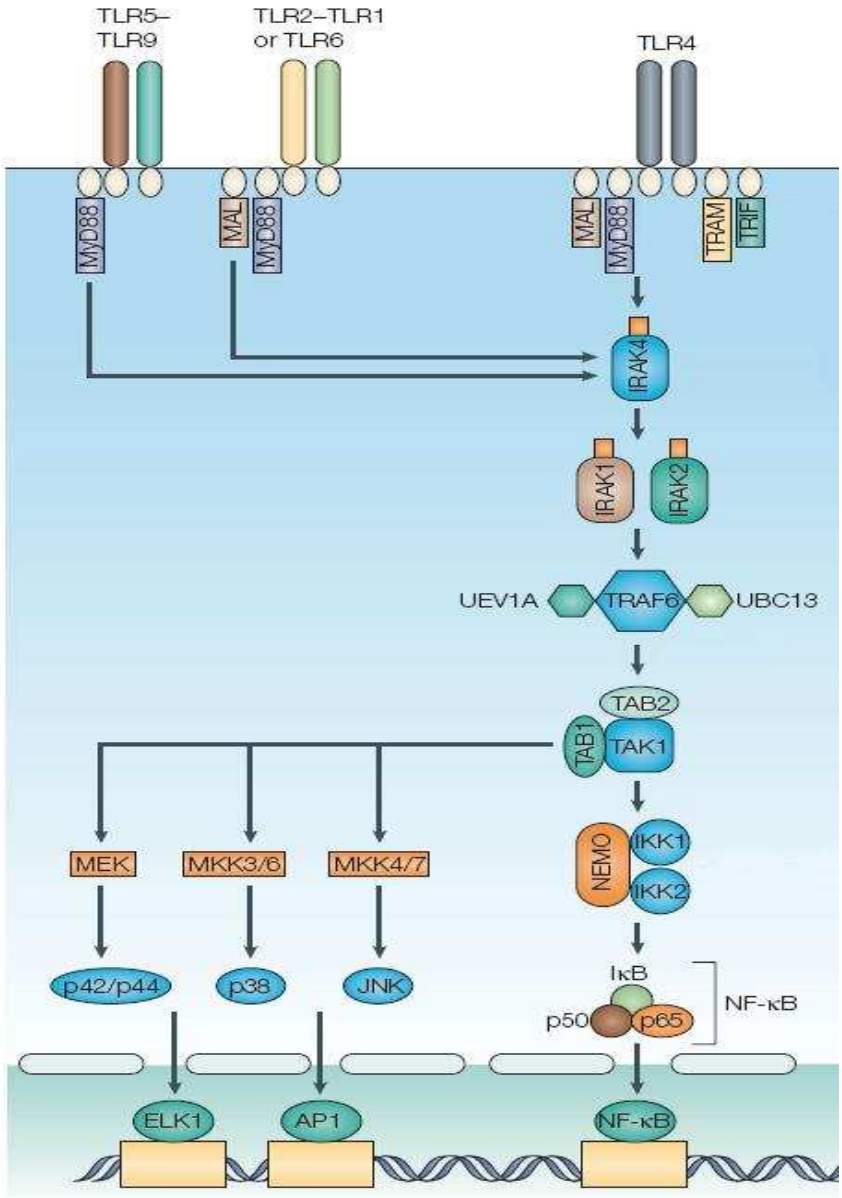

Figure 1.4: Signalling mediated by MyD88 and MAL. MyD88 is a central signalling adaptor used by all TLRs except for TLR3. It directly interacts with TIR domains and facilitates the IRAK-4 and IRAK-1 activation. IRAK-1 is now able to activate the TRAF-6. Activated TRAF-6 binds ubiquitin-conjugating enzyme 13 (UBC-13) and a UBC-like protein (UEV1A), which facilitate its ubiquitination (Ub-TRAF6). Ub-TRAF6 then engages with TGF-β-activated kinase (TAK1) and TAK1-binding proteins, TAB1 and TAB2. That ultimately results in TAK-1-dependent activation of NF-κB, and mitogen-activated protein (MAP) kinases, such as p42/p44 MAP kinase, p38 MAP kinase and Jun N-terminal kinase (JNK). MyD88-mediated signalling also promotes the activation of IRF1, IRF5, and IRF7 (not shown). In addition to MyD88, TLR2 and TLR4 also recruit MAL, which mainly functions as a bridging adaptor for MyD88 recruitment (West et al., 2006, Liew et al., 2005).

1.2.3.i.b. MAL-mediated signalling

MAL is a key component of the MyD88-dependent pathway triggered by TLR2 and TLR4 (Takeda and Akira, 2004). It has a binding domain on its N-terminus that mediates its recruitment to the plasma membrane through binding to phosphatidylinositol-4,5-biphosphate (PtdIns(4,5)P_2) (Kagan and Medzhitov, 2006). This is an important step for signalling via TLR4 and TLR2, which cannot bind directly to MyD88. Following MAL recruitment to the plasma membrane, TLR4 and TLR2 interact with MAL, which serve as a bridge to recruit MyD88 (O'Neill and Bowie, 2007) (Figure 1.4).

1.2.3.i.c. TRIF-mediated signalling

Apart from being the exclusive adaptor in TLR3 signaling, TRIF also regulates the TLR4-mediated-MyD88-independent pathway, leading to IRF3, and delayed NF-κB activation. In contrast to MyD88, its overexpression was shown to induce IFNB promoter, and its deficiency impaired both IFNβ production and IRF3 activation induced by TLR3 and TLR4, while inflammatory cytokine production by TLR2, TLR7, and TLR9 was not affected. By using knock-out MΦ, MyD88 contribution to mitogen-activated protein kinase (MAPK) activation was demonstrated to be higher than that of TRIF. As well as IRF3, like MyD88, TRIF may also be involved in the activation of IRF5 (O'Neill and Bowie, 2007).

There appears to be two distinct pathways mediating the TRIF-induced activation of NF-κB. While the first is thought to be through the TRAF-6-binding sites found on the N-terminal region of TRIF (Jiang et al., 2004, Sato et al., 2003), the second route involves the receptor-interacting protein (RIP) homotypic interaction motif (RHIM) on the C-terminus, through which TRIF was shown to recruit both RIP1 and RIP3. Among those, while RIP1 induces NF-κB activation, RIP3 was shown to negatively regulate the TRIF-RIP1-NF-κB pathway (Meylan et al., 2004) (Figure 1.5). However, it appears that both TLR3 and TLR4 may use TRIF differently, as the lack of RIP1 expression did not influence TLR4-TRIF mediated signalling (O'Neill and Bowie, 2007).

TRIF also mediates IRF activation through TRAF3. Even though the mechanism is yet not clear, it is thought to involve the recruitment of TBK-1 (a crucial upstream kinase for IRF3) via TRAF-3 and NAK-associated protein 1 (NAP1) (Sasai et al., 2005).

In addition to NF-κB and IRF3 activation, TRIF also facilitates TLR4- and TLR3-mediated signalling for apoptosis (Kaiser and Offermann, 2005, De Trez et al., 2005). Among the other signalling adaptors mentioned, TRIF is the only one with such an activity. The pathway appears to be mediated through RHIM of TRIF and involve RIP1, FADD and caspase-8 (Han et al., 2004, Ruckdeschel et al., 2004).

As in the case of MyD88 signaling, TRIF signaling can be downregulated by endogenous molecules. However most of these are not specific to TRIF, and they mainly target MyD88, TRAF6 or downstream

components of TRIF signaling. One such TRIF-specific negative regulator, that does not affect MyD88 pathway, is SARM which is the fifth TIR adaptor and will be discussed later (O'Neill and Bowie, 2007).

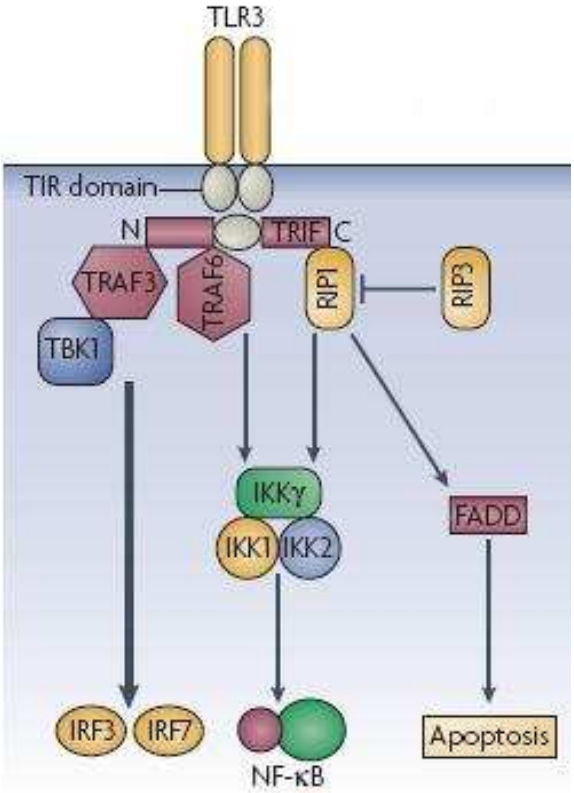

Figure 1.5: TRIF-mediated signalling. TRIF signalling is induced by both TLR3 and TLR4 and can lead to the activation of NF-κB and IRF, as well as to apoptosis. Like MyD88, it interacts with TLRs through the TIR domains. It possesses distinct motifs through which it can directly or indirectly recruit the effector proteins TBK-1, TRAF-6, and RIP-1. It is still not clear how TRIF mediates IRF3 activation through TRAF3, but it is thought to involve TBK-1 recruitment via TRAF-3 and NAP-1. The NF-κB activation is mediated through two distinct pathways which are mediated by TRAF-6 and RIP-1. Among those, RIP-1 is also involved in the FADD-mediated activation of TRIF that leads to apoptosis through caspase-8 (O'Neill and Bowie, 2007).

1.2.3.i.d. TRAM-mediated signalling

TRAM is the most restricted adaptor involved in TLR signalling and it functions exclusively in the TLR4 signalling cascade. Even though it is involved in the interaction with TRIF, its role is not TRIF-restricted as TRAM- and TRIF-deficient mice displayed different signalling phenotypes in response to LPS (O'Neill and Bowie, 2007).

There have been two biochemical modifications identified which are required for TRAM functioning. The first one is the myristoylation of the N-terminus that enables TRAM recruitment to the membrane (Rowe et al., 2006). As in the case of MAL, which recruits MyD88, TRAM functions as a bridging adaptor for TRIF, since the mutation of the myristoylation motif was enough to abolish its downstream signalling (Oshiumi et al., 2003). The second modification is the phosphorylation of serine at position 16 by protein kinase Cε (PKCε), a key component of LPS-induced signalling (McGettrick et al., 2006). The downstream elements triggered upon TRAM phosphorylation are not yet clear (Figure 1.6).

It appears that the TRAM-TRIF-mediated pathway is only initiated by endocytosed TLR4, as the inhibition of TLR4 endocytosis was reported to disrupt its signalling (Kagan et al., 2008). Today the mechanism behind this is not yet clear, however it is thought to be mediated by depletion of PtdIns(4,5)P_2 from the plasma membrane during endocytosis that results in the release of the MAL-MyD88 complex from TLR4, enabling TLR4 interaction with TRAM-TRIF in endosomes (Murphy et al., 2009).

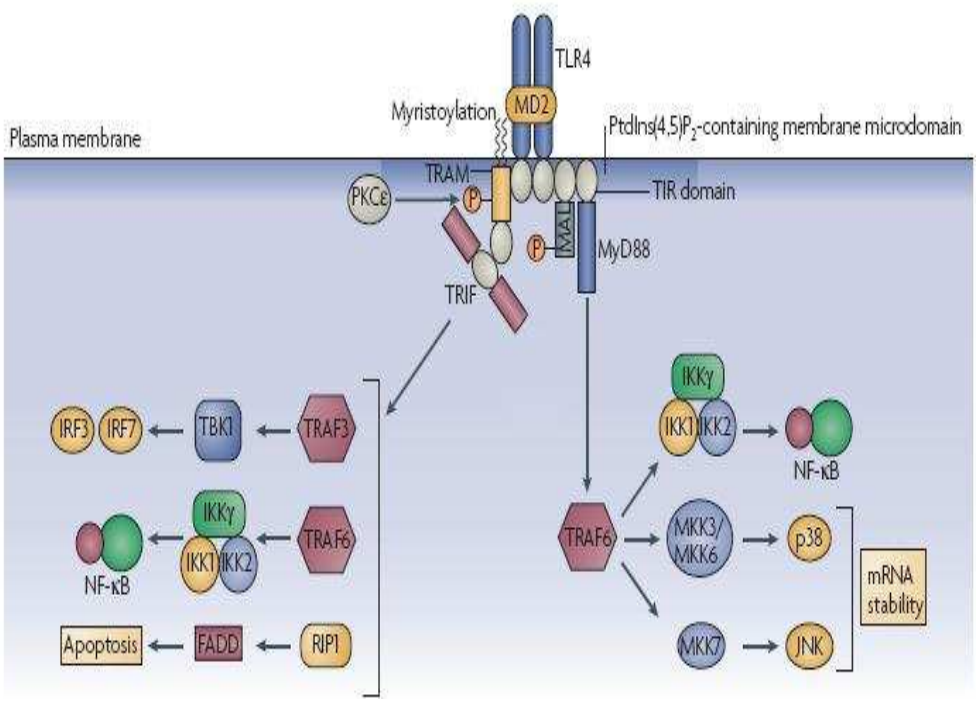

Figure 1.6: TIR-mediated signalling triggered by TLR4. TLR4-signalling is complex in terms of signalling adaptor usage as it utilises four signalling adaptors upon LPS binding. As in the case of TLR2 signalling, MAL acts as a bridging adaptor for MyD88 and is involved in the activation of NF-κB, p38 and JNK MAPK pathways. The MyD88-independent pathway involves TRAM, which like MAL, is recruited to the membrane through its attached myristate group. TRAM is a substrate for PKCε and must be phosphorylated to be active. It enables the recruitment of TRIF which promotes the activation of IRF and IRF7 through TBK-1, NF-κB through TRAF-6, and the apoptosis through RIP-1 dependent pathways. (O'Neill and Bowie, 2007).

1.2.3.i.e. SARM-mediated signalling

In contrast to other TIR-domain-containing adaptors, SARM does not induce NF-κB activation when overexpressed, and was shown to act as a negative regulator of NF-κB and IRF activation (Liberati et al., 2004). Its expression was reported to specifically inhibit TRIF-dependent signalling through direct interaction with TRIF, without having an influence on the

MyD88-dependent pathway or non-TLR signalling by TNF or RIG-1 (Figure 1.7) (Carty et al., 2006). Further, its deficiency resulted in the enhanced polyI:C- and LPS-induced chemokine and cytokine expression by primary human peripheral-bood mononuclear cells (O'Neill and Bowie, 2007).

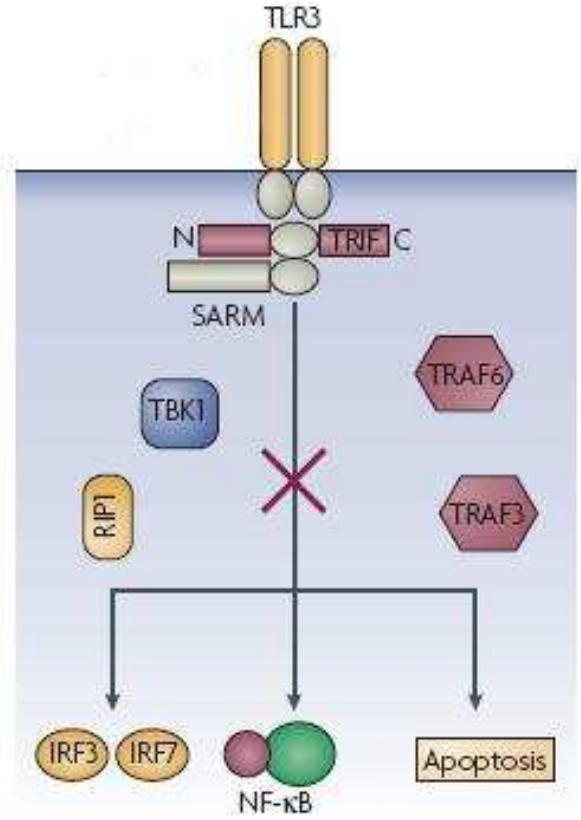

Figure 1.7: Signalling modulation by SARM. The TLR3 and TLR4 signalling also results in an enhancement in SARM expression which is involved in the inhibition of TRIF-mediated signalling probably by blocking the recruitment of TRIF effector proteins (O'Neill and Bowie, 2007).

TLR4 signalling was shown to enhance SARM expression indicating a specific negative feedback. It is thought to be regulated by the SARM N-terminus as its deletion was reported to enhance SARM expression (Chuang

and Bargmann, 2005). The TIR-domain is essential for SARM function; however how it inhibits the TRIF-mediated effects is still not yet clear. It could be because of the TRIF-SARM interaction that may block the recruitment of downstream effector proteins by TRIF (such as TRAF-6, and TBK-1), or alternatively SARM may recruit an unidentified TRIF inhibitor through its SAM motifs (O'Neill and Bowie, 2007).

1.2.3.ii. C-type lectins (CTL):

Lectins comprise a wide variety of carbohydrate-binding molecules. In 1988, Drickamer proposed to categorise animal lectins into various families and classify Ca^{2+} dependent lectins with asialoglycoprotein receptor (ASGR)-like structure as the C-type lectin family (Drickamer, 1988). After inclusion of thousands of members, the C-type lectins are today regarded as a superfamily, including both soluble and membrane-anchored proteins with immune and non-immune functions characterised by the presence of one or more C-type lectin-like domains (CLTDs) (Weis et al., 1998, Zelensky and Gready, 2005).

The domain responsible for Ca^{2+} dependent carbohydrate binding was initially identified as a globular structure in rat MBL, and accordingly named as 'carbohydrate recognition domain (CRD)' (Weis et al., 1991a). Comparing CRDs from different C-type lectins revealed a conserved sequence of ~150 amino acids facilitating the correct structural folding required for carbohydrate and Ca^{2+} binding (McGreal et al., 2004). The nomenclature of the CRD was

further modified by crystallographic studies suggesting the distinct folding structure of the domain that is different to any known protein folds; therefore CRD was replaced by 'C-type CRDs' (Weis et al., 1991b). However, these domains were then referred to as 'C-type lectin-like domains (CTLD)' due to the discovery of non-standard CRDs without any Ca^{2+} binding ability, most of which is involved in the detection of non-cabohydrate ligands (Vales-Gomez et al., 2000). Today, the term CTLD is used to identify these common folds possessed by the C-type lectin superfamily members without deducing functional similarities (Figure 1.8) (Weis et al., 1998) (McGreal et al., 2004).

Among the CTLs, only the membrane-anchored members of the (Group VI) mannose receptor (MR), (Group V) NK-cell receptors, and (Group II) asialoglycoprotein and DC receptor families were shown to facilitate direct fungal recognition by the immune cells (Zelensky and Gready, 2005). The CTL groups differ structurally, such that the group VI members have multiple CTLDs in the extracellular domain, while group II and V members have one, which is classical (requires Ca^{2+} for ligand binding) in group II and non-classical (does not require Ca^{2+} for ligand binding) in group V members.

The following section will focus on the members of Group II (i.e. DC-SIGN, dectin-2, mincle) and V (i.e. dectin-1) involved in the activation of anti-fungal immune response, and on the intracellular signalling they trigger. Finally, the remaining part of this chapter will discuss the MR, which is the only MR family member shown to participate in fungal recognition and which is the main focus of this thesis.

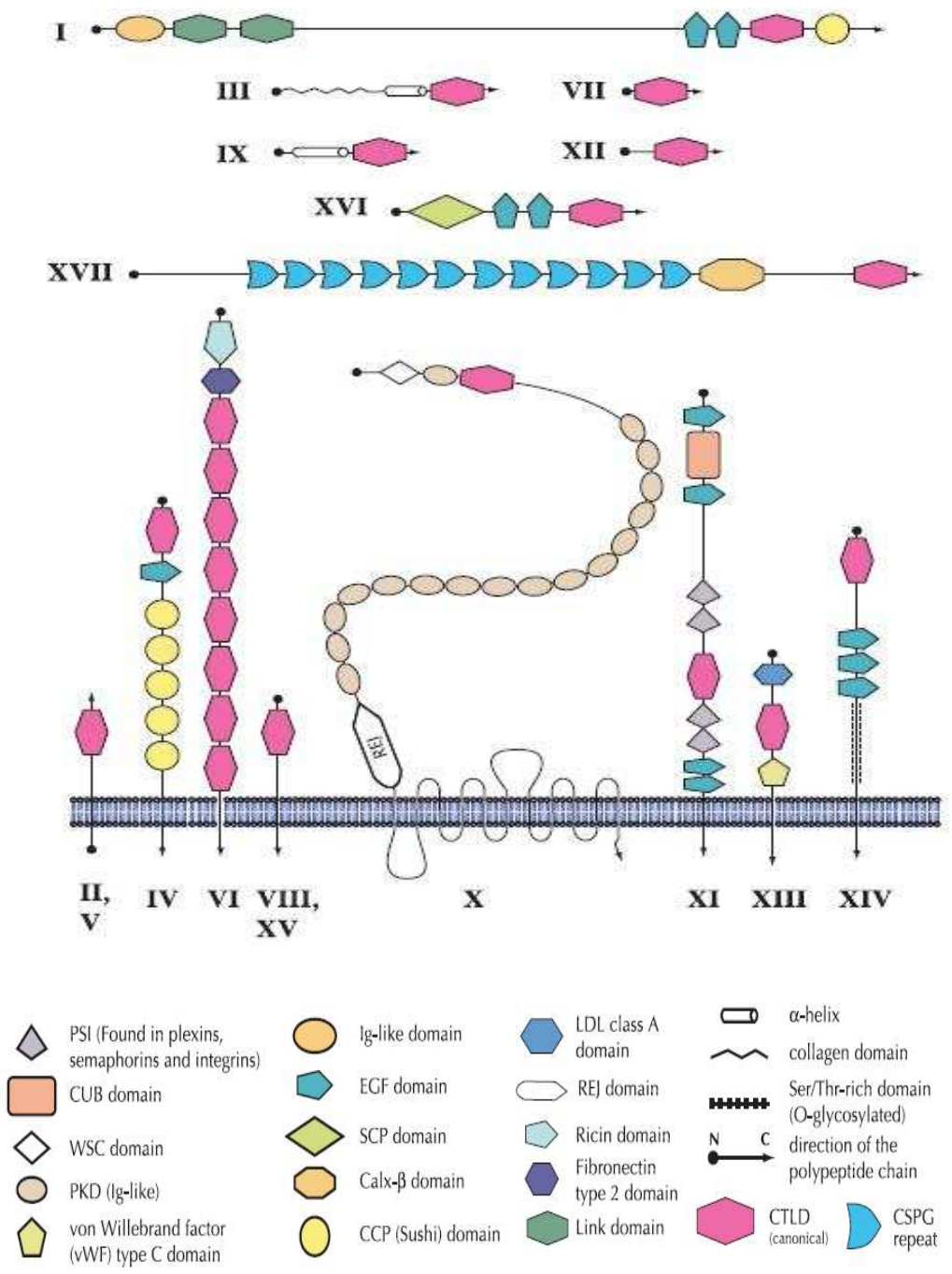

Figure 1.8: Vertebrate C-type lectins from different groups. Group I- lecticans, II- the ASGR group, III- collectins. IV- selectins, V- NK receptors, VI- the MR group, VII- REG proteins, VIII- the chondrolectin group, IX- the tetranectin group, X- polycystin I, XI- attractin, XII-Eosinophil major basic protein (EMBP), XIII- DiGeorge syndrome critical region (DGCR)2, XIV- the thrombomodulin group, XV- Bimlec, XVI- soluble protein containing SCP, EGF, EGF, and CTLD domains (SEEC), XVII- Calx-β and CTLD containing protein (CBCP) (Zelensky and Gready, 2005).

1.2.3.ii.a. DC-SIGN

DC-SIGN is primarily expressed on phagocytic cells such as DCs and MΦ (Koppel et al., 2005) (Lai et al., 2006) (Tailleux et al., 2005). Its expression is mainly upregulated by IL-4, and downregulated in response to IFN-γ, TGF-β and dexamethasone (Relloso et al., 2002). It is composed of a single CTLD followed by a neck region on the extracellular portion, a single transmembrane helix, and a cytoplasmic tail responsible for receptor internalization. Through the neck region, which contains tandem repeats of a highly conserved 23-amino-acid sequence, DC-SIGN undergoes tetrameric clustering which is important in ligand engagement (Khoo et al., 2008) (Koppel et al., 2005).

Up to today there have been eight mouse (i.e. SIGNR1, SIGNR2, SIGNR3, SIGNR4, SIGNR5, SIGNR6, SIGNR7, and SIGNR8) and two human DC-SIGN orthologues (i.e. DC-SIGN, and DC-SIGNR) identified (Powlesland et al., 2006) (Figure 1.9). In humans, DC-SIGNR displays 77% amino acid identity with DC-SIGN, and both homologues differ in their ligand specificities, such that DC-SIGN can detect both mannose- and fucose-containing glycans, while DC-SIGNR can only recognise high mannose oligosaccharides (Powlesland et al., 2006). In contrast to DC-SIGN, DC-SIGNR expression is restricted to endothelial cells in liver, lymph nodes, and placental capillaries (Li et al., 2009).

Like human DC-SIGN, mouse SIGNR1, R3, and R7 have preferential binding to fucose over mannose, while SIGNR5 and R8 displayed preferential

binding to mannose to an extent similar to that observed for human DC-SIGNR (Powlesland et al., 2006). Among those, SIGNR1 is the most extensively studied murine homologue and is also termed murine DC-SIGN (Willment and Brown, 2008). According to the study by Powlesland et al, the SIGNR2 and SIGNR6 genes are pseudogenes, as the cDNA prepared did not have the signalling sequences required for the expression of functional protein, and SIGNR4 does not have any sugar binding ability (Powlesland et al., 2006).

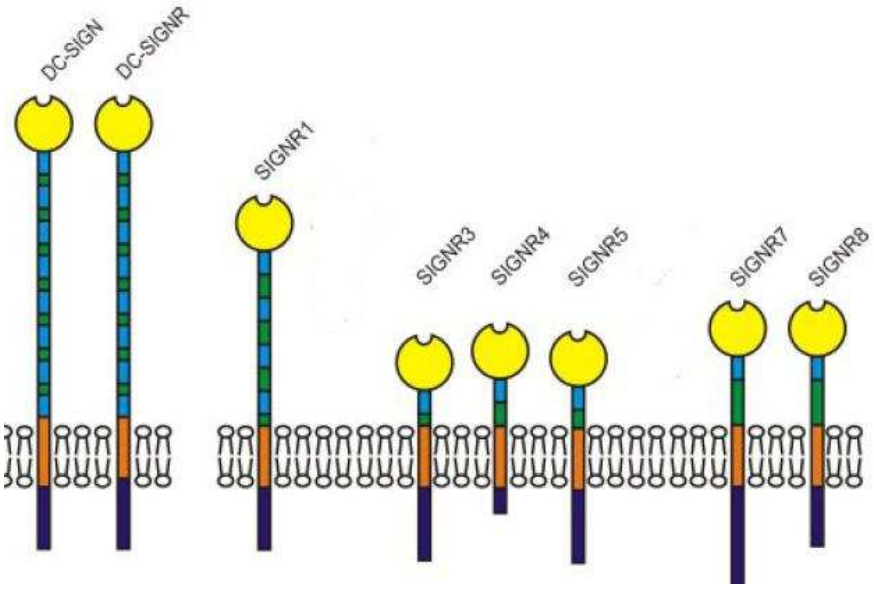

Figure 1.9: Human and mouse SIGNs. The yellow, orange, and purple correspond to CTLD, transmembrane domain and cytoplasmic tail, respectively. The light blue indicates related segments, while green represents the variable spacer domains in the neck domain (Powlesland et al., 2006).

DC-SIGN was shown to facilitate the detection of various pathogens including fungi such as Candida albicans, Aspergillus fumigatus and Chrysosporium tropicum in a Ca^{2+}-dependent manner (Khoo et al., 2008, Cambi et al., 2009) (Serrano-Gomez et al., 2004) (Serrano-Gomez et al.,

2005). DC-SIGN can internalize antigens through endocytosis (Koppel et al., 2005), while its phagocytic activity has not yet been demonstrated conclusively (Cambi et al., 2009, Willment and Brown, 2008).

Upon its engagement, DC-SIGN can trigger intracellular signalling through its cytoplasmic tail (with still an unidentified signalling motif), which results in NF-κB activation through serine/threonine protein kinase Raf-1 (Gringhuis et al., 2007). The NF-κB activation by DC-SIGN-mediated signalling was shown to modulate TLR-mediated responses at a transcription level (Figure 1.10) (Gringhuis et al., 2007) (Geijtenbeek et al., 2003). This makes DC-SIGN an important PRR for triggering an effective immune response. The signalling triggered by DC-SIGN is still poorly understood, as immune responses mediated by the detection of fucose-rich structures by DC-SIGN were reported to be Raf-1 independent (Geijtenbeek and Gringhuis, 2009).

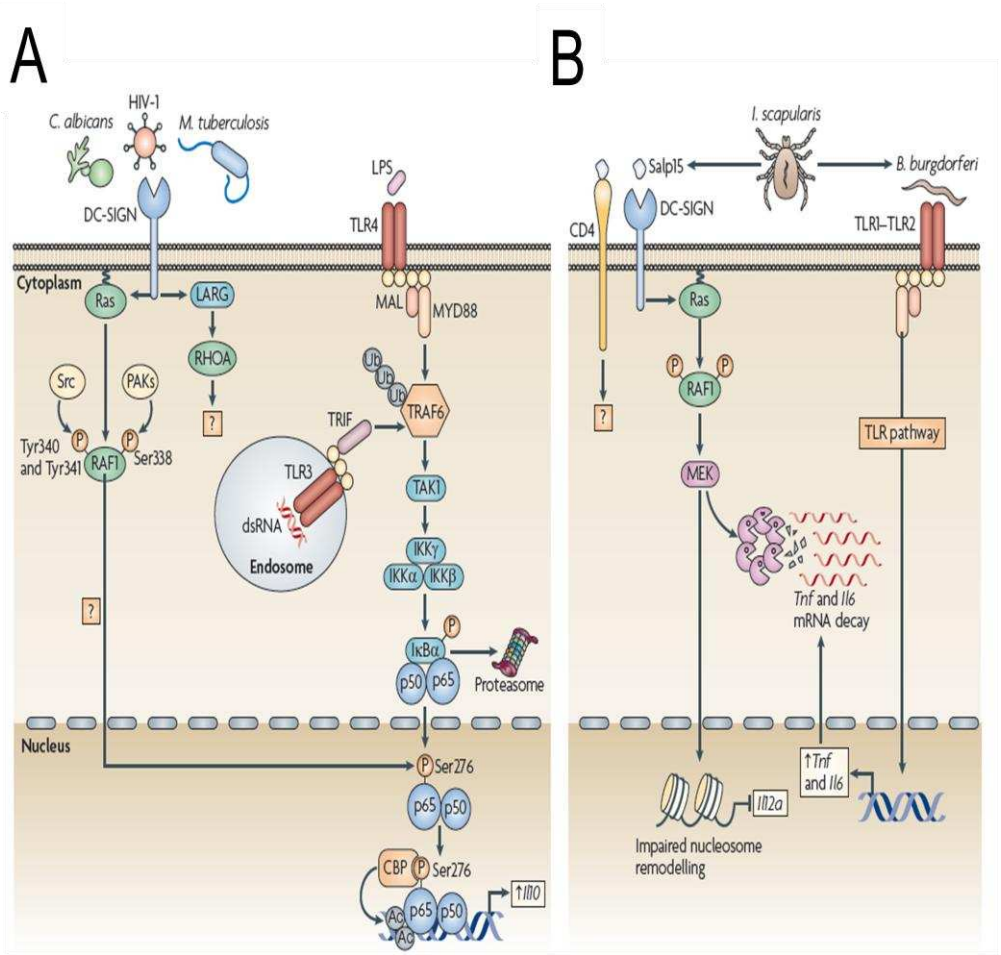

Figure 1.10: DC-SIGN utilises Raf-1-mediated signalling to modulate TLR signalling. DC-SIGN binding to pathogens results in the activation of small GTPase Ras proteins which then leads to the phosphorylation of Raf-1 kinase at residues Ser338 and Tyr340, and Tyr341 by p21-activated kinases **(**PAKs) and Src kinases respectively. The upstream elements are still unknown but thought to involve leukaemia-associated Rho guanine nucleotide exchange factor (LARG), and Ras homologue A (RHOA). Raf-1 leads to modulation of TLR-mediated NF-κB activation through an unknown mechanism that results in phosphorylation of NF-κB subunit p65 at Ser276, which then interact with histone acetyl-transferases CREB-binding protein (CBP) and p300. This leads to p65 acetylation, and to an enhanced DNA binding ability, promoting an enhanced transcriptional rate of the target genes such as Il8, Il10, Il6, and Il12b (A). The Raf-1 pathway triggered by DC-SIGN engagement with the salivary protein Salp15 from the tick lxodes scapularis is altered by the co-ligation of another receptor such as CD4 that leads to MEK (MAPK/ERK kinase) but not ERK activation. This results in reduced TLR1-TLR2-dependent pro-inflammatory cytokine production by enhancing the degradation of Il6 and Tnf mRNA, and impaired nucleosome remodelling at the Il12a promoter as observed in the case of Borrelia burgorferi-based models (Geijtenbeek and Gringhuis, 2009).

1.2.3.ii.b. Mincle

Mincle is composed of a single extracellular CTLD followed by a stalk region, a transmembrane domain and a short cytoplasmic domain. It does not have any signalling motif, but associates with FcR common γ chain (FcRγ) to induce signalling (Figure 1.11) (Graham and Brown, 2009). Its interaction with FcRγ is mediated by the positiviely charged arginine in the transmembrane domain (Yamasaki et al., 2008). Mincle expression in peritoneal MΦ was upregulated by LPS, IFN-γ, IL-6 or TNF-α (Matsumoto et al., 1999), and bone marrow (BM)-derived MΦ exposed to C. albicans were reported to have enhanced levels of mincle expression (Wells et al., 2008).

Mincle was shown to facilitate the immune response against fungal species such as S. cerevisiae, C. albicans and Malassezia (Wells et al., 2008) (Yamasaki et al., 2009). Even though it has a mannose-binding motif (EPN) in its recognition domain, the interaction appears to detect the specific geometry of α-mannosyl residues, since the mincle-Ig construct displayed specific binding to the α-mannose-polyacrylamide conjugate but not to the mannan spots in the glycoconjugate microarray (Yamasaki et al., 2009). Soluble mannan was also not able to block mincle-mediated NFAT (nuclear factor of activated T-cells) activation in response to Malassezi (Yamasaki et al., 2009).

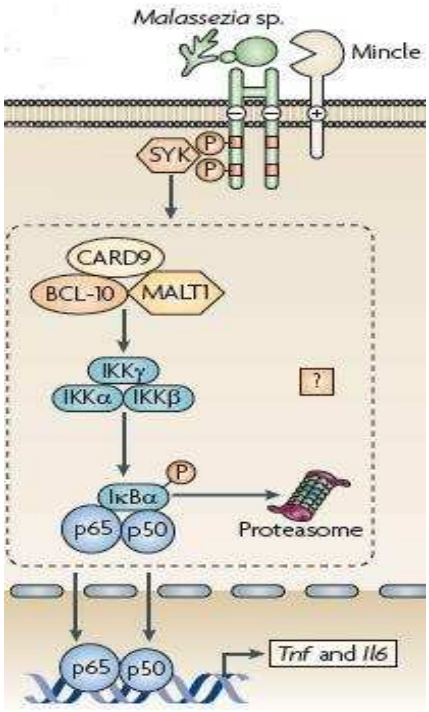

Figure 1.11: Mincle-mediated signalling. Mincle, like dectin-2, interacts with the FcRγ signalling adaptor through positively charged amino acids. Phosphorylation of the ITAM motif (on the FcRγ cytoplasmic tail) creates a docking site for Syk that triggers a signalling pathway involved in the modulation of cytokine expression in a TLR-independent manner. Both CLRs are thought to couple Syk to NF-κB activation through CARD9-BCL-10-MALT1 complex (Geijtenbeek and Gringhuis, 2009).

1.2.3.ii.c. Dectin-2

Dectin-2 is widely expressed by tissue MΦ, langerhans cells and DCs and is up-regulated during immune responses (Ariizumi et al., 2000a) (Taylor et al., 2005c). It shares the same ligand specificity with DC-SIGN, and displayed mannose and Ca^{2+}-dependent binding to A. fumigatus, C. albicans, Saccharomyces serevisiae, M. tuberculosis, Microsporum audounii, Trichophyton rubrum, Paracoccoides brasiliensis, Histoplasma capsulatum

and Cryptococcus neoformans (Graham and Brown, 2009) (Barrett et al., 2009) (Graham and Brown, 2009). In the case of fungal infections, dectin-2 appears to be mainly involved in the recognition of hyphal, rather than conidial forms (McGreal et al., 2006) (Sato et al., 2006). Quite recently, dectin-2 was also demonstrated to facilitate the production of cysteinyl leukotrienes (an important mediator for allergic inflammation in lungs) in response to the house dust mite (Barrett et al., 2009).

Dectin-2 shares the same structure with mincle, and triggers intracellular signalling through FcRγ (Figure 1.11) (Graham and Brown, 2009). Strikingly in contrast to other receptors that signal through FcRγ, its association with FcRγ was reported to be through a short region of cytoplasmic domain close to the transmembrane region (Sato et al., 2006).

In humans, a shortened dectin-2 isoform, which lacks most of the transmembrane domain and all cytoplasmic domain, was also identified (Gavino et al., 2005). Its exact function is not known yet, however, it may act as an antagonist to full-length dectin-2 after being secreted (Graham and Brown, 2009).

The engagement of the full-length dectin-2 results in the phosphorylation of the ITAM motif on FcRγ that enables the subsequent recruitment of spleen tyrosine kinase (Syk) (Yamasaki et al., 2008, Barrett et al., 2009). The downstream signalling is largely unknown, however it is thought to be similar to that triggered by other FcRγ-associated receptors (such as mincle) that promote NF-κB activation through the Syk-CARD9-BCL10-

MALT1 complex (Hara et al., 2007) (Figure 1.11). Even though dectin-2-signalling was also shown to induce NF-κB activation, a role for Syk-CARD9-BCL10-MALT1 complex has not been confirmed yet (Sato et al., 2006) (Barrett et al., 2009).

The NF-κB activation suggests an important role of dectin-2 in regulating the adaptive immune response. This is further supported by data showing the TLR-independent expression of TNF and IL-6 upon dectin-2 recognition of C. albicans, Trichophyton rubrum and Microsporum audouinii, and house dust mite-mediated production of cysteinyl leukotrienes through dectin-2-induced Syk activation (Sato et al., 2006, Barrett et al., 2009). Additionally, the dectin-2 role in the induction of T_H-17 immune responses was recently identified in a systemic C. albicans infection model (Robinson et al., 2009).

1.2.3.ii.d. Dectin-1

Dectin-1 is a phagocytic receptor expressed on DCs, MΦ, monocytes, neutrophils, microglia and weakly on subsets of murine T-cells, human B-cells, mast cells and eosinophils (Taylor et al., 2002, Brown, 2006). Its high expression levels at the possible pathogen entry sites, such as lungs and intestines, correlate with its crucial role in triggering the immune response (Taylor et al., 2002, Reid et al., 2004). Dectin-1 expression can be influenced by various cytokines, steroids and microbial stimuli. Cytokines such as those

involved in alternative MΦ activation and T_H-2 immune responses including IL-4 and IL-13, enhance dectin-1 cell surface expression while dexamethasone and LPS suppress its expression (Willment et al., 2003).

It is involved in the detection of β-glucan, which makes up to 50% of the fungi cell wall, and accordingly it was shown to have a central role in anti-fungal immune responses such as those against Pneumocystis carinii, P. brasiliensis, C. albicans, Coccidioides posadasii and A. fumigatus (Brown, 2006).

Dectin-1 is composed of an extracellular non-classical CTLD that can detect β-glucan in a Ca^{2+}-independent manner, followed by a stalk, a transmembrane domain, and a cytoplasmic tail. The stalk region is the region that is most commonly spliced out in functional dectin-1 isoforms in both human and mouse. In mice, dectin-1 mRNA is alternatively spliced to generate two functional dectin-1 isoforms; dectin-1A and dectin-1B, among which dectin-1B lacks the stalk region. In contrast to mice, humans have at least eight dectin-1 isoforms. Among those, isoforms A&B are the only functional isoforms involved in β-glucan recognition and have a structural similarity to murine isoforms (Willment et al., 2001).

Upon ligand engagement, the intracellular signalling is mediated by an ITAM-like motif on the cytoplasmic tail, named as "hem-ITAM" (Figure 1.12). The nomenclature is derived from its unusual characteristic behaviour in triggering Syk-dependent intracellular signalling by phosphorylation of a single YXXL motif, instead of two (Figure 1.12). The cytoplasmic tail also

contains a highly charged tri-acidic motif (DED) in the hem-ITAM motif that is required for particle uptake (Underhill et al., 2005) (Brown, 2006).

Dectin-1-mediated Syk activation was also demonstrated to be required for dectin-1 cross-talk with TLR signalling. The cross-talk is important for some dectin-1 responses such as TNF-α, and IL-12 production (Gantner et al., 2003) (Brown et al., 2003) (Ferwerda et al., 2008). Other then Syk, sor far, MyD88, (Dennehy et al., 2008), Raf-1 kinase (Gringhuis et al., 2009) and CARD9 (Hara et al., 2007) were found to be essential in this collaborative cellular response.

In addition to the Syk kinase pathway, some of the dectin-1-mediated cell responses are regulated by the Syk-independent pathway. The Syk-independent pathway is still largely uncharacterized and was recently shown to involve Raf-1 kinase-mediated non-canonical NF-κB activation (Figure 1.12) (Gringhuis et al., 2009).

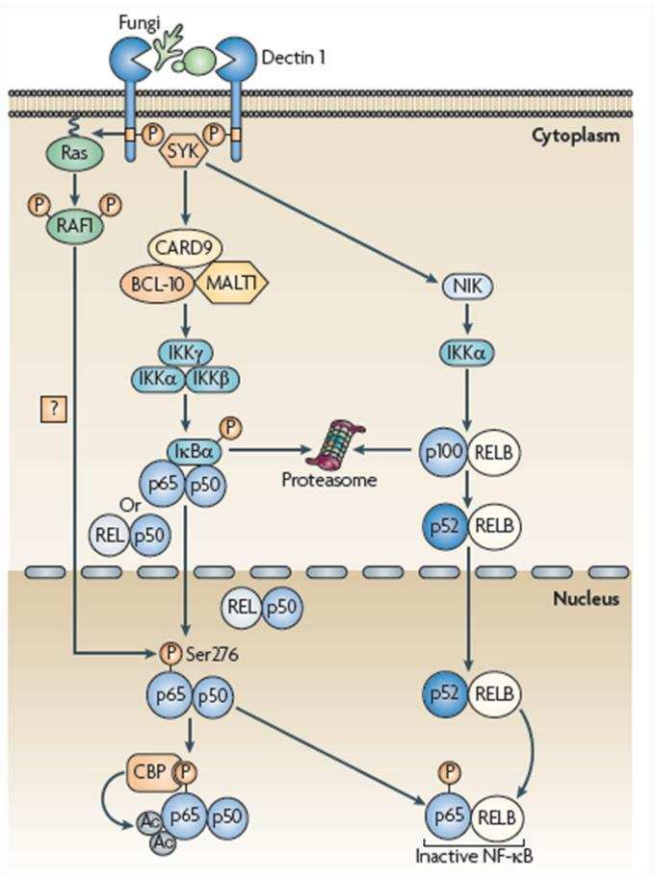

Figure 1.12: Dectin-1 signalling through Raf-1 and Syk-dependent pathways. Fungi recognition by dectin-1 induces phosphorylation of the YXXL (X is any amino acid) in its cytoplasmic tail that results in Syk recruitment. The nature of the Syk binding is still unknown, as dectin-1 uses only one ITAM-motif for signalling and Syk recruitment is thought to bridge two dectin-1 molecules. Activation of Syk leads to the formation of a signalling complex comprised of CARD9, BCL10 and MALT1 which promote the activation of the IκK complex through a yet unrecognised pathway. The inhibitor of NF-κB (IκBα) is phosphorylated by IκκB that targets it to be degraded by proteasomes. Thereby NF-κB, which is composed of either p65-p50 or REL-p50 dimers, becomes free to translocate into the nucleus (canonical NF-κB pathway). In addition, Syk-mediated signalling can also promote a non-canonical NF-κB pathway that involves NF-κB inducing kinase (NIK) and IκK-α, which target p100 for proteolytic processing to p52. This eventually leads to the nuclear translocation of RELB-p52 dimers. Dectin-1 can also signal in a Syk-independent manner, which is mediated by the Raf-1 kinase activated by Ras proteins. The signalling leads to the phoshprylation of p65 at Ser276 that acts as a binding site for the histone acetyltransferases CBP, or p300 required for the acetylation of p65 at different lysine residues. Phosphorylated p65 also attenuates the RELB-mediated transcription by forming an inactive dimer with RELB that cannot bind to DNA (Geijtenbeek and Gringhuis, 2009).

1.3. Mannose Receptor (MR)

The MR was initially identified in the late 1970s as a 175 kDa endocytic receptor on rabbit alveolar MΦ involved in the clearance of endogenous glycoproteins (Wileman et al., 1986). It was the first member discovered of the MR family of C-type lectins, which was also shown later on to include the M-type phospholipase A_2 receptor ($mPLA_2R$), DEC-205, and the Endo-180 (East and Isacke, 2002, Zelensky and Gready, 2005).

The MR family members are unique within the C-type lectin superfamily since these alone possess multiple CTLDs within a single polypeptide backbone. They are also characterised by an N-terminal cysteine-rich (CR) domain, which acts as a second lectin domain only in MR, followed by a single fibronectin type II (FNII) domain responsible for collagen detection by all family members. Their cytoplasmic tails contain motifs that help them to transport the bound antigen into the cellular endocytic machinery (East and Isacke, 2002).

Even though these receptors share structural similarities, differing only in the number of CTLDs within a single polypeptide backbone (eight in the case of MR, PLA_2R and Endo 180 and ten in the case of DEC205), they possess different ligand binding properties, and therefore each family member has a different range of functions (East and Isacke, 2002). For instance, the PLA_2R is involved in the internalization of PLA_2 enzymes, DEC-205 can internalize antigen for presentation to T-cells, Endo-180 can facilitate

extracellular matrix remodelling, while MR has a role in the innate and adaptive system (East and Isacke, 2002).

1.3.1. MR structure, expression, and specificity

MR is a type I membrane protein with a single transmembrane domain and a cytoplasmic domain that mediates receptor internalization and recycling. The three types of domains at the extracellular region have different ligand specificity; the CR domain is capable of Ca^{2+}-independent binding to sulphated sugars terminated in SO_4-3-galactose (Gal) or SO_4-3/4-N-acetylgalactosamine (GalNAc) (Taylor et al., 2005a), the FN II which is involved in collagen binding especially collagen type I, II, III, and IV (Martinez-Pomares et al., 2006, Napper et al., 2006), and eight tandemly arranged CTLD responsible for Ca^{2+}-dependent binding to sugars terminated in D-mannose, L-fucose or N-acetylglucosamine (GlcNAc) (Figure 1.13) (Taylor et al., 2005a). Two independent studies by Napper et al., and Boskovic et al., suggested a structural model for MR which includes two different conformations: an extended form and a bent form which was created as a result of the interaction between the CR domain and the CTLD4 region, and was suggested to facilitate projecting ligand binding competent domains closer to their possible substrates (Napper et al., 2001) (Boskovic et al., 2006).

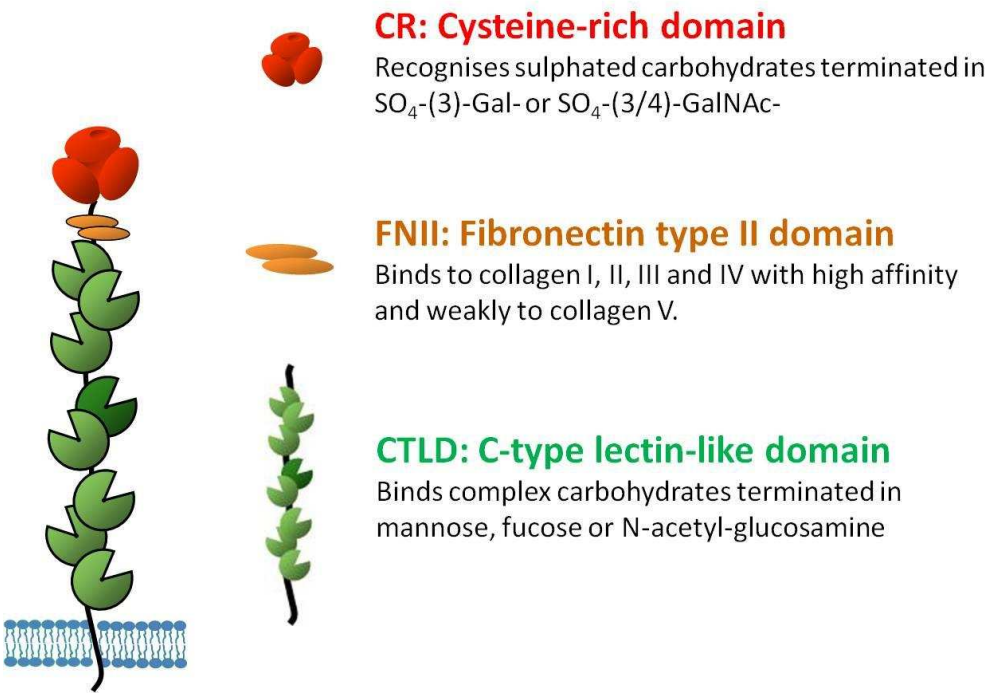

Figure 1.13: MR structure. The MR extracellular domains including CR domain (red), FN II domain (brown), and CTLD (green) were shown with their predicted N-linked glycosylation sites. Among the CTLDs, CTLD4, which is the domain mostly responsible for sugar binding, was shown in dark green.

Initially MR was thought to be expressed only on MΦ, however, it is now known that its expression is not MΦ specific and can also be detected in tracheal smooth muscle cells, retinal pigment epithelium, hepatic and lymphatic endothelia, kidney mesangial cells, human monocyte-derived DCs, and on the subpopulation of murine DCs (Lew et al., 1994, Linehan et al., 1999, Shepherd et al., 1991, Avrameas et al., 1996, Engering et al., 1997, Sallusto et al., 1995, McKenzie et al., 2007). The MR promoter region was suggested to have binding sites for the transcription factors PU.1, which is

necessary for the proper development of myeloid progenitors, and SP.1 which is ubiquitously expressed in all cell types (Egan et al., 1999).

MR is up-regulated by cytokines IL-10, IL-4 and IL-13 and therefore expressed at high levels by MΦ in alternatively-activated and de-activated states (Martinez-Pomares et al., 2003) (Doyle et al., 1994). Other anti-inflammatory agents such as prostaglandin E (PGE) and dexamethasone were also shown to enhance its expression, which is in contrast to the effect induced by pro-inflammatory IFN-γ that stimulates classical activation of MΦ and was shown to down-regulate MR expression (Cowan et al., 1992) (Schreiber et al., 1990, Harris et al., 1992). This suggests a role for MR in restricting self-damage and resolution of inflammation. Accordingly, MR was reported to mediate the removal of lysosomal enzymes, neutrophil-derived myeloperoxidase and tissue plasminogen activator and its deficiency was shown to lead to enhanced lysosomal hydrolases in serum (Shepherd and Hoidal, 1990) (Noorman et al., 1995) (Lee et al., 2002).

As an endocytic receptor, MR is constitutively internalised and sent back to the plasma membrane even in the absence of any ligand. The majority of MR is expressed intracellularly, in a way that the cell surface expression corresponds to only 10-30% of total expression (Taylor et al., 2005a, East and Isacke, 2002). Therefore, besides enhancing total MR expression, extracellular signalling may also regulate MR activity by stimulating the recycling process without affecting the rate of protein synthesis as was observed in the case of

collectins (surfactant proteins (SP)-A and-D)-induced MR activity in alveolar MΦ (Kudo et al., 2004).

Moreover, surface expression can be further regulated by a yet uncharacterised mechanism that results in metalloprotease mediated cleavage of membrane-anchored MR (cMR) into functional soluble MR (sMR) that is comprised of extracellular domains of intact MR (Martinez-Pomares et al., 1998). The cleavage does not result to any change in ligand binding capacity since, like cMR, sMR was also able to interact with carbohydrates, heat-killed C. albicans and zymosan, in a mannose- and fucose- dependent manner (Martinez-Pomares et al., 1998). As with total cellular expression, sMR expression also seems to be up-regulated by IL-4, while IFN-γ and LPS are involved in its down-regulation (Martinez-Pomares et al., 1998).

1.3.1.i. Cysteine-rich domain

The lectin acivity of the CR domain is unique within the family and it can bind to sulphated sugars such as those present on the pituitary hormones lutropin and thyrotropin, chondroitin sulphates A and B, and sulphated oligosaccharides of blood group Lewis^{a} and Lewis^{x} types (Fiete et al., 1998, Leteux et al., 2000). To date, there is no exogenous CR domain ligand identified and therefore this domain is mainly involved in maintaining haemostasis and resolution of inflammation. Additionally, domain binding to

chondroitin sulphate-associated extracellular matrix proteoglycans could play a part in promoting adhesion of cells to the ECM (Taylor et al., 2005a).

Moreover, additional CR domain ligands (sulphated glycoforms of sialoadhesin and CD45) have been identified in MΦ subpopulations in secondary lymphoid organs proximal to B-cells, and follicular dendritic cells during the germinal centre reaction. This suggests a putative role for the soluble receptor in delivering mannosylated antigens to secondary lymphoid organs (Martinez-Pomares et al., 2005).

1.3.1.ii. Fibronectin type II domain

The FNII domain, the most conserved domain among the MR family members (East and Isacke, 2002), is responsible for collagen recognition (Martinez-Pomares et al., 2006). Despite the highly conserved nature of the FN II domain, each family member detects different types of collagen. For instance, PLA_2R binds to type I and type IV collagen, Endo-180 interacts with collagen types I, IV, and V, whereas the MR is involved in the detection of collagen type I, II, III, and IV, but not type V (Martinez-Pomares et al., 2006) (East and Isacke, 2002).

Type I and type III collagens are the major fibrillar collagens found in connective tissues throughout the body, and type IV collagen is highly abundant in the basal lamina. Therefore the collagen-binding activity of MR suggests a crucial role in mediating the clearance of collagen fragments during

tissue re-modelling and wound healing. Furthermore, a role for the MR in cell migration was demonstrated in BM-MΦ (Sturge et al., 2007) and myeloblast cells (Jansen and Pavlath, 2006). In both studies, MR-deficient cells displayed a migration defect.

1.3.1.iii. C-type lectin-like domains (CTLDs)

Initially, the MR family term was used to identify receptors with multiple C-type lectin or C-type carbohydrate recognition domains (CRDs). Today, it is known that only a few of these domains possess lectin activity, which is why they are rather termed CTLD (Zelensky and Gready, 2005). As mentioned before, the MR CTLDs are responsible for the binding to sugars terminated in mannose, fucose or N-acetyl glucosamine (Taylor et al., 2005a).

Each CTLD share a common structure with MBL, made up of two α-helices and two small anti-parallel beta sheets linked together with both covalent and non-covalent interactions. The overall folded structure is stabilised by the two conserved disulphide bonds, and the interactions between hydrophobic amino acids that lead to the formation of a hydrophobic core. Hydrophobic core formation in functional CTLDs facilitates bringing the residues required for the Ca^{2+} ions and sugar residues come into contact (Weis et al., 1992) (Apostolopoulos and McKenzie, 2001) (East and Isacke, 2002).

In MR, only CTLD4 and CTLD5 domains have residues required for Ca^{2+} dependent sugar binding, albeit only CTLD4 can bind to monosaccharide

in isolation. Since this interaction is rather weak, multiple CTLDs are required to increase avidity and the CTLD4-8 construct was shown to interact with multivalent ligands with the same affinity as the whole MR (Taylor et al., 2005a) (Apostolopoulos and McKenzie, 2001).

The MR CTLD4 requires two Ca^{2+} ions for sugar binding; one is placed in conserved binding site two and interacts directly with sugars, and the other one binds to the residues located on an extended loop unique to the MR CTLD4, providing an interaction between β-strands two and three, and positioned away from the domain core (Figure 1.14). The loop is thought to have a crucial role in releasing MR-bound carbohydrates in the endosomal compartment by inducing structural change through mediating pH-dependent Ca^{2+} release upon acidification (Taylor et al., 2005a).

Like the CR domain, the CTLD region is also involved in regulating the levels of endogenous ligands such as thyroglobulin and lutropin hormones (Simpson et al., 1999) (Linehan et al., 2001), as well as in resolving inflammation through its interaction with enzymes such as myeloperoxidase, and lysosomal hydrolases.

In contrast to the CR and FNII domains, the CTLDs also help the MR to function as a PRR. Mannose and N-acetylglucosamine are not commonly found as terminal residues in mammalian glycoproteins, but they are frequently found on the glycoproteins that decorate the surface of many microorganisms. The pathogens recognised by MR include; C. albicans (Marodi et al., 1991, Martinez-Pomares et al., 1998), Leishmania (Chakraborty

et al., 2001, Chakraborty et al., 1998), M. tuberculosis (Tailleux et al., 2003), HIV (Nguyen and Hildreth, 2003), P. carinii (Ezekowitz et al., 1991, O'Riordan et al., 1995), selected strains of Klebsiella pneumoniae (Zamze et al., 2002), Cryptococcus neoformans (Dan et al., 2008), Streptococcus pneumoniae (Zamze et al., 2002), and Dengue virus (Miller et al., 2008).

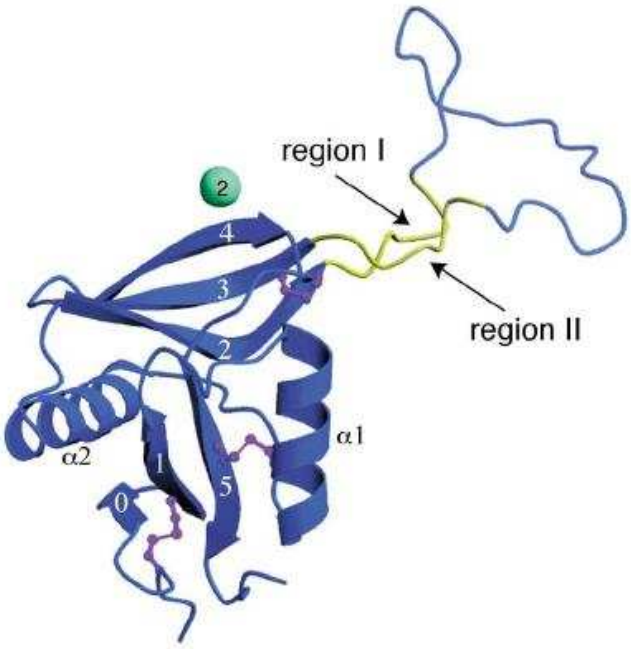

Figure 1.14: Ribbon diagram of MR CTLD4. The disulphide bonds are shown in pink while the yellow represents the two segments (regions I and II) connecting the extended loop to the core of the CTLD. The principal Ca^{2+} is shown as a green sphere (East and Isacke, 2002).

1.3.1.iv. Cytoplasmic domain

The MR is constitutively internalised into early endosomes and sent back to the plasma membrane (East and Isacke, 2002) (Taylor et al., 2005a). Internalization of the MR is mediated by the tyrosine residue in the FENTLY

sequence motif similar to that present in the low density lipoprotein receptor-and involves clathrin-coated vesicles (Schweizer et al., 2000). The correct endosomal sorting of MR depends on the di-aromatic Y-F motif in which Y belongs to the endocytosis motif (Schweizer et al., 2000) (Figure 1.15). The transmembrane domain was also suggested to have a role in receptor trafficking, since its substitution did impair receptor internalisation (Kruskal et al., 1992). In the early endosomes, the receptors are targeted to be sent either back to the plasma membrane, or into the late endosomes, which are characterised by abundant intravesicular membranes and the presence of active lysosomal hydrolases.

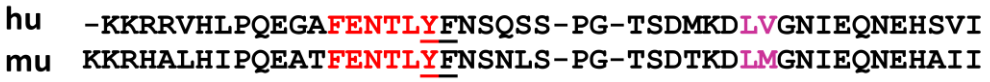

Figure 1.15: Comparison of cytoplasmic domains of human (hu) and mouse (mu) MR. The amino acid sequences are written in single letter code. The FENTLY (red) and di-aromatic YF (underlined) sequences are important for receptor internalization and correct endosomal sorting, respectively. The conserved di-hydrophobic chain of LV in human, and LM in mouse MR are shown in purple.

Furthermore, a distinct di-hydrophobic chain (LV in human, and LM in mouse MR) was identified, as in the case of other receptors, including Fc receptor (FcR), mannose-6-phosphate receptors and IFN-γ receptors (East and Isacke, 2002) (Figure 1.15). An acidic residue in the -4 position to this di-hydrophobic motif was conserved among all family members, and the

equivalent acidic residue in other receptors was shown to be necessary for intracellular trafficking from the early endosomes and, in some cases, for receptor internalization (Pond et al., 1995, East and Isacke, 2002).

To date, no intracellular signalling motifs have been identified on the MR cytoplasmic tail. However, in several studies MR engagement was shown to induce intracellular signalling leading to the production of several inflammatory mediators. This suggests that MR forms a receptor complex with a signalling-motif associated receptor, as revealed by Tachado et al, in which MR was shown to interact with TLR2 during the detection of P. carinii (Tachado et al., 2007) (discussed below).

1.3.2. The role of MR in immunity

As discussed previously, since mannose, fucose and N-acetylglucosamine are not commonly found as terminal residues in mammalian glycoproteins, MR can function as a PRR through its CTLD. MR was shown to recognise various pathogens such as C. albicans (Marodi et al., 1991, Martinez-Pomares et al., 1998), Leishmania (Chakraborty et al., 2001, Chakraborty et al., 1998), M. tuberculosis (Tailleux et al., 2003), and P. carinii (Ezekowitz et al., 1991, O'Riordan et al., 1995).

However despite its demonstrated role in pathogen recognition, the importance of MR in immune response is not clear since MR-deficient mice displayed unaltered susceptibility to infections with C. albicans, P. carinii, and

leishmaniasis (Lee et al., 2003) (Akilov et al., 2007, Swain et al., 2003). This can be due the compensation of MR by other receptors sharing the similar pattern of ligand binding (e.g. DC-SIGN, and dectin-2). The following section will focus on the current data describing the role of the MR in phagocytosis, antigen processing and presentation, and intracellular signalling triggered response to pathogen recognition.

1.3.2.i. Phagocytosis

The literature on the role of the MR in phagocytosis still includes contradictory data. Even though the MR has been shown to be involved in the phagocytosis of pathogens, such as M. tuberculosis (Kang et al., 2005), Francisella tularensis (Schulert and Allen, 2006) and C. albicans (Marodi et al., 1991), CHO cells expressing human MR were found unable to phagocytose Mycobacterium kansasii or mannosylated latex beads, despite the occurrence of mannosylated glycoprotein endocytosis (Le Cabec et al., 2005). In support of this, MR knock-out (KO) mice did not display impaired uptake of C. albicans, Leishmania donovani and Leishmania major (Lee et al., 2003) (Akilov et al., 2007), and MR was absent from the early stages of C. albicans containing phagosome formation (Heinsbroek et al., 2008). Additionally, the MR was demonstrated to provide a safe-route of entry for pathogens, since its engagement resulted in the suppression of phagosome-lysosome fusion and phagosome maturation after phagocytosis of both pathogenic and non-

pathogenic mycobacteria (Astarie-Dequeker et al., 1999), and Mycobacterium avium (Shimada et al., 2006).

In some of these studies, the role of MR in phagocytosis was confirmed by using mannan, which was being traditionally used as a specific MR inhibitor. However, the discovery of other mannan binding receptors, such as DC-SIGN and dectin-2, revealed the unreliability of mannan as a MR inhibitor. Nevertheless, MR-expressing J774-E MΦ ingested F. tularansis more efficiently than MR-negative cells and the MR role was confirmed by using an MR-specific antibody, as well as soluble mannan, as inhibitors (Schulert and Allen, 2006). Additionally, non-phagocytic COS-1 cells were reported to phagocytose C. albicans and P. carinii upon transfection with MR (Ezekowitz et al., 1991, Ezekowitz et al., 1990). When compared with the data obtained from CHO cells (mentioned above), these data suggest that the effect of the MR on phagocytosis and phagosome maturation might depend on the cell-type used.

Additionally, research on the mechanism of MR-phagocytosis indicates that the cytoplasmic tail is crucial in phagocytosis. Mutation of the single tyrosine caused reduction, but not complete inhibition, of the particle uptake (Kruskal et al., 1992). The MR phagosomes were found to be associated with F-actin, talin, PKCα, MARCKS and myosin I, but in contrast to FcRγ and CR3-phagsosomes, vinculin and paxilin are not recruited to MR phagosomes (Kerrigan and Brown, 2009). Further studies also revealed the requirement of F-actin polymerisation, Cdc42 and Rho activation, PAK1 activity, and Rho

effector molecule ROCK, but not Rac, for MR-mediated phagocytosis (Kerrigan and Brown, 2009, Zhang et al., 2005b).

1.3.2.ii. Antigen processing and presentation

Intracellular MΦ MR was reported to be dominantly expressed in $Rab5a^+$ early endosomes. However, in conditions known to induce MR expression (e.g. in the presence of IL-4, and PGE), this expression pattern was further extended to include the $Rab11^+$ recycling endocytic compartment, as well as the $Rab7^+$ late endosomes (Wainszelbaum et al., 2006). This suggests a MR role in the transportation of the bound ligand into the late endocytic compartment required for antigen presentation, under these conditions.

Similar co-localisation results suggesting a crucial role for MR in antigen presentation in the context of MHC class II and CD1b were also observed by Engering et al, Sallusto et al, Prigozy et al (Engering et al., 1997) (Prigozy et al., 1997) (Sallusto et al., 1995). Among those, in contrast to Prigozy et al which observed MR co-localization with CD1b and lipoarabinomannan (LAM) in the MIIC (the compartment where antigen is loaded on MHC class II molcules), Engering et al, reported distinct localization of the MR and MHC class II molecules, which is thought to be because of the recycling nature of MR that results in antigen release in the early endosomal compartment. This contradictive data can be explained by the type of the MR ligand used (mannosylated-bovine serum albumin (BSA) vs

LAM), and the possible contribution of other PRRs, such as DC-SIGN and dectin-2, that share similar ligands with MR (discussed above). However, MR participation in antigen presentation with MHC class II molecules was further supported by the studies of Desgupta et al and McKenzie et al, which proposed MR involvement in the therapeutic factor VIII antigen presentation (Dasgupta et al., 2007), and the generation of antibody isotype switching upon immunization with anti-MR antibody (McKenzie et al., 2007) respectively.

MR was shown to present exogenous proteins in context with MHC class II molecules, as well as to promote cross-presentation of internalized antigens by Burgdorf et al. This was in contrast to pinocytosed lucifer yellow, and scavenger receptor-internalized antigen, which were detected in MHC class II positive lysosomal compartments. The MR role in cross-presentation was further confirmed by Ab25-D1.16 antibody staining specific to the OVA-derived peptide SIINFEKL in the context of the class I MHC molecule H-2b, which was negative in the case of MR-KO APCs in contrast to wt cells. This, however, contradicts with the study by Berlyn et al who reported enhanced $CD4^+$ T-cell activation upon antigen mannosylation, without any effect on the $CD8^+$ branch of the immune response (Berlyn et al., 2001).

Other studies showing MR influence on MHC class I antigen presentation involve the work by Apostolopoulos et al (Apostolopoulos et al., 1995). The study showed that the tumour-associated antigen MUC1, linked to oxidised mannan, was more efficiently directed to the class I pathway than the one associated with reduced mannan (Apostolopoulos et al., 1995). This

selective passage appears to occur after the internalization step, and depends on the aldehyde groups on the oxidised form of the antigen, as revealed by the same group (Apostolopoulos et al., 2000).

In spite of being contradictive, these data overall suggest the MR role in directing the bound ligands to the compartments associated with antigen presentation (either through class I or class II MHC molecules), which is probably determined by the nature of the antigen and the activation states of the cells used. In support, among endotoxin-free and endotoxin-contaminated OVA, only the latter was effectively presented with MHC class I molecules after internalization via MR. This is in correlation with the translocation of the peptide transporter TAP (transporter associated with antigen processing) to the endosomal compartments in conditions contaminated with endotoxin (Hotta et al., 2006) (Norbury et al., 2004). The recruited TAP facilitates the re-import of the processed antigen into the early endosomes to be loaded onto MHC class I molecules and transported to the cell surface (Burgdorf et al., 2008).

1.3.2.iii. Intracellular signalling

MR has also been suggested to play a crucial role in intracellular signalling leading to the regulation of gene expression in several studies (Zhang et al., 2004, Tachado et al., 2007, Yamamoto et al., 1997, Fernandez et al., 2005, Lopez-Herrera et al., 2005, Zhang et al., 2005a, Chieppa et al., 2003). However, it appears to require the assistance of other receptors in order

to trigger any signalling cascade, due to the absence of any signalling motifs in its cytoplasmic domain (Shibata et al., 1997) (Zhang et al., 2005a) (Tachado et al., 2007). In support, in contrast to phagocytosable mannose-coated beads and chitin which stimulated TNF-α, IFN-γ, and IL-12 production by murine spleen cells, non-phagocytosable beads did not induce any change in cytokine production. Additionally, treatment with soluble mannan did not cause any change in the levels of the above cytokines, while it was able to inhibit chitin-mediated IFN-γ release. One candidate receptor that the MR is thought to cross-talk with is TLR2, as revealed by Tachado et al, which showed the requirement of co-expression of both TLR2 and MR for IL-8 secretion in response to P. carinii by HEK-293 cells. It appears that both receptors interact with each other forming a functional complex on the cell surface during pathogen recognition, as demonstrated by the co-precipitation studies in the presence of P. carinii (Tachado et al., 2007).

In a different model, Chieppa et al suggested a direct role of the MR in mediating T_H-2 and T_{reg}, but not T_H-1, chemokine and cytokine induction, by using MR specific monoclonal antibodies (Chieppa et al., 2003). Strikingly, in the same work, not all MR ligands had the same effect; while mannan, and thyroglobulin treatments did not induce any significant change in cytokine or chemokine production, mannose-capped LAM and biglycan treatment resulted in enhanced IL-10 and reduced IL-12 levels in LPS-maturing DCs.

One possible mechanism through which MR can deliver a negative signal to block pro-inflammatory cytokine release was recently revealed by

Pathak et al that showed IRAK-M up-regulation upon MR-engagement (Pathak et al., 2005). IRAK-M is a negative regulator for TLR signalling and acts through blocking the dissociation of IRAK-1 and IRAK-4 from MyD88 (Figure 1.16).

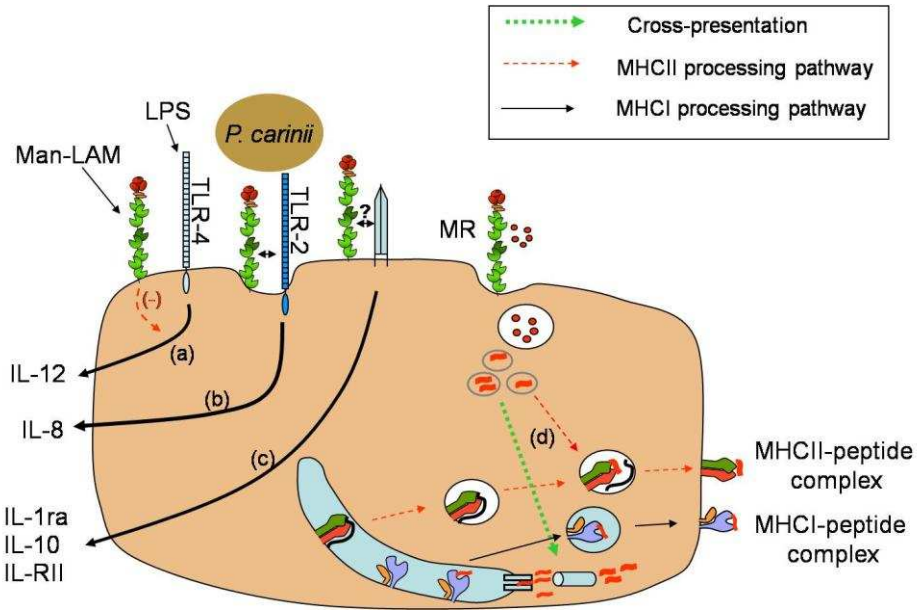

Figure 1.16: MR as a complex regulator of immunity. MR is speculated to cooperate with other signalling receptors in the modulation of cytokine secretion. It has been shown that MR engagement by Man-LAM has a negative effect on the production of IL-12 in response to LPS in human DC (a). On the other hand, co-expression of MR and TLR2 is required for IL-8 production in response to P. carinii (b). Additionally, engagement of MR by a specific mAb or selected ligands leads to the production of anti-inflammatory mediators (c). Finally, there is strong evidence in support of MR-mediated internalization favouring cross-presentation (d) in addition to MHC class II-mediated presentation of exogenous antigens (Gazi and Martinez-Pomares, 2009).

1.4. Aims and Objectives

The aim of this research was to characterize the role of MR in MΦ biology. In particular the focus was on the regulation of MR expression (in both cell-associated and soluble forms), and function as an endocytic receptor. The following were the objectives:

- Determining whether MR can facilitate cellular adhesion to collagen-rich surfaces which may influence the endocytic function of MR. This was achieved by monitoring the level of MR-mediated endocytosis, and MR expression at both protein and mRNA levels after overnight incubation of MΦ on collagen I or IV-coated surfaces.

- Assessing whether the previous observation of enhanced MR-shedding in response to P. carinii can also be induced by other fungi species and whether the enhanced sMR production has an effect on MR-mediated endocytosis and fungi-mediated cytokine release by MΦ.

- Identifying the main fungal PRR and the associated intracellular signaling responsible for the induction of sMR production. This was done by using knock-out mice and intracellular signalling inhibitors.

- Determining whether MR-shedding can also be triggered by stimuli other than fungi. This was achieved by the incubation of MΦ with house dust mite or fixed Staphylococcus aureus.

2. THE EFFECT OF EXTRACELLULAR MATRIX ON MR FUNCTION AND EXPRESSION

2.1. Introduction

Extracellular matrix (ECM) constitutes the extracellular part of animal tissues, providing structural support and elasticity, as well as cellular organisation. In addition, by sequestering growth factors, it can also be involved in cellular growth and differentiation. The expression of ECM macromolecules exhibit a tissue-specific distribution which results in tissues displaying different forms of extracellular medium; ranging from hard structures of bone and teeth, to the transparent matrix of the cornea.

ECM contents are produced and secreted by the underlying cells, and mainly by fibroblasts. There are three main types of molecules abundant in ECM; collagen fibres, highly viscous proteoglycans, and matrix adhesive glycoproteins. Among those, collagen, which constitutes one-third of all protein in mammalian tissues, is the most abundant protein in the animal kingdom. To date, more than 20 types of collagen, and at least 30 different collagen genes, have been identified. Type I, II and III in connective tissues constitute 80-90% of collagen in the body. Collagen fibers provide mechanical strenght and flexibility to tissues (Lodish, 2003).

Collagen has also been shown to facilitate various cellular responses, such as, cellular adhesion, cell survival, and migration (Leitinger and Hohenester, 2007). To achieve this wide-range of functions, cells express various collagen receptors involved in direct collagen detection, which include integrins, discoidin domain receptors, glycoprotein IV, and leukocyte-associated IG-like receptor-1 (Leitinger and Hohenester, 2007).

MR was recently added to the list by the discovery of its collagen-binding property through its FNII domain (Napper et al., 2006) (Martinez-Pomares et al., 2006). MR was reported to be the only receptor able to mediate collagen internalization by bone marrow (BM)-MΦ and have high binding affinity to collagen types I, II, III, and IV (Martinez-Pomares et al., 2006) (Napper et al., 2006, Gazi and Martinez-Pomares, 2009). As collagen is the most abundant protein in animals, it was suggested that MR might also serve as an ECM adhesion receptor, and accordingly, MR deficiency was shown to result in migration defects in BM-MΦ and myeloblast cells (Sturge et al., 2007) (Jansen and Pavlath, 2006).

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2.1.1. The aim of the study

The possible involvement of MR in cellular adhesion suggests that its activity as an endocytic receptor would be influenced in collagen-rich tissues. The aim of this study was to investigate any effect of MΦ-collagen adhesion on MR expression and function as an endocytic receptor in MΦ (Figure 2.1).

This was done by plating BM-MΦ on ECM protein (namely collagen I, collagen IV and fibronectin(FN))-coated wells overnight (O/N), and screening the level of MR-mediated endocytosis by flow-cytometry. MR expression was monitored at both mRNA and protein levels. The effect of ECM adhesion on overall MΦ activity was also studied by screening the zymosan-induced TNF-α mRNA expression, and the level of acetylated low-density-lipoprotein (LDL) endocytosis.

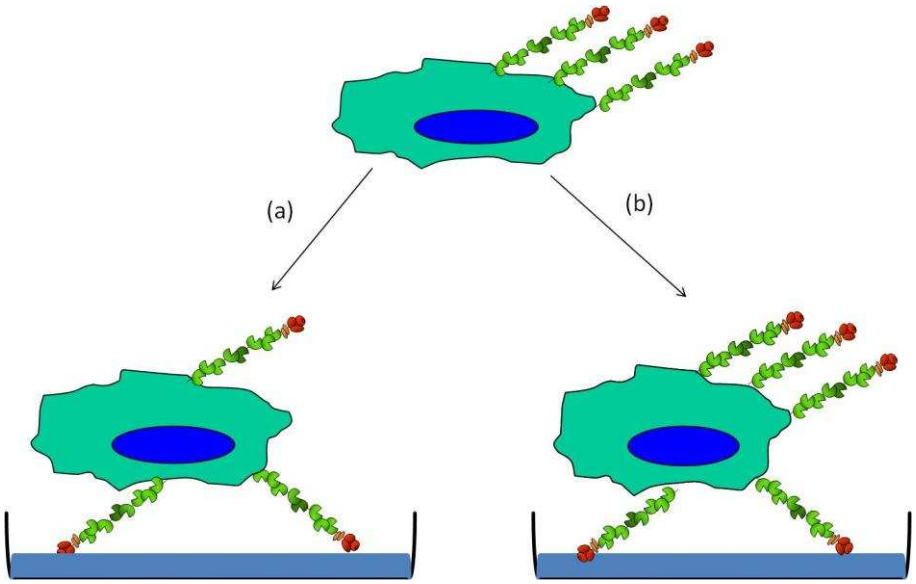

Figure 2.1: The hypothesis. It was hypothesized that if the MR is involved in cellular adhesion, after O/N incubation on collagen-coated plates MΦ would have either reduced MR expression available for receptor-mediated endocytosis (a), or enhanced MR expression, which would enable the MR to facilitate both cellular adhesion and receptor-mediated endocytosis (b).

2.2. Materials and Methods

2.2.1. Animals

Wild-type (wt) C57 BL/6 mice were supplied from Charles River, UK, and MR knock-out (MR-KO) C57 BL/6 mice were bred at the Biomedical Services Unit (BMSU) at the University of Nottingham. Animals were handled according to institutional and UK Home Office guidelines and kept under specific pathogen-free conditions. They were used at 10-12 weeks of age.

2.2.2. Preparation of L929-conditioned media (LCM)

The L929 cell line was cultured in DMEM medium supplemented with 10% (v/v) heat inactivated foetal bovine serum (HI FBS, Sigma Aldrich) or foetal calf serum (HI FCS, Harlem), 2 mM L-glutamine (Gln, Sigma Aldrich), 100 U/ml penicillin (Sigma Aldrich) and 100 μg/ml streptomycin (Sigma Aldrich), on a T-75 tissue culture flask (Nunc) at 37°C for two days after which the cells were sub-cultured into four T-75 flasks. After four days of incubation at 37°C (reaching 100% confluency), the cells were sub-cultured into ten T-225 flasks. The media was then refreshed after three days of incubation. After 10-12 days, the L929 conditioned media supernatant (LCM) was collected, filtered and frozen at -80°C.

2.2.3. Preparation of mouse BM-MΦ

The BM cells collected from wt C57 BL/6 mice were cultured in RPMI medium 1640 (Sigma Aldrich) supplemented with 10% (v/v) HI FBS or HI FCS, 2 mM Gln (Sigma Aldrich), 100 U/ml penicillin (Sigma Aldrich), 100 μg/ml streptomycin (Sigma Aldrich), 10 mM Hepes (Invitrogen) and 15% (v/v) LCM (R-10) on a 150 mm Petri dish (Falcon) for seven days at 37°C. Following maturation, BM-MΦ were harvested using PBS containing 10 mM EDTA (Sigma Aldrich), and 20 mg/ml Lidocaine (Sigma Aldrich) and plated on PBS or ECM-protein coated plates in R-10, or serum-free Opti-MEM with 50 ng/ml M-CSF (R&D) overnight (O/N).

2.2.4. Preparation of non-tissue cultured Bacteriologic Plastic (BP) plates coated with ECM proteins

Type I collagen from rat tails (BD Biosciences), mouse type IV collagen (BD Biosciences) and human FN (BD Biosciences) were diluted in 0.02 N acetic acid, 0.05 N hydrochloric acid (HCl) or PBS (Sigma Aldrich) respectively, at a final concentration of 50 μg/ml; 400 μl and 1.5 ml of each suspension was dispensed into 12-well and 6-well non-tissue cultured BP plates (Falcon) respectively. After 1 hour of incubation at room temperature the wells were washed three times with PBS (Sigma Aldrich), prior the adherence of MΦ.

The ECM protein (collagen I, collagen IV and FN)-coated and uncoated 6-well tissue culture (TC) plastic plates were purchased from BD Biosciences.

2.2.5. Preparation of protein lysates

BM-MΦ plated on PBS or ECM-coated 6-well non-tissue culture BP plates (at a concentration of $1x10^6$ cells/well) were washed three times with PBS and lysed by incubation with ice-cold lysis buffer (2% (v/v) Triton X-100, 10 mM Tris-HCl, pH 8, 150 mM NaCl, 10 mM NaN_3, 2 mM EDTA) plus protease inhibitors (Roche Molecular Biochemicals) (300-400 μl per well of a 6 well plate) for 45 min at 4°C. The lysates and the media were centrifuged at 2 000 rpm at 4°C for 5 minutes using a bench top centrifuge. Supernatants were further centrifuged at 13 000 rpm for 30 mins at 4°C, collected and stored at –20°C. Protein concentration was determined using the BCA protein assay kit (Pierce) following the manufacturer's protocol.

2.2.6. Western blotting

Cell lysate (3 μg for MR-detection or 5 μg for CD68 detection) and an equivalent proportion of supernatant preparation were loaded and electrophoresed in a non-reducing 6% (w/v) sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel and Tris-Glycine-SDS Buffer (TGS) 1X

(Bio-Rad). Proteins were transferred overnight to Hybond-C extra nitrocellulose membrane (GE Healthcare) using transfer buffer; Tris-Glycine Buffer Solution (TG) 1X concentration (Bio-Rad) plus 20% (v/v) methanol. After being incubated for 1 hour in blocking buffer (5% (w/v) non-fat milk in PBS 0.1% (v/v) Tween20), MR and CD68 were visualised by using a combination of anti-MR (clone 5D3, 2 μg/ml) or anti-CD68 mAb (clone FA-11, 5 μg/ml), HRP-conjugated anti-rat IgG (Chemicon), and an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech), high performance chemiluminence film (Hyperfilm, Amersham) and Biomax Cassette (Kodak).

2.2.7. Cell surface biotinylation assay

The cells were washed three times in PBS (Sigma Aldrich) at 4°C and labelled with 0.5 mg/ml sulpho-NHS-LC-Biotin (Pierce) in Ca^{2+} Mg^{2+} containing PBS (Invitrogen) for 30 minutes on ice. The reaction was stopped by incubating the cells in RPMI medium at 4°C for 1-2 minutes. After being washed with ice-cold Ca^{2+} Mg^{2+} containing PBS, the cells were lysed as described in section 2.2.5. Biotinylated proteins were selected by incubating the half the volume of cell lysates with immobilised avidin beads (40 μl of 50% suspension, Pierce) for at least 2 hours at 4°C on a rotating tube rack. The samples were then centrifuged at 13 000 rpm for 2 mins and the supernatant was discarded. The pellet was washed in 1 ml lysis buffer containing protease inhibitor for two times by centrifugation at 13 000 rpm for 2 mins before being suspended in 50 μl 2X SDS Loading buffer. The volume to be loaded on a

SDS-PAGE gel was calculated by using the protein concentration of the non-avidin-captured fractions of the same sample, determined by using the BCA protein assay kit.

2.2.8. Internalization assays

BM-MΦ plated on ECM protein-coated 12-well non-tissue culture BP plates (at concentration of $7x10^5$ cells/well) were washed with serum-free RPMI or Opti-MEM medium and incubated for 30 min at 37°C in the same serum-free media. The cells were then treated with 5 μg/ml of the FITC-labelled sugar polyacrylamide (PAA) substrates (D-mannose-PAA-FITC, SO_4-3-galactose-PAA-FITC) (Lectinity), or acetylated LDL (low-density lipoprotein)-FITC (Invitrogen) for 30 minutes in serum-free media. After incubation, the cells were washed with PBS, harvested using 1X trypsin-EDTA (Sigma Aldrich), and fixed in 1% (v/v) formaldehyde solution in PBS. The internalisation was then analysed using flow cytometry and Weasel software.

2.2.9. Lectin ELISA

All washings were performed in lectin Buffer (10 mM Tris-HCl, pH 7.5, 10 mM Ca^{2+}, 154 mM NaCl and 0.05% (v/v) Tween-20) (250 μl /well).

Sugar PAA substrates (Lectinity), ultrapure mouse laminin (BD Biosciences), laminin from human placenta (Sigma Aldrich) and human FN

(BD Biosciences) were used to coat the ELISA plate wells (Nunc; Maxisob) by incubation in PBS at 4°C overnight (50 µl/well, covered with a Parafilm). After coating, the plates were washed three times with lectin Buffer. The MR domain–Fc construct(s) (Chavele et al., published online in 2010) (Zamze et al., 2002) (Martinez-Pomares et al., 2006) diluted in lectin Buffer were added and left for incubation for 1 hour at room temperature (50 µl per well). During inhibition assays, this step was done in the presence of 12.5 mM mannose or galactose, and 1 M NaCl. The binding was then detected by incubation with alkaline phosphatase-conjugated goat anti-human IgG (gamma-chain specific) (Sigma) diluted 1:1000 in lectin Buffer (50 µl per well) after washing the plate three times with lectin buffer. After being washed with lectin buffer three times and two times with alkaline phosphatase developing buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$), the plates were developed with p-nitro-phenyl phosphate substrate (Sigma-Aldrich) in alkaline phosphatase developing buffer (100 µl per well, 1 mg/ml). Absorbance was measured at 405 nm.

2.2.10 Zymosan treatment

BM-MΦ cultured overnight on 6-well tissue culture-treated, were washed three times with 1 ml PBS to eliminate non-adherent cells. The adherent cells were then treated with zymosan particles (Molecular Probe) at a concentration of 50 particles per cell suspended in Opti-MEM for the indicated time period under serum-free conditions.

2.2.11. MR immunolabeling

All washes between each incubation periods were performed in PBS.

BM-MΦ incubated over different ECM component coated multi-well slides (Nunc) overnight, were fixed with 2% (v/v) paraformaldehyde in Hepes-buffered saline for 10 minutes and washed with PBS before being permeabilised by incubation with PBS containing 0.1% (v/v) Triton X-100 for 10 minutes. The slides were then blocked by incubation with 5% (v/v) normal donkey serum (Sigma Aldrich) in PBS for 30-45 minutes. MR was detected by incubation with 10 μg/ml rat anti-mouse MR (5D3) antibody for 30 minutes in blocking buffer. The same concentration of IgG2a antibody isotype was used as the control staining. The binding was then detected by Alexa 488-labelled donkey anti-rat secondary antibody (Molecular Probes) incubation for 30 minutes in blocking buffer. Slides were mounted using DAKO fluorescent mounting media (Dakocytomation).

2.2.12. Real-time quantitative PCR (qPCR)

RNA was extracted and purified from BM-MΦ, using the RNeasy mini kit (Quiagen). The DNase digestion was performed using the RNase-free DNase set (Quiagen); 500-1000 ng of total RNA was used in the RT reactions. QPCR was performed by using and following the protocol supplied with Brilliant SYBR Green QPCR master mix (Strategene). Primers used for specific PCR were as follows:

Table 2.1: Forward and reverse primer sequences used in qPCR experiments.

Gene	Forward oligonucleotide sequence (5'→ 3')	Reverse oligonucleotide sequence (5'→ 3')
TNF-α	TCTTCTCATTCCTGCTTGTGG	GGTCTGGGCCATAGAACTGA
MR	AGAAAATGCACAAGAGCAAGC	GGAACATGTGTTCTGCGTTG
HPRT	GTAATGATCAGTCAACGGGGGAC	CCAGCAAGCTTGCAACCTTAACCA

2.2.13. Statistical analysis

The results were displayed as the mean ± standard deviation (SD) or mean ± standard error of the mean (SEM) from three independent experiments. Statistical analysis (unpaired t-test) was performed by using GraphPad QuickCalcs software. Differences were considered significant if P-values were < 0.05. Asterisks indicate the corresponding P-values: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

2.3. Results

2.3.1. The MΦ MR can bind to laminin but not to fibronectin

MR is involved in the detection of two different ECM macromolecule classes: chondroitin sulphates of proteoglycans detected by its CR domain and collagen detected by its FNII domain (Gazi and Martinez-Pomares, 2009). However, up to date, there was no study on MR interaction with the third ECM macromolecule class, matrix adhesive glycoproteins.

FN and laminin are the best known examples of adhesive glycoproteins, and therefore were chosen for the binding assays. In order to eliminate any false reading coming from entactin, which forms a very tight non-covalent complex with laminin, entactin-free ultrapure laminin preparations from human placenta were preferred (Niquet and Represa, 1996). Possible MR interaction with FN or laminin was investigated by lectin ELISA using different MR constructs: CR-Fc binds to sulphated sugars through CR domain, CR-FNII-CTLD1-Fc possess sulphated sugar and collagen binding abilities, CTLD4-7-Fc is involved in the recognition of sugars terminated in D-mannose, L-fucose, or N-acetyl glucosamine. The mannan and SO_4-3-galactose coated substrata were used as positive controls. Among the two glycoproteins examined, only laminin was able to bind to MR (Figure 2.2).

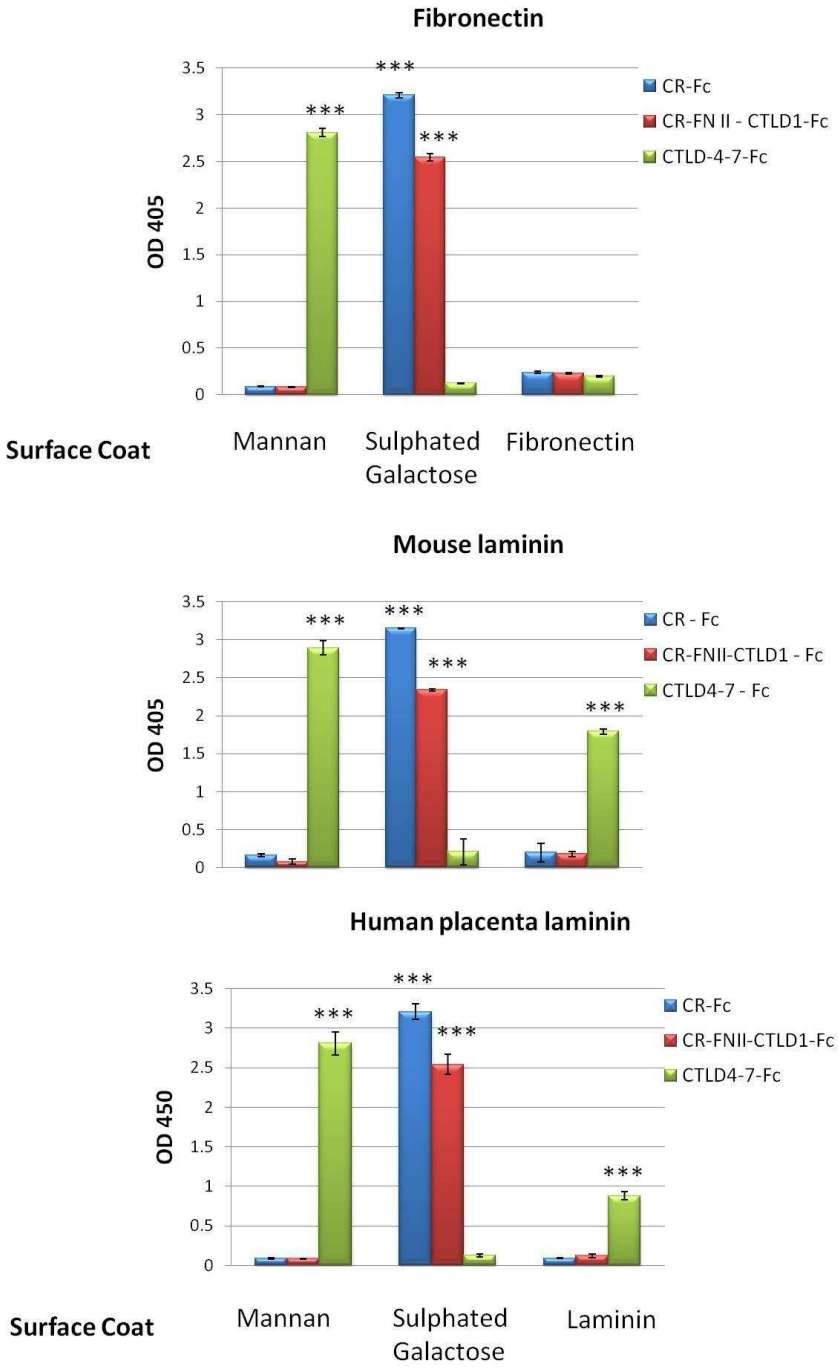

Figure 2.2: MR binds to laminin, but not FN. Analysis by lectin ELISA of the binding of FN (A), mouse laminin (B) and human placenta laminin (C) to the MR-domain-Fc constructs: CR-Fc, CR-FNII-CTLD-1-Fc, and CTLD4-7-Fc. The data represent the mean optical density (OD) at wavelength of 405 nm ± SEM (error bars) obtained from three independent experiments. Asterisks indicate the significant increase of MR construct binding (t-test; *** $P < 0.001$).

The MR interaction with laminin appears to be mediated by the domains involved in mannosylated carbohydrate recognition, which reflects the mannosylated nature of laminin (Cooper et al., 1981) (Figure 2.2 and Figure 2.3). The same binding was also reported when laminin from another source (mice) was used, suggesting that the MR binding, and the mannosylated nature of laminin do not differ between different sources (Figure 2.2).

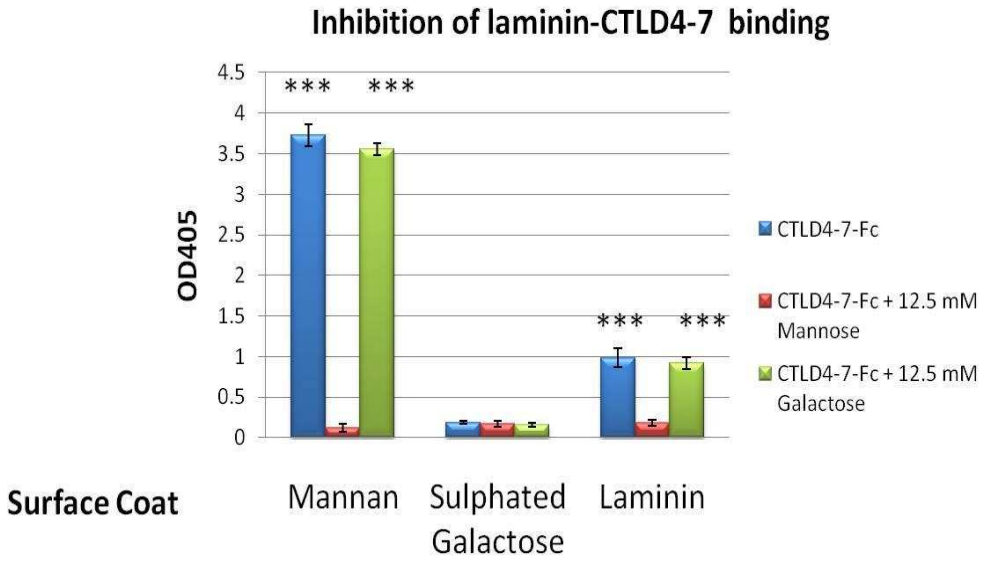

Figure 2.3: Binding of MR to laminin is mannose-mediated. Analysis by lectin ELISA of the binding of human placenta laminin to the CTLD4-7-Fc construct in the presence and absence of mannose or galactose. The data represent the mean OD ± SEM obtained from three independent experiments. Asterisks indicate the significant increase of MR construct binding (t-test; *** $P < 0.001$).

The mannose dependence of laminin-MR interaction was further confirmed by an inhibition assay in which the binding was competed with mannose or galactose (control). In this assay, in contrast to the lectin ELISA mentioned above, the CTLD4-7-Fc construct suspension contains either 12.5

mM galactose or 12.5 mM mannose. The laminin-CTLD4-7 binding was inhibited by the presence of mannose, but not galactose, confirming that the laminin interaction is mediated through the mannose binding domains of MR (Figure 2.3).

2.3.2. Morphologies of BM-MΦ adhered to different ECM components in the presence of serum

MR function and expression upon MΦ adhesion to ECM components was studied by using BM-MΦ matured in R-10 supplemented with LCM for 7 days. Following their maturation, BM-MΦ were plated on uncoated, and ECM protein (collagen I, collagen IV, and FN) coated plates. BM-MΦ were followed at regular time-intervals to detect any significant morphological changes. Among the ECM proteins used both collagen types are known to be MR-ligands. On the other hand, FN was used as a negative control, since, as discussed above, it cannot bind MR.

During the first 5 hours of incubation, BM-MΦ adhered to uncoated and collagen IV- and FN-coated plates relatively faster than to collagen I-coated plates. After 5 hours, the BM-MΦ which adhered to uncoated BP, collagen IV and FN largely displayed slightly spread cell morphology, in contrast to BM-MΦ that adhered to collagen I which exhibited a rather round morphology (data not shown).

However, after 18-24 hours of incubation, the morphologies displayed by MΦ populations on each condition were found to be different. In spite of the relatively slow adhesion of MΦ to collagen I, in comparison to untreated wells, during the first 5 hours of incubation, both MΦ populations showed almost identical spread cell morphology. In the case of collagen IV- and FN-adhered MΦ, however, the spreading was more extensive, with collagen IV-adhered MΦ possessing a more "MΦ-like" morphology and FN-adhered MΦ a "fibroblast-like" morphology (Figure 2.4).

To determine if MR has any role in displaying these morphologies, the appearances of ECM protein adhered wt and $MR^{-/-}$ BM-MΦ were compared. MR does not seem to be important for the morphologies displayed, as both cell types showed the same appearances on the ECM component adhered (data not shown).

The media used to culture the cells for O/N incubation were supplemented by FCS (purchased from Harlem). However, at that time questions regarding the suitability of the serum used for these studies were raised as, thioglycollate-elicited MΦ would not adhere to tissue culture plastic in the presence of this serum, but did in the presence of a different batch of the same serum supplied by a different manufacturer (FBS from Sigma Aldrich). Therefore, the possible effect of FBS on MΦ adhesion and displayed morphology was examined.

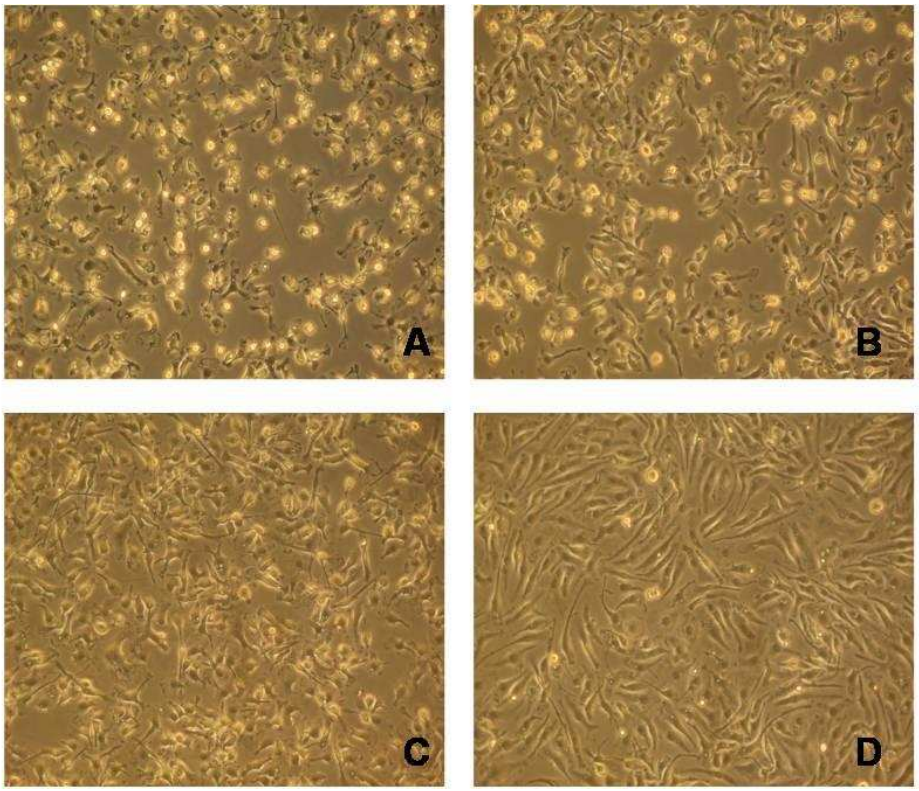

Figure 2.4: Representative MΦ morphology after O/N incubation on different ECM-protein-coated surfaces. The 7-day old BM-MΦ were cultured on PBS (control, A), collagen I (B), collagen IV (C) or FN (D) coated plates in R-10 media with FCS (original magnification X 20) (n > 3).

BM-MΦ also appeared to adhere better to the coated and un-coated plates when grown in FBS-supplemented media. The BM-MΦ on uncoated and collagen IV coated wells adopted a much more fibroblast-like morphology, as observed on FN-coated wells, and the BM-MΦ on collagen I-coated plates, displayed slightly better adherence upon replacement of FCS by FBS (Figure 2.5).

The cellular activation states were previously suggested by Koyama et al (Koyama et al., 2000) to have a crucial impact on cellular adhesion on

different ECM components. In order to examine if the BM-MΦ were being activated by FCS or FBS, the levels of TNF-α (an important cytokine mediator of immune regulation and inflammation) in the extracellular milieu were analyzed using TNF-α cytokine ELISA from zymosan-treated and untreated MΦ.

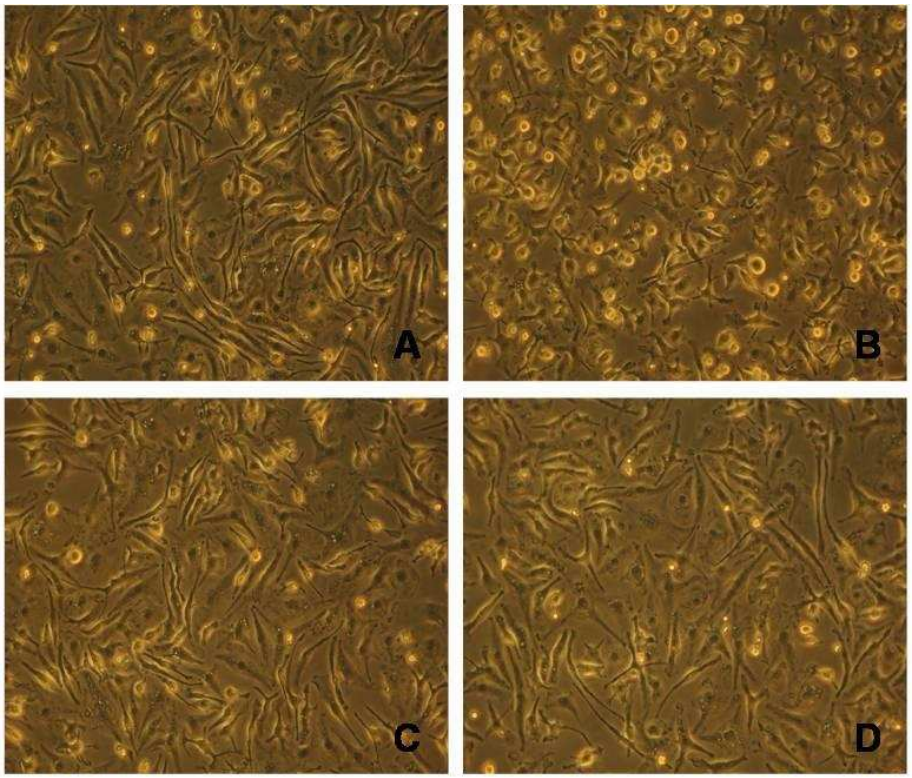

Figure 2.5: Adhesion induced morphological changes in MΦ plated in media supplemented with FBS. 7-day old BM-MΦ were cultured on PBS (control, A), collagen I (B), collagen IV (C) or FN (D) coated plates. In contrast to the previous MΦ populations, these MΦ were matured and used in R-10 media supplemented with FBS in place of FCS (original magnification X 20) (n > 3).

Data showed that the MΦ incubated in the presence of FCS were already activated, as the cell culture supernatant from untreated FCS-cultured

cells possessed significantly higher amounts of TNF-α in comparison to that from untreated FBS-cultured MΦ (Figure 2.6). This reveals that the activation of BM-MΦ by FCS ingredients is responsible for the morphologies displayed upon adhesion to plastic and ECM components shown in Figure 2.4 and Figure 2.5.

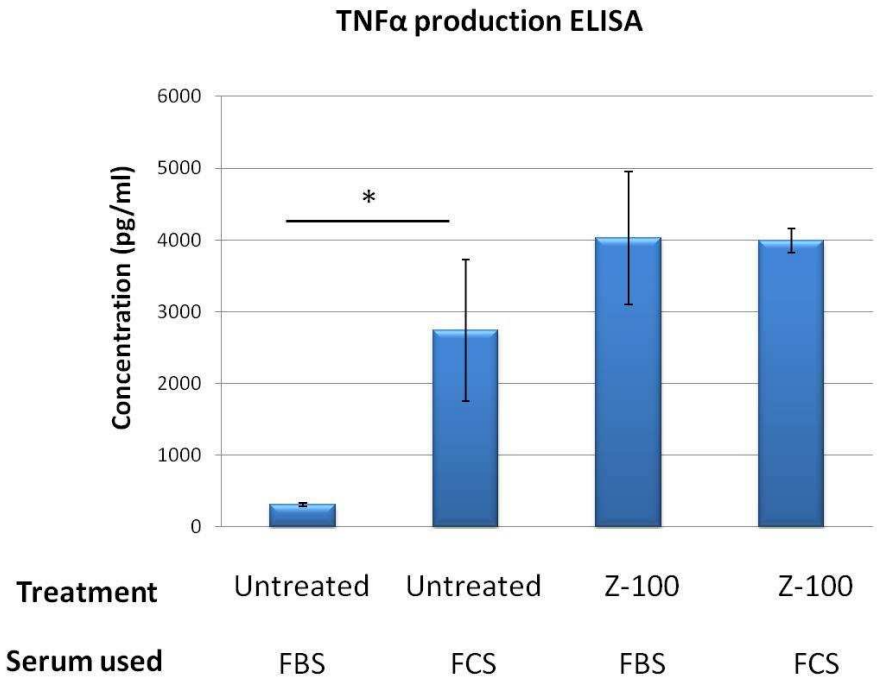

Figure 2.6: BM-MΦ matured in FBS posses significant TNF-α production even in the absence of any immunologic activator. Capture ELISA analysis of the TNF-α production by MΦ left untreated or treated with zymosan particles (at a concentration of 100 particles/cell, Z-100) under the conditions in which the supplementary serum was either FCS or FBS. The data represent the mean TNF-α concentration detected ± SEM obtained from three independent experiments. Asterisks indicate a statistically significant increase of TNF-α production compared to FBS-cultured unstimulated cells (t-test; * $P > 0.05$).

2.3.3. Morphologies of BM-MΦ adhered to different ECM components in serum-free conditions

To increase the influence of the ECM components on the MΦ, the morphology of BM-MΦ plated on ECM-protein-coated wells was also examined in serum-free Opti-MEM media supplemented with 50 ng/ml M-CSF, instead of LCM which contains serum.

It seems that the inclusion of serum modulates the effects that ECM components have on MΦ appearance. The “fibroblast like” morphology of uncoated, collagen IV or FN-adhered MΦ observed in the presence of FBS, was completely lost and, together with collagen-I-adhered BM-MΦ, they had a “MΦ-like” morphology (Figure 2.7).

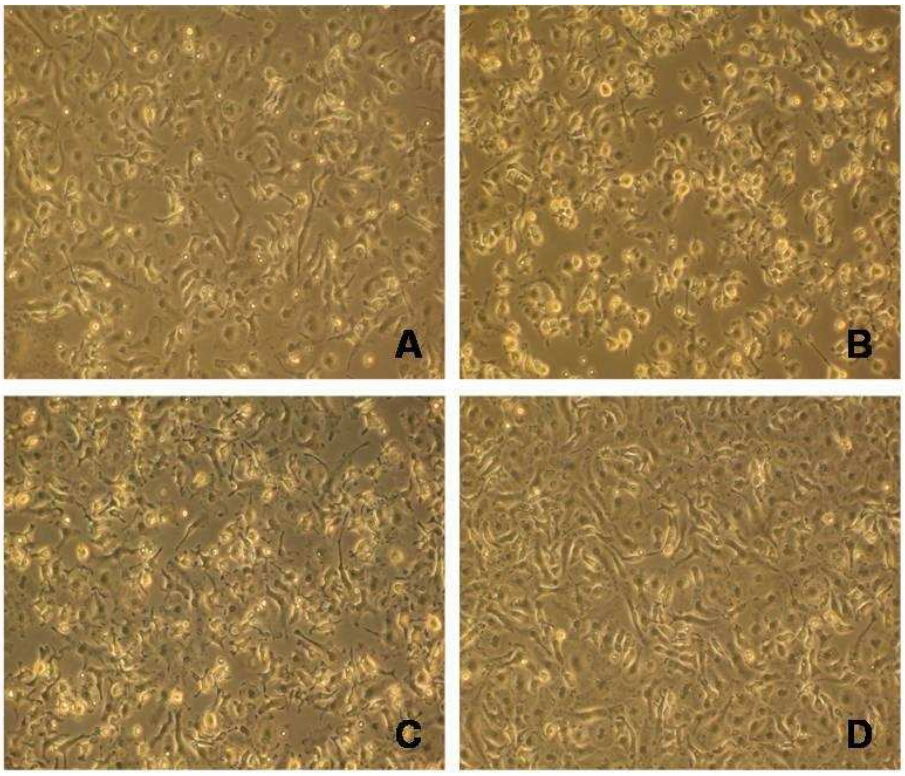

Figure 2.7: In the absence of serum, BM-MΦ exhibit different cellular morphology upon adhesion to different ECM proteins. The 7-day old BM-MΦ were cultured O/N on PBS (control, A), collagen I (B), collagen IV (C) or FN (D) coated plates in serum-free Opti-MEM suppmeneted with M-CSF (original magnification X 20) (n > 3).

Similar differences can also be marked when compared to the morphologies observed in FCS-containing conditions. In contrast to MΦ cultured in the presence of FCS, MΦ in serum-free conditions displayed firmer adhesion to uncoated wells: the spreading of the adhered MΦ were more extensive and the incidence of round non-adhered cells was lower. MΦ also displayed better adhesion to collagen-I-coated wells in serum-free conditions. On the other hand, the MΦ on FN-coated surfaces lost their fibroblast like

morphology, and had more MΦ-like morphology in comparison to the MΦ adhered to FN under serum-containing conditions (Table 2. 2).

Table 2. 2: The cell-spreading displayed by BM-MΦ plated on ECM-protein-coated wells changed depending on the presence and the type of serum used during plating. The + indicates MΦ with low spreading, while +++++ implies MΦ with extensive spreading and fibroblast-like morphology, as observed under light microscopy.

Plating Conditions	Adhesion Surface			
	PBS	Collagen I	Collagen IV	Fibronectin
FCS	+	+	+++	+++++
FBS	+++++	++	+++++	+++++
Serum-free	++++	++	+++	++++

2.3.4. ECM adhesion does not alter MR-dependent or –independent ligand endocytosis and zymosan-induced cytokine mRNA expression

The recent discovery of the collagen-binding property of MR, suggested that MR might also serve as an ECM adhesion receptor, which in turn may influence its activity as an endocytic receptor. Therefore, the MR-mediated endocytic activities of ECM protein-adhered BM-MΦ were screened

by using FITC labelled MR ligands; SO_4-3-galactose-PAA and BSA-mannose. In order to verify that the change, if any, in MR-mediated endocytosis was MR-specific, uptake of FITC labelled acetylated LDL (ac-LDL) was also monitored. To exclude any possibility that the manual surface coating of BP plates would have artificial effects on MΦ, ECM protein-coated TC plates (purchased from BD Biosciences) were used under serum-free conditions.

The level of internalized endocytic tracers, both MR- and non-MR ligands, by BM-MΦ was not influenced by the ECM component in all conditions mentioned (Figure 2.8). This indicates that ECM adhesion does not affect the cellular endocytic machinery, including the MR-mediated pathway, and that the collagen binding ability of MR does not interfere with the MR endocytic function.

In addition to monitoring the uptake of endocytic tracers of both MR and non-MR-ligands, the possible effect of ECM adhesion on MΦ function was further investigated by investigating zymosan-induced TNF-α expression at the mRNA level. Zymosan is derived from the S. cerevisea cell wall and is mainly composed of β-glucans and mannan. It is known to activate several MΦ receptors such as dectin-1, complement receptor (CR)-3, MR, TLR2 and TLR4.

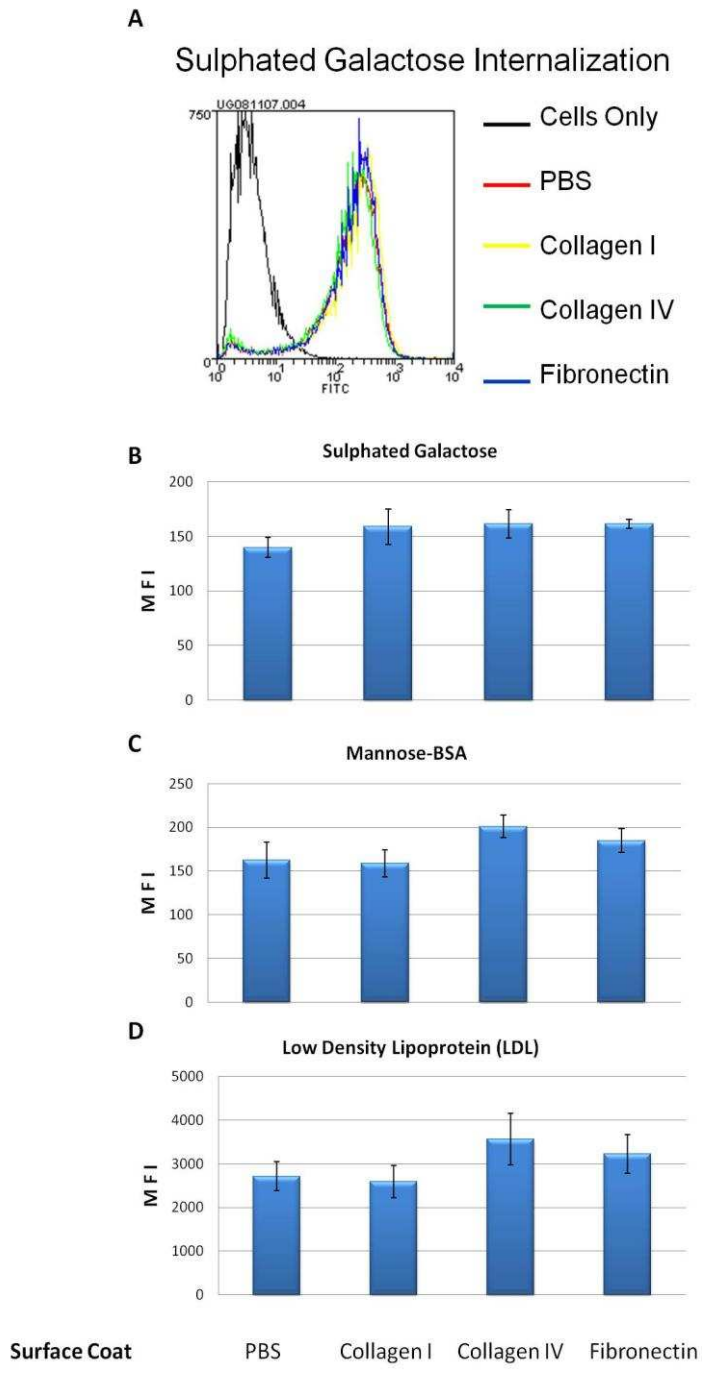

Figure 2.8: ECM adhesion does not influence MR- and non-MR-mediated endocytic machinery. Flow cytometry analysis of sulphated galactose (A-B), mannose-BSA (C), and ac-LDL (D) internalization by BM-MΦ cultured O/N on PBS, collagen I-, collagen IV- or FN-coated plates. The histograms represent the mean MFI value ± SEM obtained from three independent experiments. Same results were reported in the distinct conditions mentioned above (i.e. inclusion or exclusion of serum during plating of MΦ, and using already or manually coated plates).

After O/N incubation on the ECM component coated substrata, BM-MΦ were treated with zymosan particles for 6 hours to monitor the TNF-α mRNA expression levels. Like the receptor-mediated endocytic machinery, the mRNA levels of both cytokines were found to be independent of the adhesive surface used, in both serum- and serum-free conditions (Figure 2.9).

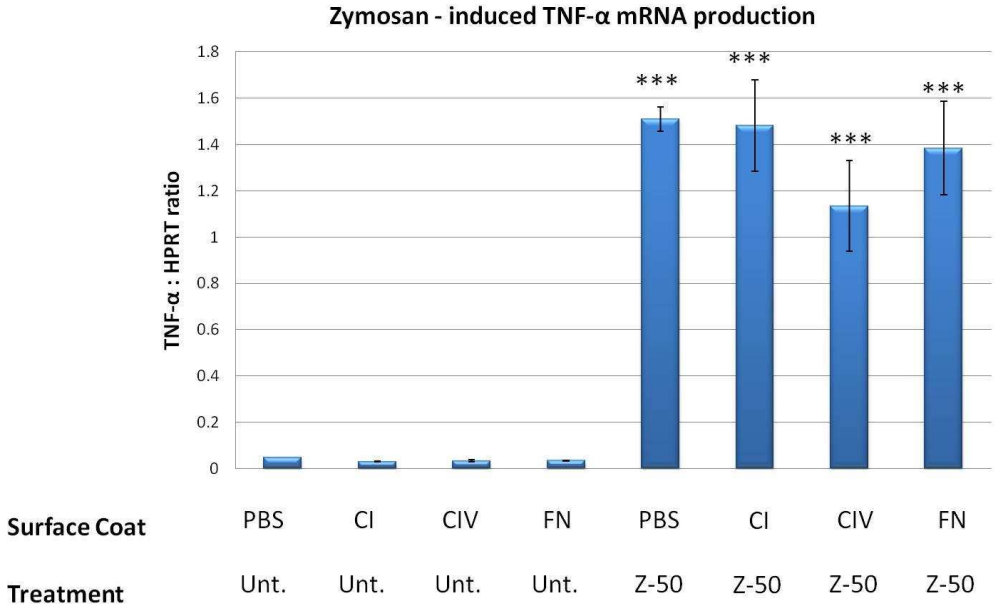

Figure 2.9: ECM adhesion does not influence zymosan-induced TNF-α mRNA induction. QPCR analysis of TNF-α mRNA expression levels by BM-MΦ left untreated (Unt.) or treated with zymosan particles at a concentration of 50 particles/cell (Z-50). The data represent the mean ratio of mRNA expression of TNF-α to HPRT ± SEM obtained from three independent experiments. Asterisks indicate a statistically significant increase of TNF-α production compared to unstimulated cells (t-test; *** $P < 0.001$). Same results were reported in the distinct conditions mentioned above.

2.3.5. ECM adhesion does not influence MR expression

The lack of effect of collagen-adherence on the MR-endocytic activity may result from the enhanced levels of MR expression that would enable MR to participate in both cellular adhesion and endocytosis. This led us to examine the MR expression in MΦ adhered to the ECM component by using different approaches including immunocytochemistry, western blot, cell surface biotinylation and qPCR.

In addition to the cell-associated form, MR is also expressed as soluble MR (sMR), which is produced as a result of the proteolytic cleavage of cell-associated MR (cMR), and was initially discovered in mouse-serum (Martinez-Pomares et al., 1998). To this day, apart from P. carinii, there had been no exctracellular signalling identified to regulate the sMR expression specifically (Martinez-Pomares et al., 1998, Fraser et al., 2000). Therefore in addition to cMR, sMR expression level was screened at the protein level by using cell culture supernatants collected from the same set of BM-MΦ.

As can be seen in Figure 2.10, both q-PCR and western blot results revealed that as well as sMR, cMR expression at both protein and mRNA levels is not affected by the MΦ adhesion to ECM components, including collagen I and IV (Figure 2.10A-B). Additionally, in correlation with the internalization assay results (Figure 2.8), surface biotinylation data showed that all MΦ populations had similar expression levels of surface MR available

for MR-ligand endocytosis (Figure 2.10A). However, this data may not be conclusive due to the presence of FA-11 positive bands (CD68 macrosialin, a late endosomal glycoprotein (Kogelberg et al., 2007)) in the surface of biotinylated samples, indicating the possible biotinylation of intracellular proteins. On the contrary, CD68 cell surface expression was also reported in an independent study by Kurushima et al. (Kurushima et al., 2000).

This led us to examine if the distribution of cMR expression is affected by collagen adhesion such that it becomes available for both cellular adhesion to collagen-coated surfaces and MR-ligand endocytosis. The immunocytochemistry data shows that the cMR in MΦ are spread all-around the cell, and this was not altered when the adhesive surface was changed (Figure 2.10C).

Overall, these data suggests that the lack of effect of collagen-adherence on the MR-endocytic activity is not as a result of the changes in the MR expression and distribution.

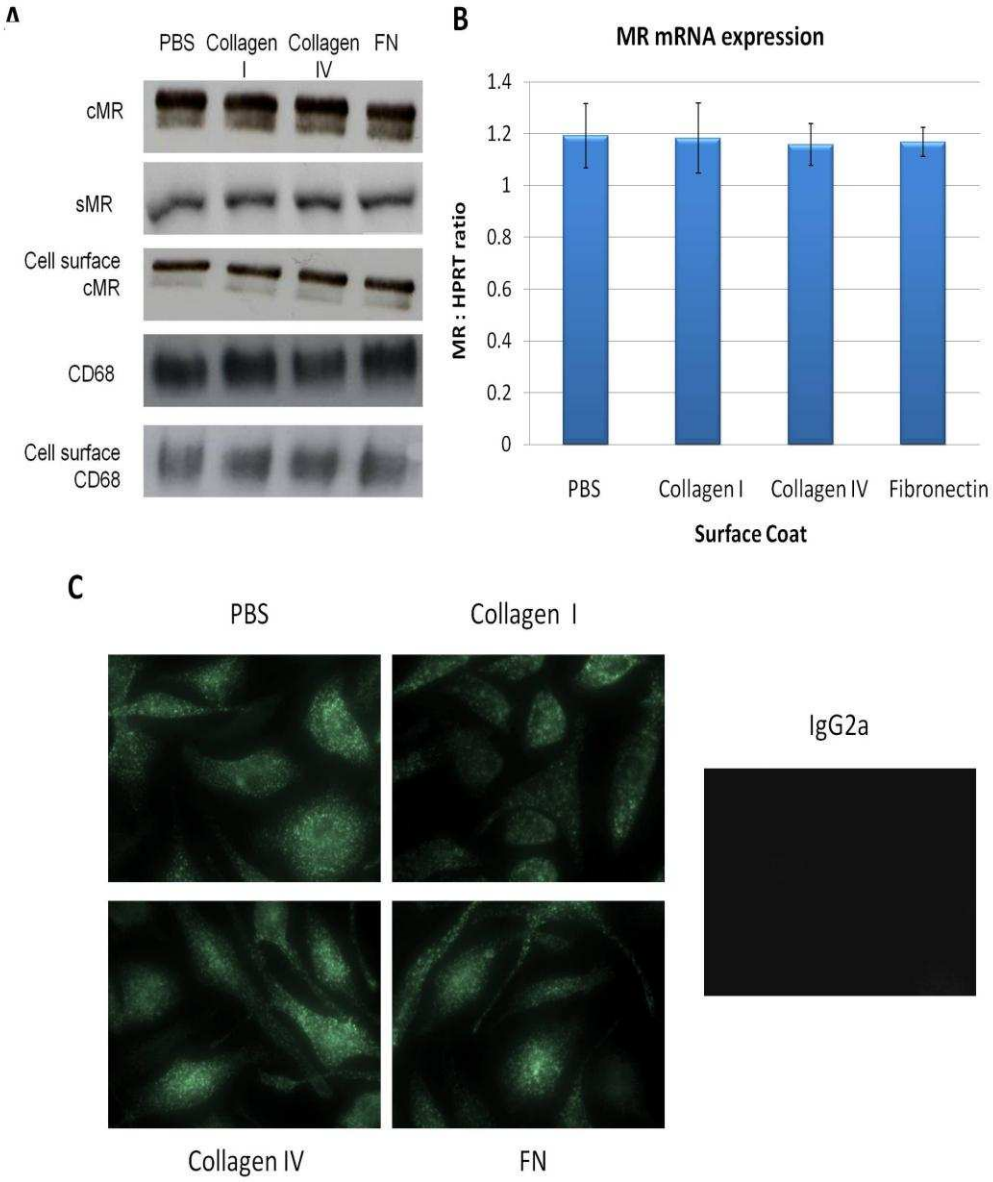

Figure 2.10 : **ECM adhesion does not affect MΦ MR expression.** Western blot analysis of cMR, cell surface cMR, sMR, CD68, and cell surface CD68 expression by BM-MΦ after O/N incubation on un-coated and ECM-component coated plates (A). QPCR analysis of MR mRNA expression by adhered BM-MΦ (B). The data represent the mean ratio of mRNA expression of MR to HPRT ± SEM obtained from three independent experiments in the distinct conditions mentioned above. Fluorescence microscopy analysis of cMR expression by BM-MΦ (original magnification X 100) (C). Signals from FITC conjugated 5D3 were detected by green channel, and IgG2a antibody isotype was used as the control staining. Same western blot and qPCR results were reported in all conditions mentioned above. The immunocytochemistry data was only performed on manually coated slides in serum-free and serum-containing conditions.

2.4. Discussion

The MR is an example of a pattern recognition receptor involved in the recognition of various pathogens, including C. albicans, L. donovani, and M. tuberculosis, as well as in the maintenance of haemostasis through the binding to endogenous ligands, such as pituitary hormones lutropin and thyrotropin, tissue plasminogen activator, and hydrolytic enzymes (Gazi and Martinez-Pomares, 2009). It was also suggested to participate in tissue remodelling via its association with ECM macromolecules, such as chondroitin sulphate-associated proteoglycans and collagen.

However, due to its high abundance in animal tissues, the discovery of the collagen binding property of MR raised the question of how the MR endocytic function will be affected in tissues rich in collagen. Therefore, in this study, the possible effect of the MR-collagen interaction on MR function and expression was investigated by using 7-day-old BM-MΦ incubated O/N over surfaces covered with collagen I, IV, or FN. Together with an uncoated surface, FN was also used as a control since, as shown in the Figure 2.2, it cannot bind MR.

After O/N incubation on ECM-protein coated substrata in FBS-containing conditions, collagen I-adhered MΦ displayed a different, slightly less adhered, morphology to that shown by MΦ on uncoated, collagen IV and FN-coated plastic. Similar non-adhesiveness to collagen-I was also reported previously for other MΦ types such as U937, J774-1 and peritoneal exudate MΦ (Koyama et al., 2000), indicating that it is not specific to BM-MΦ. As

suggested for U937 cells by Koyama et al, low α_2 integrin subunit expression (of $\alpha_2\beta_1$ involved) may, at least partly, explain the observed effect. Other receptors involved in the detection of collagen I, but not collagen IV, include integrin $\alpha_{11}\beta_1$, DDR2, and LAIR-1. Among these, LAIR-1 can send inhibitory signals via its ITIM signalling motif on the cytoplasmic tail.

The BM-MΦ morphology was also influenced by the batch of the serum (FCS from Harlem or FBS from Sigma Aldrich) used to supplement the media for O/N incubation. A possible explanation is the significant level of TNF-α production in cell culture supernatants collected from the untreated BM-MΦ cultured in media supplemented with FCS. This implies that before being plated on ECM-component coated plates, the FCS-cultured MΦ were already activated; something that was previously suggested to influence ECM adhesion (Koyama et al., 2000). Differences between the two serum batches could be caused by variation in animals, handling and country of origin.

The cell appearance underwent a further change when the serum was removed, confirming a serum-mediated effect on the morphology shown by MΦ. During O/N incubation, serum components may cover the cell surface and interfere with the cellular adhesion to ECM components by interacting with MΦ surface receptors, before the cells establish a strong adhesion with the adhesive surface (e.g. serum FN can interact with FN receptors on BM-MΦ). In this way, the trapped serum contents would not be removed by the PBS washes, and would have an effect on the cell morphology displayed.

The endocytosis assay using both FITC-labelled MR- and non-MR-ligands showed that both MR-dependent and –independent endocytic capacity of the MΦ were not affected by the adhesive surface, including collagen I and IV which are known to be MR-ligands. The observed lack of effect was not because of any change in the MR expression level which would enable the receptor to participate in both adhesion and endocytosis. This was demonstrated by both western blot and q-PCR data showing no change in the expression levels of MR mRNA and protein levels in the BM-MΦ populations compared. Furthermore, in correlation with the internalization assay results, surface biotinylation assay showed no alteration in the surface MR expression, available for MR-mediated endocytosis.

As an alternative approach to the endocytic studies, the MR expression pattern was also investigated by immunocytochemistry. Adhesion to collagen I or IV may trigger intracellular signalling which would divert the MR expression in a way that it would become concentrated on both MΦ surfaces involved in adhesion and ligand endocytosis. The immunocytochemistry data showed no obvious changes in the expression pattern of MR, by the BM-MΦ incubated on ECM-protein coated substrata for O/N.

However, this does not exclude the possibility of altered MR expression on both MΦ surfaces, as the observed lack of change may be due to the detection of out-of-focus light emitted as a result of the excitation of the whole specimen. This results in the detection of the fluorescence coming from all parts of the MΦ, rather than specific ones. The out-of-focus light can be

eliminated by using confocal microscopy, which is able to isolate and collect a plane of focus from within a sample, and in this way it can detect light emitted only from the focused parts of the cell. Future studies involving confocal microscopy can further help to analyse the MR expression on MΦ surfaces involved in the adhesion to ECM protein-coated wells.

Apart from receptor-mediated endocytosis, the possible effect of ECM adhesion on MΦ cellular responses was further examined by monitoring zymosan-induced TNF-α mRNA expression levels. In contrast to Rosas et al., who did not observe any TNF-α production by BM-MΦ in response to particulate β-glucan (the active component of zymosan) (Rosas et al., 2008), the data shown demonstrates a significant enhancement in the level of TNF-α mRNA synthesis upon incubation with zymosan particles. The observed difference can be explained by the involvement of TLRs, which are also stimulated by zymosan particles. Accordingly, BM-MΦ from mice with MyD88 (central adaptor molecule for TLR signalling) deficiency were shown to lack TNF-α production in response to zymosan particles (Gantner et al., 2003). Nevertheless, the level of enhancement in the TNF-α mRNA synthesis did not differ in BM-MΦ adhered to ECM protein-coated and uncoated surfaces.

Overall, it was suggested that the role of the collagen binding ability of MR may be to mediate the clearance of collagen fragments, rather than cellular adhesion. MR interaction with collagen, in addition to other ligands of endogenous origin such as lysosome enzymes, makes MR a key receptor

involved in the resolution of inflammation, tissue remodelling, and wound-healing. In fact, MR is used as a marker for MΦ populations, known as alternatively-activated MΦ (AA-MΦ), which are mainly involved in the resolution of inflammation and maintaining heaemostasis once the pathogen is neutralized (Daigneault et al., published online in 2010).

Apart from collagen, another ECM component that MR was shown to interact with was chondroitin sulphate-associated proteoglycans. This list was further extended in this study which was the first to show the interaction between MR and the matrix adhesive glycoprotein class of ECM macromolecules. Among the best characterised members of this family, laminin, but not FN, was shown to engage with MR in a mannose-dependent manner, through the CTLD4-7 region. This is in correlation with the previous study by Cooper et al that showed the mannosylated nature of laminin (Cooper et al., 1981).

The other study which emphasizes the importance of MR-ECM interaction was Sturge et al., which showed enhanced random migration of MR $^{-/-}$ BM-MΦ (Sturge et al., 2007). In support to the data presented, this suggests a putative role for MR in the function of podosomes, which are subcellular structures used by myeloid cells for migration, as well as internalization, of receptor-bound antigens- e.g. collagen and other matrix components detected by MR. The MR function in cell migration was further highlighted by the enhanced MΦ recruitment in the lungs of MR-KO mice infected with P.carinii (Swain et al., 2003).

To conclude, in this chapter, it was shown that, in spite of inducing distinct cell morphologies, substrata coated with ECM component (including collagen I and IV) did not have a major effect on murine BM-MΦ function including zymosan-induced cytokine mRNA expression, MR- and non-MR-mediated endocytosis as well as MR expression. Therefore MR is proposed to function mainly as an endocytic rather than an ECM adhesion receptor, and the collagen binding ability of MR might be simply crucial for tissue remodelling and wound healing during which MR expression is enhanced by the released cytokines such as IL-10, IL-4, and IL-13. With this study, the range of ECM components recognized by MR is extended further to include laminin as it was shown to bind MR in a mannose-dependent fashion.

3. ENHANCED MR-SHEDDING IN RESPONSE TO FUNGI SPECIES AND HOUSE DUST MITE

3.1. Introduction

In addition to the cell-associated form (cMR), a soluble form of MR (sMR) has been detected previously in the supernatants from cultured human and mouse MΦ, and in human and mouse serum (Taylor et al., 2005a, Martinez-Pomares et al., 2006, Martinez-Pomares et al., 1998, Jordens et al., 1999). sMR comprises the extracellular region of the receptor and is produced as a result of proteolytic cleavage of cMR by a metalloprotease (Martinez-Pomares et al., 2006). Previously Fraser et al observed that P. carinii enhanced sMR production, and reported the formation of sMR-coated fungi that could not be phagocytosed by MΦ (Fraser et al., 2000).

Fungi constitute a part of normal flora in healthy individuals, and the innate immune response provides an effective defence against all fungal species that are encountered. Fungi-associated diseases in humans are mainly caused by opportunistic fungi that represent a small subset and cause infection once the immune system is compromised. Because of the increased incidences of immunocompromised individuals as a result of HIV infection, and immunosuppressant administration to transplant recipients and cancer patients,

the clinical relevance of fungal diseases has augmented sharply. Today the average mortality from opportunistic fungal infections is still more than 50% and it is as high as 95% in bone-marrow transplant recipients (Romani, 2004).

To date, almost all of the fungal PAMPs involved in anti-fungal immunity were found to be carbohydrate structures that make up to 90% of the cell wall (Figure 3.1). The associated PRRs are mostly those that can detect mannose-rich matrix component, and β-glucan of the inner skeletal component. Membrane-anchored receptors involved in the recognition of mannosylated macromolecules include C-type lectins; MR, dectin-2, DC-SIGN, and mincle, and TLRs; TLR2, and TLR4. The β-glucan component was shown to be detected by at least four β-glucan receptors: lactosylceramide, scavenger receptors, complement receptor-3 (CR3) and dectin-1. While the physiological role of the former two is still unclear, the latter two are the primary receptors responsible for β-glucan recognition. Today the PRR involved in chitin detection is still not yet clear (Netea et al., 2008).

3.1.1. The aim of the study

The aim of this study was to investigate if the enhanced shedding of MR was fungi- rather than P. carinii-specific and was triggered by the β-glucan component of the fungi cell wall, which was shown to have a central role in the initiation of the anti-fungi immune response (Taylor et al., 2007), (Werner et al., 2009, Brown et al., 2003).

MR-shedding was detected by western blot and there were several attempts to identify the protease responsible. The effect of enhanced sMR production on MΦ function was also examined by screening the levels of fungi-induced cytokine production and MR- and SR-mediated endocytic internalization.

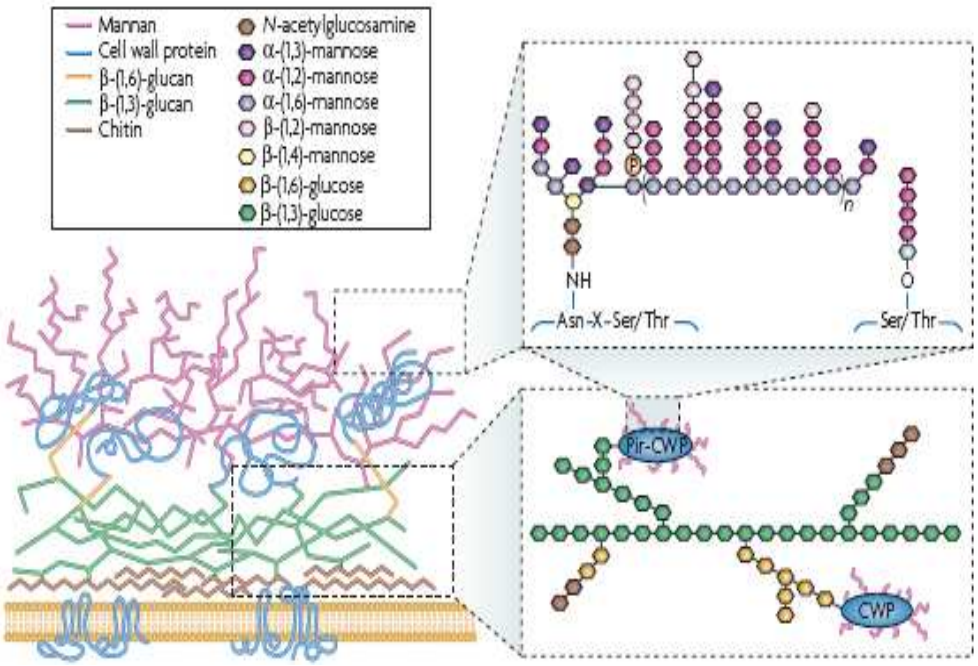

Figure 3.1: The Candida albicans cell wall structure. Fungal cell walls are composed of two components: highly immunogenic inner skeletal components (i.e. chitin and β-glucan) which are found in proximity to the cell membrane in an inner layer; and the matrix components which, in contrast, are located towards the outside of the cell wall. The matrix component is rich in highly mannosylated glycosylphosphatidylinositol-linked (GPI)- and Pir- cell wall proteins (CWP) which are anchored to the inner skeleton through GPI linkages with β-(1,6)-glucan and alkali-sensitive linkages with β-(1,3)-glucan, respectively (Netea et al., 2008). Even though they are mostly hidden deep inside the cell wall, the surface expression of chitin and β-glucan can be detected in restricted regions such as bud scars, as a result of cell wall modelling during budding (Ruiz-Herrera et al., 2006).

3.2. Materials and Methods

3.2.1. Animals

The wt and MR-KO mice were bred and handled as described in section 2.2.1.

3.2.2. Cells

Thioglycollate-elicited mouse MΦ (thio-MΦ) were obtained by intraperitoneal injection of 4% (w/v) Brewer's thioglycollate broth (Sigma Aldrich) 4 days before harvest. Mice were sacrificed and the peritoneal cavities were rinsed with PBS (Sigma Aldrich) containing 5 mM EDTA. MΦ were cultured O/N on 6-well tissue culture plates ($1.25x10^6$ cells per well, BD Biosciences) in RPMI medium 1640 (Sigma Aldrich) supplemented with 10% (v/v) HI FBS (Sigma Aldrich), 2 mM Gln (Sigma Aldrich), 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma Aldrich), 10 mM Hepes (Invitrogen) (R-10 media) and washed three times with PBS prior use.

3.2.3. Experimental conditions

Thio-MΦ were treated with particulate house dust mite (HDM) (Allergon), zymosan (Molecular Probes), *A. fumigatus* ATCC 13073 strain (a kind gift from Gordon Brown from the University of Aberdeen), Heat-killed

(HK) and paraformaldehyde-fixed C. albican ATCC 18804 strain (gift from Sigrid Heinsbroek from the University of Amsterdam), Staphylococcus aureus RN6390B strain (kindly contributed by Alan Cockayne, from the University of Nottingham) at concentrations of ~50 particles per cell in serum-free Opti-MEM with GlutaMAX (Invitrogen) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin for 3 hours at 37°C, 5% CO_2. Before treatment with fixed stimuli, free aldehyde groups were quenched by incubating with 0.1 M glycine in PBS, followed by three washes in PBS.

For inhibition assays, thio-MΦ were pre-incubated with β-glucan phosphate (kindly contributed by David L. Williams, from East Tennessee State University, USA), mannan (Sigma Aldrich), z-VAD-fmk (Calbiochem), GM6001 or control for GM6001 (Calbiochem) at indicated concentrations for 1 hour before treatment, and were present during the incubation with the stimuli.

3.2.4. Collection of culture media and preparation of cell lysates

Before starting cell lysate preparation, cell culture supernatants were collected after the addition of protease inhibitors (1X final concentration) (Roche Molecular Biochemicals), 10 mM EDTA, and 1 mg/ml BSA (Sigma Aldrich) to diminish any protein loss due to protease activity or non-specific adhesion to the collection tube. Following PBS washes to remove any non-

adhered cells, MΦ were lysed and protein concentration was quantified by using the same protocol described in section 2.2.5.

3.2.5. Western blotting

Equivalent proportions of cell lysates (3 μg) and supernatants were electrophoresed under the same conditions as described in section 2.2.6.

3.2.6. Internalization assay

Thio-MΦ were plated for 2 hours at 37^{o}C 5% CO_2 in non-tissue culture treated plastic in Opti-MEM with GlutaMAX (Invitrogen) containing 100 U/ml penicillin (Sigma Aldrich) and 100 μg/ml streptomycin (Sigma Aldrich). After 2 hrs Opti-MEM was replaced with R-10 media and cells were maintained at 37^{o}C, 5% CO_2 O/N and MΦ selected by washing three times with cold PBS. Following a 3-hour treatment with zymosan in the presence and absence of GM6001 or GM6001 control, the MΦ were then incubated for 30 minutes in serum-free Opti-MEM media containing 5 μg/ml of SO_4-3-Gal-PAA-FITC (Lectinity) or Alexa-488 conjugated acetylated low-density lipoprotein (Ac-LDL, Invitrogen). The MΦ were collected by scraping, using 1X trypsin-EDTA (Sigma Aldrich) and fixed in 1% (v/v) formaldehyde solution in PBS. The internalisation was then analysed using Beckman Coulter Epics Altra and Weasel software.

3.2.7. Cytokine assays

Levels of TNF-α, MCP-1 and KC were determined by using the mouse TNFα DuoSet, mouse CCL2/JE/MCP-1 DuoSet and mouse CXCL1/KC DuoSet (R&D systems) respectively.

3.2.8. Cytokine profiles

The cytokine profiles of treated MΦ were determined by using mouse cytokine array panel A array kit (R&D systems) and following the manufacturer's protocol. At the end of the protocol, the cytokine array data on the developed X-ray film was quantified by utilising a transmission-mode scanner (BD Biosciences).

3.2.9. Preparation of liver necrotic cell extracts

Mouse liver (12 weeks old) was dissected into minute pieces in a sterile environment before been homogenized in sterile PBS by using a Dounce homogenizer. The liver homogenate was centrifuged at 2 000 rpm for 5 minutes and the supernatant was collected.

3.2.10. Statistical analysis

Statistical analysis was performed as described in section 2.2.13.

3.3. Results

3.3.1. MR shedding is promoted by the non-opsonic recognition of fungal particles

In order to investigate if other pathogens in addition to P. carinii (Fraser et al., 2000) could promote MR shedding, thio-MΦ were treated with zymosan, fixed A. fumigatus, and fixed and heat-killed (HK) C. albicans, under serum-free conditions. However, initially the incubation time for the treatments was determined by using different time points: 1.5 hours, 3-hours and O/N. In Fraser et al, enhanced sMR production was detected after O/N incubation with P. carinii.

After treatment with zymosan particles, cell culture supernatants and cell lysates were analysed, for sMR and cMR levels respectively, by western blot. As shown in Figure 3.2A, enhanced levels of sMR were detected in cell culture supernatants collected from all time intervals. Since, MR bands detected after each time-interval correspond to independent exposures the band intensities are not comparable.

Shorter incubations were preferred to minimise the chances of having new protein synthesis. It appears that the sMR accumulation after 1.5 hours was not high enough since, in comparison to 3-hour-treated samples, it required longer exposure times to give a detection signal as strong as that

detected from 3-hour treated samples. Therefore, it was chosen to treat thio-MΦ with fungal particles for 3 hours.

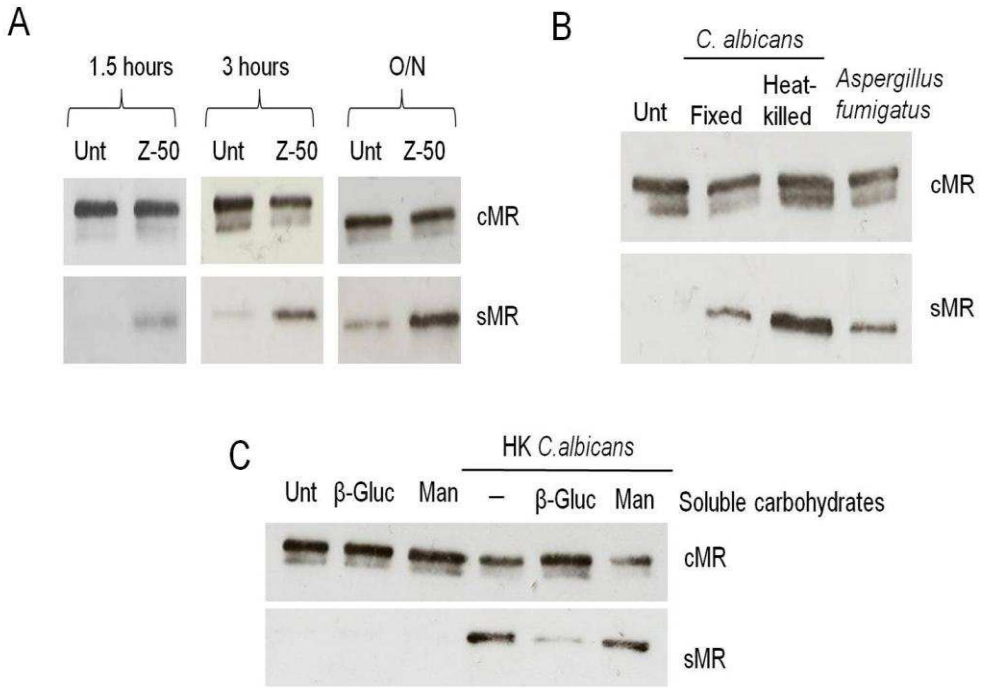

Figure 3.2: MR shedding is induced by zymosan, C. albicans and A. fumigatus, and requires β-glucan-recognition. Western blot analysis of cMR and sMR expression by thio-MΦ incubated with zymosan particles at concentration of 50 particles/cell (Z-50) for 1.5 hours, 3-hours or O/N (A). The level of cMR and sMR expression by thio-MΦ treated with fixed A. fumigatus, or fixed or HK C. albicans for three hours (B). MR shedding in response to HK C. albicans could be inhibited by soluble β-glucan-phosphate (β-Gluc) but not mannan (Man) pre-treatment (C). Data are representative of three independent experiments.

Enhanced MR-shedding was also evident after 3-hour treatment with other fungi species examined, i.e. fixed A. fumigatus, and fixed and HK C. albicans (Figure 3.2B). However, the method of fungi processing appeared to have an effect on the level of sMR production as HK C. albicans consistently induced more MR shedding than the fixed fungal particles. Due to their

complex composition, C. albicans, like other fungi species, mediate their effect on MΦ through a variety of receptors. Among those, β-glucan has been previously shown to be the active component involved in fungal detection. As during heat-inactivation, the β-glucan component is thought to become more exposed on the outer surface (Wheeler and Fink, 2006), it was hypothesised that enhanced β-glucan exposure could be responsible for the differences observed between HK- and fixed fungi, and that the recognition of β-glucan in the fungal cell wall could be responsible for their effect on MR cleavage. This possibility was investigated by performing the treatment with HK C. albicans in the presence of soluble glucan phosphate. Figure 3.2C shows that glucan-phosphate, but not mannan treatment considerably blocked sMR expression, which suggests that fungi-induced MR shedding is mainly mediated through the recognition of the β-glucan component of the cell wall. These results also demonstrate that the recognition of soluble β-glucan, or mannan per se, are insufficient for inducing sMR production.

3.3.2. Fungi-induced MR shedding is blocked by a MMP/ADAM inhibitor but not by caspase inhibition

In an attempt to characterize the protease activity responsible for the enhanced MR shedding, the effect of caspase inhibiton was examined. Previously Gross et al., showed the activation of inflammasome for anti-fungal

host defence (Gross et al., 2009). An inflammasome is a multiprotein complex of more than 700 kDa whose central effector molecule is the cysteine protease caspase-1 that mediates the cleavage, activation and secretion of cytosolic pro-IL-1β, pro-IL-18 and pro-IL-33 (Pedra et al., 2009). Nevertheless, caspases do not appear to have a major role in the fungi-mediated MR shedding as their inhibition by z-VAD-fmk did not result in a significant change in the amount of sMR produced in response to HK and fixed C.albicans (Figure 3.3A).

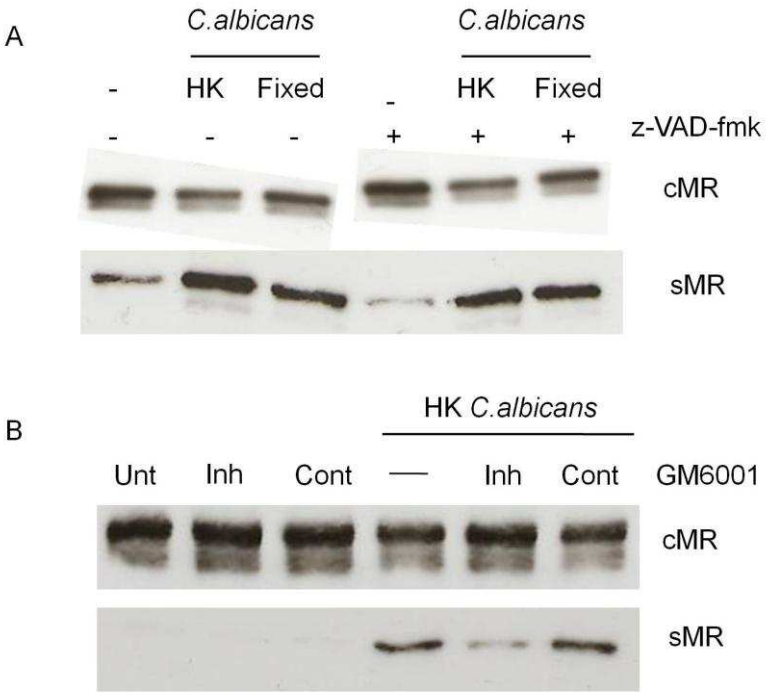

Figure 3.3: MR shedding is mainly mediated by MMP/ADAM-, but not by caspase-mediated responses. Western blot analysis of cMR and sMR expression by thio-MΦ treated with fixed or HK C. albicans in the presence and absence of z-VAD-fmk caspase inhibitor (A). In contrast to caspase inhibitor, pre-treatment of thio-MΦ with GM6001 metalloprotease inhibitor (Inh), but not GM6001 control (Cont), reduced the level of HK C. albicans-induced MR shedding (B). The data is representative of three independent experiments.

Another protease family examined for its role in the induction of MR shedding was the metalloproteases. In fact, the steady state MR shedding detected in the supernatants of murine primary MΦ was revealed to be mediated by a MMP or ADAM activity (Martinez-Pomares et al., 1998). By using GM6001, a wide spectrum MMP/ADAM inhibitor, it was investigated wheather fungi detection leads to a similar mechanism promoting MR shedding. Pre-treatment of MΦ with GM6001 significantly inhibited sMR production, which suggests that the fungi-induced MR shedding, like in the case of steady-state sMR production, is MMP or ADAM dependent (Figure 3.3B).

3.3.3. Zymosan treatment reduces MR-mediated endocytosis which cannot be restored by inhibition of MR-shedding

Under steady state conditions, sMR was previously shown to be produced by the proteolytic cleavage of pre-existing full-size cMR (Martinez-Pomares et al., 1998). This raised the question if MΦ endocytosis of MR ligands is affected upon fungi-mediated MR shedding. Zymosan particles were used as an MR shedding inducer, and MR-mediated endocytosis was monitored by using SO_4-3-Gal-PAA-FITC, which has a higher MR-specificity than the other MR-ligands which can also be detected by non-MR receptors (e.g. detection of mannose-rich residues by dectin-2, and collagen by

integrins). The level of internalized Ac-LDL, a ligand of SRs, was also monitored in order to verify if any observed effect is MR-specific.

As expected, the zymosan treatment diminished the level of MR endocytosis. Nonetheless, the reduction is not mediated through MR shedding, since GM6001 was not able to restore the level of SO_4-3-Gal-PAA-FITC internalization, albeit it inhibits sMR production (Figure 3.4A). Therefore it appears that the MR-shedding is not the only way through which zymosan can diminish MR endocytosis, and it can also trigger other distinct signalling pathway(s) independent of metalloprotease activity to target MR functionality.

This effect however is not solely restricted to MR, since similar zymosan-induced reduction (that cannot be reversed by addition of GM6001) was also observed in Ac-LDL-internalization (Figure 3.4B). That led us to consider if the Ac-LDL-receptors could also undergo ectodomain-shedding.

Modified LDL are mainly detected by SRs (Pluddemann et al., 2007). Among the family members, most of the MΦ population express SR-A, which as well as detecting lipoproteins and other ligands of endogenous origin, was also shown to have an important role in establishing an effective immune response (Haworth et al., 1997) (Kobayashi et al., 2000) (Pluddemann et al., 2007).

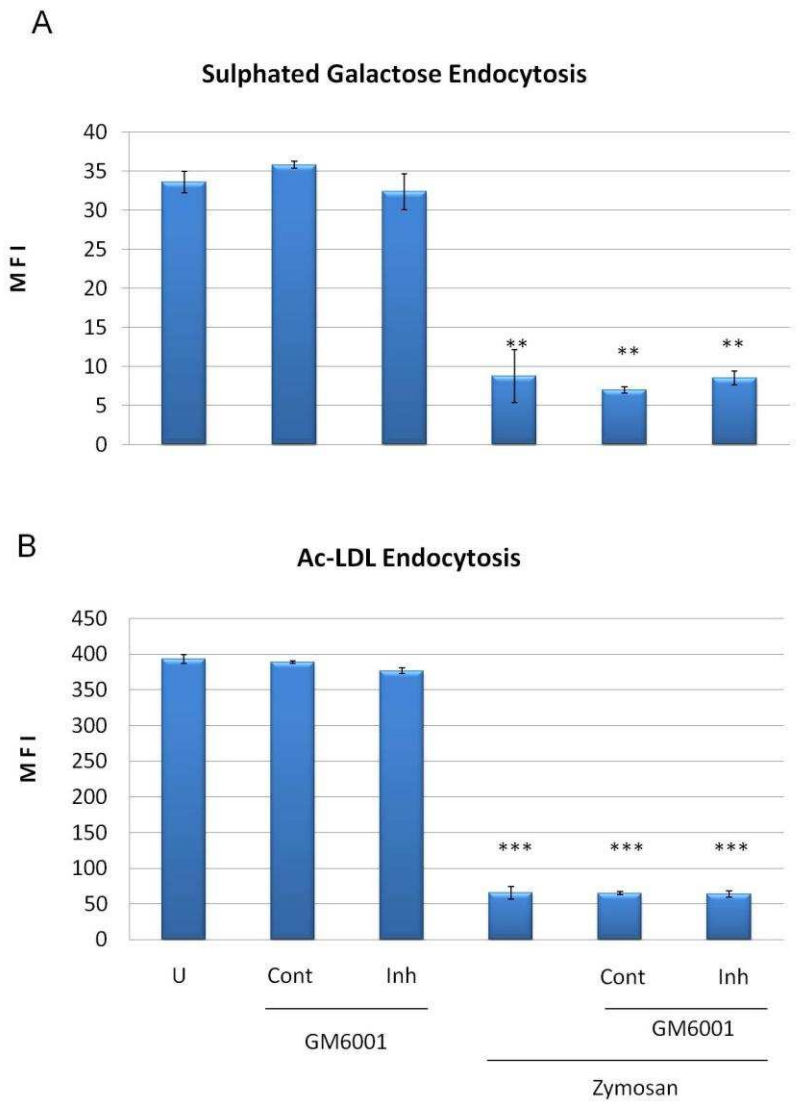

Figure 3.4: Zymosan-induced inhibition of endocytic machinery can not be prevented by GM6001. Flow cytometry analysis of sulphated galactose (A), and ac-LDL (B) internalization by thio-MΦ left untreated or treated with zymosan particles for three hours. The data represent the mean MFI value ± SEM obtained from three independent experiments. Asterisks indicate a statistically significant decrease in the level of endocytosis in comparison to unstimulated cells (t-test; ** $P < 0.01$, *** $P < 0.001$).

SR-A shedding was examined by using both cell lysates and cell culture supernatants collected from the treated cells. However, the rabbit

polyclonal antibody against MΦ SR (purchased from Abcam) failed to give any specific band regardless of the changes introduced to the western blot protocol which included longer incubation periods, higher concentrations of primary and secondary antibodies, and BSA as blocking buffer (data not shown). Hence it is still not yet clear whether the ectodomain shedding effect induced by fungal particles is MR-specific.

Overall these data suggest that zymosan-treatment has a negative effect on MΦ endocytic machinery, and that the reduction in MR endocytosis is independent of ectodomain shedding. This may be mediated by the reduced expression levels of MR and SR on the cell surface due to the disruption of the receptor recycling between intracellular compartments and cell membrane (which will be discussed further on the next chapter). Alternatively, it can also be explained by a possible lack of membrane availability due to phagosome formation.

3.3.4. Inhibition of MR-shedding does not influence cytokine production induced by fungi

Ectodomain shedding can have multiple, functional roles, including down-regulating receptor and adhesion molecules to inhibit receptor-ligand interactions, inducing cellular repulsion of interacting cells through the disposal of the receptor-ligand complex, and pro-protein processing of cytokines such as TNF-α. Additionally, as in the case of CD44, following

receptor shedding, the cleaved cytoplasmic tail can migrate to the nucleus inducing transcription of several genes (Garton et al., 2006).

Even though there had been no signalling motif identified on MR cytoplasmic tail, the literature is full of studies suggesting a MR role in intracellular signalling. In order to examine whether the ectodomain-shedding is a way of MR to perturb intracellular signalling pathway(s) leading to cytokine release (e.g. through the release of its cytoplasmic tail that may interact with intracellular signalling molecules), the supernatant levels of HK C. albicans-induced IL-1β, IL-6, KC, and CCL2 (or MCP-1, monocyte chemoattractant protein-1) in the presence and absence of GM6001 were monitored by cytokine ELISAs. These cytokines were chosen, since previous studies showed cMR involvement in their production (except for KC) in response to C. albicans (Yamamoto et al., 1997) (Heinsbroek et al., 2008). TNF-α production also served as a control since its cellular release is known to be ADAM-17 mediated and GM6001-sensitive.

Among these cytokines, the supernatant levels of IL-1β and IL-6 were below the detection level, and as expected, the amount of fungi-induced TNF-α was significantly reduced in the presence of GM6001, but not affected by GM6001 control (GM6001 cont). On the other hand, the level of fungi-induced KC production did not change in the presence of GM6001 and GM6001 cont, implying lack of MR shedding effect on cytokine production by MΦ during fungal infections. This was in contrast to the fungi-induced MCP-1 levels which displayed a significant change upon the introduction of GM6001.

However, the observed effect appears not to be as a result of the inhibition of MR shedding, as treatment with GM6001 cont which is a negative control for the GM6001 inhibitor, also resulted in a significant increase in the MCP-1 level (Figure 3.5C).

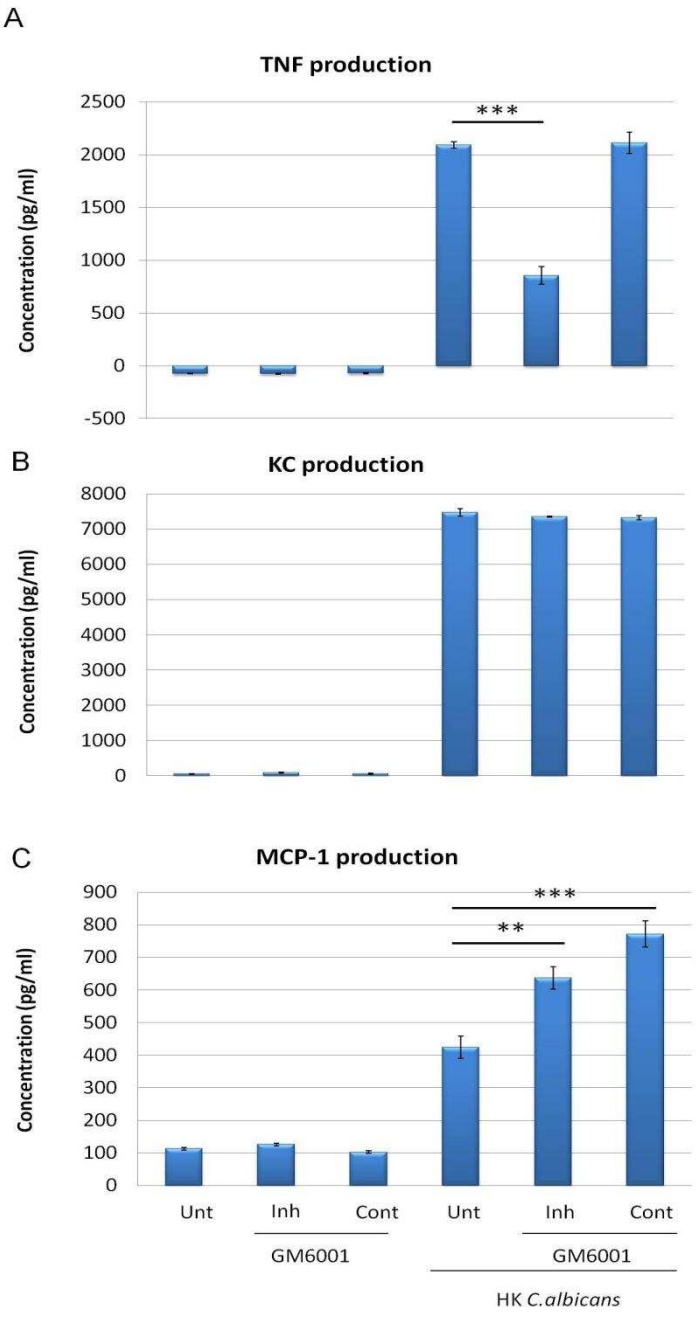

Figure 3.5: Inhibition of fungi-induced MR shedding does not influence the cytokine production examined. Capture ELISA analysis of the TNF-α (A), KC (B), and MCP-1 (C) production by MΦ left untreated or treated with HK C. albicans in the presence and absence of GM6001 metalloprotease inhibitor (Inh) or control (Cont). The data represent the mean cytokine concentration detected ± SEM obtained from three independent experiments. Asterisks indicate a statistically significant change in cytokine production compared to MΦ treated with HK C. albicans in the absence of GM6001 and GM6001 control (t-test; ** $P < 0.01$, *** $P > 0.001$).

3.3.5. Cytokine production in response to zymosan or necrotic cells is not affected by MR deficiency

The effect of MR on cytokine production was further examined by comparing the cytokine profiles of wt and MR-KO MΦ. For this purpose, both MΦ populations were either left untreated or treated with different stimuli (i.e. zymosan or necrotic cells) O/N in serum-free Opti-MEM before their supernatants were collected, centrifuged, and analyzed by using commercially available nitrocellulose membranes spotted with capture antibodies specific to a wide-range of cytokines. In Figure 3.6, only the cytokines with detectable signal are shown. The other released factors that were assayed but could not be detected on the membrane included CXCL13, G-CSF, GM-CSF, CCL1, CCL11, CD54, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-13, IL-12 p70, IL-16, IL-17, IL-23, IL-27, CXCL10, CXCL11, M-CSF, CCL12, CXCL9, CCL5, CXCL12, CCL17, tissue inhibitor of metalloproteases (TIMP)-1, and triggering receptor expressed on myeloid cells (TREM)-1.

Initially the importance of MR in zymosan-induced cytokine production was examined. In support of the data presented in Figure 3.5, MR-KO and wt MΦ did not differ in the cytokines they released in response to zymosan (Figure 3.6A). However, the profile displayed by the untreated cells showed slight variation upon MR deficiency: MR-KO MΦ appeared to release more IL-1ra, KC, and MIP-2 in the absence of any stimuli (Figure 3.6A).

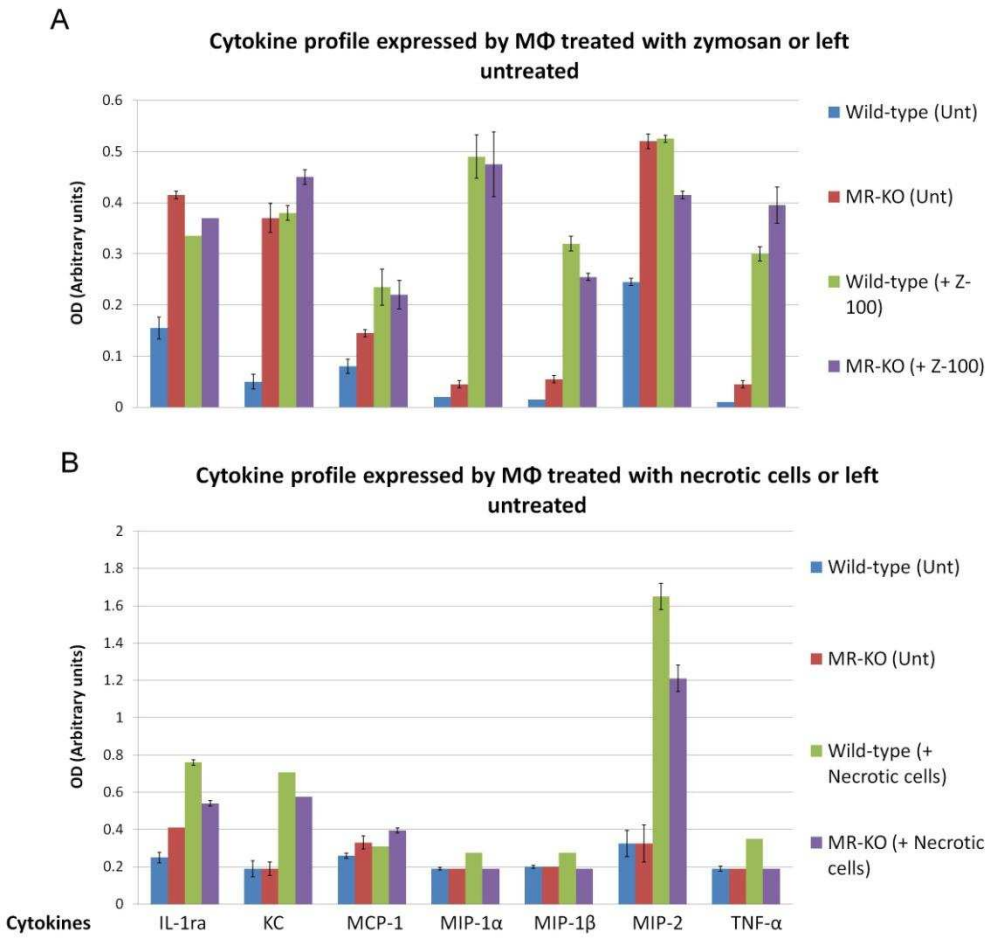

Figure 3.6: MR deficiency did not influence the cytokine production in response to zymosan or necrotic cells. Cytokine profile analysis of wt and MR-KO thio-MΦ left untreated (Unt) or treated with zymosan particles (100 particles/cell, Z-100) (A) or necrotic cell extracts (1/25 dilution) (B) for O/N. The data represent the mean arbitrary OD values detected ± SD obtained from duplicate cultures of a single experiment.

These cytokines were previously shown to facilitate the infiltration of leukocytes following the injection of necrotic cells into the peritoneal cavity (Tanimoto et al., 2007). Therefore, the observed difference was thought to be due to the presence of necrotic cells in the cell suspensions and MR role in the regulation of the cytokine release. This was investigated by incubating both wt

and MR-KO MΦ populations with freshly prepared liver necrotic cells O/N. However no change was detected in the cytokine profile displayed by wt and MR-KO (Figure 3.6B).

Even though both of these results indicate that MR may not be involved in the cytokine release in response to zymosan or damaged cells, further studies are required to verify the conclusion, as both studies were performed only once.

3.3.6. Allergen, but not fixed bacteria, can induce MR shedding

In an attempt to investigate if the enhanced MR ectodomain shedding is fungi specific, the levels of sMR production were analysed after MΦ treatment with other inducers, such as Staphylococcus aureus, which is not known to have any detectable β-glucan, or house dust mite (HDM, Dermatophagoides pteronyssinus) that was previously shown to induce an immune response through β-glucan recognition (Nathan et al., 2009). It appears that the ability to induce MR shedding is not a fungi-specific effect and can also be induced upon incubation with particulate HDM, but not by soluble HDM (data not shown) or S. aureus (Figure 3.7).

Since HDM is also known to have β-glucan in its structure (Nathan et al., 2009), the observed induction of MR shedding by HDM further supports the importance of β-glucan recognition. However, the proteolytic cleavage

responsible appears to be distinct, since in contrast to fungi-treated samples, HDM-treated MΦ released two distinct populations of sMR with different molecular weights (~165 and ~130 kDa) (Figure 3.7). Among the two sMR populations, the heavy sMR (sMR_H) appears similar to the one induced by fungi.

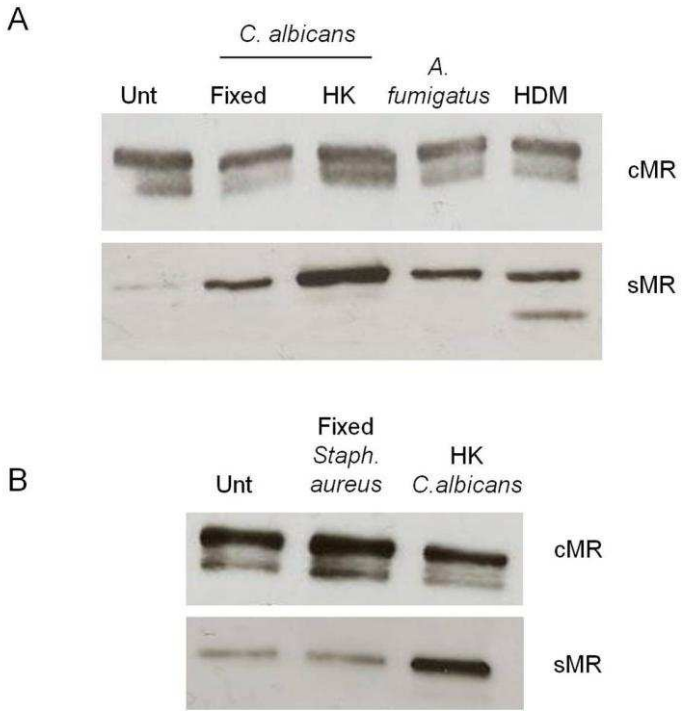

Figure 3.7: In addition to fungi, particulate HDM, but not S. aureus can also induce MR shedding. Western blot analysis of MR expression by thio-MΦ incubated with particulate HDM, fixed A. fumigatus, or fixed or HK C. albicans (A). The cMR and sMR expression levels by MΦ treated with fixed S. aureus (B). Data are representative of two independent experiments.

As well as being MΦ-derived, the proteases responsible may also come from the HDM used, since allergens are generally rich in proteolytic enzymes that allow their access to internal tissues (Shakib et al., 2008). Among those

Der p 1 is an immunodominant allergen found in the faeces of HDM and is involved in destroying tight junctions between the epithelial cells via its cysteine protease activity (Shakib et al., 2008, Shakib et al., 1998).

3.4. Discussion

In this chapter of results, it was shown that (i) the induction of MR shedding is an early event as it was evident after 3 hours of treatment, (ii) enhanced sMR production is not P. carinii specific and can also be induced by other fungal particles such as zymosan, A. fumigatus and C. albicans, as well as particulate HDM, (iii) the fungi-mediated sMR production depends on β-glucan recognition and GM6001-sensitive MMP/ADAM activity, but not on caspase, (iv) the inhibition of enhanced MR shedding by GM6001 does not influence the levels of cytokines released and does not block the reduction in the levels of MR- and SR-mediated endocytosis in response to fungi particles, (v) in contrast to fungi, HDM leads to the production of two sMR fragments of different molecular weights (MW).

Enhanced MR shedding was initially described in response to P. carinii after O/N incubation by Fraser et al (Fraser et al., 2000). By using a rather shorter incubation period (i.e. 1.5 and 3 hours), it was shown that MR shedding is an early event. It was considered that at these early points there would be less chances of having new protein synthesis, and therefore de novo protein expression may not be required for initial sMR release upon induction. Future studies including transcriptional and translational inhibitors may further help to understand this area.

By using zymosan, A. fumigatus, and both heat-killed and fixed C. albicans, it was shown that MR shedding can also be induced by other

opportunistic pathogens. In correlation with previous studies which showed that β-glucan recognition is the main requirement for inducing anti-fungi immune responses (Taylor et al., 2007), (Werner et al., 2009, Brown et al., 2003), it was demonstrated that the enhanced sMR production is mostly induced by the β-glucan component of the fungi cell wall. This clearly implies the participation of dectin-1, the main myeloid β-glucan receptor, which will be examined in the next chapter.

Having significant, but not complete, inhibition of HK C. albicans-induced sMR production by β-glucan phosphate pre-treatment, implies the possible involvement of other MΦ receptors. Apart from β-glucan, another key PAMP responsible for triggering the anti-fungal immune response are the mannoproteins, which together with β-glucan, make up to 90% of the fungal cell wall (Poulain and Jouault, 2004) (Chaffin et al., 1998) (Wheeler and Fink, 2006).

The lack of obvious change in the level of fungi-mediated MR shedding in the presence of soluble mannan suggests that mannoprotein detection may not be responsible for the β-glucan-independent C. albicans-induced MR shedding. However, the commercial mannan used in these studies is derived from S. cerevisiae and it differs from the C. albicans mannan in structure and MW that may influence its interaction with PRRs (Williams DL, unpublished data). Therefore in the presence of soluble mannan (purchased from Sigma Aldrich), there may still be PRRs involved in the detection of C. albican mannan that can trigger sMR production. Further studies are required

to understand the underlying mechanism responsible for the β-glucan-independent MR shedding.

The attempt to identify the enzyme(s) responsible for the proteolytic cleavage of MR revealed that the ectodomain shedding was mainly mediated by a GM6001 sensitive MMP/ADAM activity. However, as with β-glucan-phosphate pre-treatment, the inhibition was not complete, which suggests the possible involvement of other GM6001-insensitive mechanisms.

MMPs are zinc-dependent endopeptidases belonging to a subfamily of metzincins. Initially they were thought to be responsible solely for the turnover and degradation of the ECM, and were named accordingly. However, today they are known to have a wide-spectrum of functions, including the release of pro-inflammatory cytokines and chemokines (Webster and Crowe, 2006), as well as facilitating the shedding of various surface receptors including CD44 (Yu et al., 1997), ErbB2 receptor (Codony-Servat et al., 1999), and TNF-receptor (Lombard et al., 1998).

ADAMs are transmembrane proteases and, like MMPs, belong to the metzincin family of metalloproteases. They are known as the major protease family responsible for the ectodomain shedding affecting functionally diverse proteins including cadherins, Fas ligand, and epidermal growth factor (EGFR) ligands as well as TNF-α (Huovila et al., 2005).

Within this family, ADAM-17 was the first ADAM sheddase to be identified. It was initially identified by its activity as a TNF-α convertase, but

following studies revealed its participation in the shedding of several other cytokines (e.g. CXCL-1, TRANCE) and receptors (e.g. IL-1 receptor II, IL-6 receptor). The other important ADAM sheddase is ADAM-10 which, like ADAM-17, can target a wide-range of proteins including cytokines (e.g. CXCL-16, and CX3CL-1), growth factors (e.g. Epidermal growth factors, and betacellulin) and receptors (e.g. IL-6 receptor).

Previous data by Martinez-Pomares et al. showed that the sMR is produced as a result of a proteolytic cleavage of the cell-associated MR, and accordingly, the flow-cytometry data revealed a significant reduction in the MR-mediated internalization by MΦ (Martinez-Pomares et al., 1998). However, this effect was not reversed upon GM6001 treatment, even though it was able to inhibit MR shedding. One possible explanation for the observed data includes the reduction of surface MR expression through disrupting its recycling between the endocytic compartments and the plasma membrane, such that it becomes trapped intracellularly without affecting its cellular expression level. The flow cytometry analysis of surface MR expression will be discussed in the next chapter.

The observed reduction in the level of endocytic internalization is not MR specific, as the same effect was also observed in Ac-LDL uptake. As in the case of MR endocytosis, this was thought to be as a result of ectodomain shedding of receptor(s) involved in its internalization. In an attempt to clarify the mechanism behind this, the level of soluble form of SR-A was screened in the supernatants collected from the treated cells. However, the antibody failed

to give any specific band, regardless of the changes introduced to the western blot protocol. Therefore, it is still open to debate whether the ectodomain shedding induced by fungal particles is MR-specific or not. In the next chapter, this will be further examined by monitoring the level of another cell surface receptor CD44 (a known target for MMPs and ADAMs) in the supernatants collected from MΦ treated with purified β-glucan particles.

On the other hand, the inability to block the reduction in the level of Ac-LDL internalization by GM6001 treatment implies the same type of machinery responsible for the decline in the level of MR-endocytic uptake. Apart from the reduced expression levels of MR and SR on the cell surface, this decrease in the level of endocytosis can also be explained by a possible lack of membrane availability due to phagosome formation.

The importance of MR in fungal infections is not yet clear, as the literature is full of conflicting results. For instance, even though the MR-KO mice were shown to have a normal host defence during systemic candidiasis (Lee et al., 2003), in the studies by Yamamoto et al and Heinsbroek et al, MR was shown to mediate IL-1β, IL-6, MCP-1, and TNF-α, in response to infection with C. albicans (Heinsbroek et al., 2008, Yamamoto et al., 1997). However, MR lacks any known intracellular signalling motif on the cytoplasmic tail that would enable it to contribute to cytokine production. This is why MR is thought to form a receptor complex with other receptor(s) (e.g. TLR2 (Tachado et al., 2007)) that participate in intracellular signalling.

sMR production has previously suggested to assist pathogens to escape the immune response (Fraser et al., 2000). In this study, formation of sMR-coat

around the fungal pathogen was shown to block its removal by phagocytosis (Fraser et al., 2000). That led us to examine if MR-shedding has also any effect on cytokine production. This was thought to, at least partially, explain the conflicting results for the MR role in cytokine production.

The effect of enhanced MR shedding on cytokine production was investigated by monitoring the levels of IL-1β, IL-6, MCP-1, and KC in the supernatants collected from MΦ treated with HK C. albicans in the presence and absence of GM6001 or GM6001 cont. Among these cytokines, the levels of IL-1β, and IL-6 were below the detection level. This was surprising since previously Yamamoto et al showed a C. albican- induced expression of these cytokines by thio-MΦ after 1 hour of treatment. However, the levels were screened at the mRNA level, and therefore the lack of detection in protein level may be as a result of post-transcriptional or post-translational modifications controlling their release. On the contrary, in a similar model, Heinsbroek et al could not detect any IL-6 expression in the supernatant collected from C. albicans-treated thio-MΦ (Heinsbroek et al., 2008).

Among the rest of the cytokines examined, the fungi-mediated release of KC did not change upon the inhibition of MR shedding by GM6001. In contrast, fungi-mediated MCP-1 release was altered upon the introduction of GM6001. However, this seems to be nonspecific since a similar increase was also detected in samples treated with HK C. albicans in the presence of GM6001 cont, which lacks any inhibitory activity. These results suggest the lack of MR-shedding effect on the fungi-mediated cytokine release. Nevertheless, a 3-hour incubation period may not be enough for C. albicans to

be coated with sMR as observed previously (Fraser et al., 2000), and therefore the possible effect of sMR production on cytokine release in the later stages of fungal infection cannot be excluded.

In an independent approach, MR-KO thio-MΦ were used to examine any MR effect on the cytokine release in response to zymosan, or liver necrotic cells. The necrotic cells were chosen as a stimuli inducer, since in the previous attempt, untreated MR-KO MΦ supernatants were reported to have enhanced levels of IL-1ra, KC and MIP-2; the cytokines shown to facilitate the infiltration of leukocytes following the injection of necrotic cells into the peritoneal cavity (Tanimoto et al., 2007). As both MR-KO and wt MΦ were handled the same way, the enhanced level of cytokine release was thought to be due to the MR role in the regulation of cytokine production in response to endogenous signals, such as damaged cells.

Both wt and MR-KO MΦ displayed the same cytokine profile in response to zymosan and liver necrotic cells, suggesting no MR role in cytokine production and release in both conditions. However further studies are required to confirm this conclusion since this study was only performed once. Additionally, as there are not many free zymosan particles after 3-hours of incubation, this data does not exclude the possible effect of sMR in the later stages of infection.

By using other stimuli it was showed that the MR-shedding is not fungi-specific, and it can also be induced by particulate preparations of Dermatophagoides pteronyssinus (house dust mite, HDM). The reason behind HDM-induced MR shedding is not yet clear. However it may be involved in

the allergic immune response by targeting sMR-bound allergens (Deslee et al., 2002) to the CR-Fc^+ cells surrounding the white pulp of the spleen, or follicular regions in lymph nodes (Martinez-Pomares et al., 1996).

The proteolytic cleavage responsible for the HDM-induced MR shedding appears to be quite different to that triggered by fungi. In contrast to the fungi-mediated shedding which produced a single sMR population (~165 kDa), HDM treatment resulted in two sMR populations with molecular weights of ~165 (sMR_H) and ~130 kDa (sMR_L). The observed sMR_H production is in correlation with a very recent study by Nathan et al, which showed a β-glucan-mediated activation of the innate immune response by HDM, that was inhibited by using β-glucanase, which digest β-glucan structures or other β-glucan moieties, such as laminarin or zymosan (Nathan et al., 2009).

The reported ~35 kDa difference between the two sMR populations may be due to the cleavage of the N-terminal CR domain, as it was previously suggested to result in the production of an additional cMR population (~40 kDa lighter) in small intestine tissues (Su et al., 2009). Today, the CR domain is known to recognise antigens of endogenous origin only. Therefore its removal may enhance MR specificity for non-self molecules, and sMR bound antigen may be immediately regarded as a foreign material to be destroyed by the immune system. Future studies to identify any immune cells that are able to recognise sMR_L, and determine sMR_L functionality, may further help to examine this possibility.

As well as being MΦ-derived, the protease(s) responsible for this distinct sMR population, may also come from the allergen, since the mite extracts are generally rich in proteolytic enzymatic activity that allow their access to internal tissues. Their effects are mediated by breaking down the connective tissues and destroying tight junctions between the epithelial cells, and involve the cleavage of surface receptors that may also help modulating the immune response. For instance, Der p 1 is known to cleave CD23, CD25, lung surfactant proteins (SP)-A and D, CD40, DC-SIGN, and DC-SIGNR (Shakib et al., 2008).

Overall, these data suggest a putative role for β-glucan recognition and ADAM/MMP activity in the induction of sMR production, which was shown to help P. carinii to evade the host immune response (Fraser et al., 2000). In contrast to Fraser et al, sMR production did not have any effect on the fungi-mediated cytokine release. This may be due to the short incubation periods used, since it would not be enough for the formation of a sMR coat around the fungi that would influence its interaction with MΦ (Fraser et al., 2000). The next chapter will be on the identification of the PRR responsible for the β-glucan-mediated MR-shedding. The first candidate is dectin-1; the main myeloid β-glucan receptor.

4. THE ROLE OF DECTIN-1 IN FUNGI-MEDIATED MR-SHEDDING

4.1. Introduction

Dectin-1 is a member of the group V transmembrane C-type lectin family encoded by the natural killer gene complex (NKC) (Huysamen and Brown, 2009). It consists of a type II transmembrane domain, a single extracellular CTLD that can detect the β-glucan component of the fungi cell wall in a Ca^{2+}-independent manner, a stalk, and a cytoplasmic tail with a hem-ITAM motif. The stalk region is the region that is most commonly spliced out in functional dectin-1 isoforms in both human and mouse (Brown, 2006, Ariizumi et al., 2000b). As discussed in section 1.2.3.ii.d, dectin-1 can trigger both Syk-dependent and Syk-independent-Raf-1-mediated signalling which can act independently as well as in cooperation with the TLR pathway.

Dectin-1 was shown to detect several fungi species including P. carinii, S. cerevisiae, C. albicans, and A. fumigatus, and their recognition by dectin-1 triggers various protective responses such as phagocytosis, killing via respiratory burst, and the production of a number of cytokines and chemokines such as TNF, CXCL2, IL-1β, IL-1α, IL-6, CCL3, GM-CSF, and G-CSF (Brown, 2006). The dectin-1 role as a crucial fungi recognition receptor was also examined in vivo with mixed results, which are probably due to the

genetic background of the mice, differences in the fungal strains and/or routes of infection used (Netea and Marodi, published online in 2010).

The importance of dectin-1 engagement was further supported by the evidence that pathogens may utilise mannoproteins to mask their β-glucan in order to evade the immune response (Gantner et al., 2005) (Wheeler and Fink, 2006). For instance, hyphal forms of C. albicans cannot be detected by dectin-1 and accordingly, do not trigger the immune response, as they do not have a detectable surface-exposed β-glucan (Gantner et al., 2005). Nevertheless, the condition was reversed by disrupting the mannoprotein outer layer which would enhance the β-glucan expression on the surface (Wheeler and Fink, 2006). Likewise, whereas A. fumigatus resting conidia and hyphae do not possess surface β-glucan expression, swollen conidia and early germlings can be detected by dectin-1, due to exposed β-glucan (Hohl et al., 2005) (Gersuk et al., 2006) (Steele et al., 2005). Additionally, the β-glucan component of Histoplasma capsulatum was recently shown to be hidden under a layer of α-glucan, and Paracoccidioides switches from β-glucan to α-glucan, to avoid detection by dectin-1 (Rappleye et al., 2007).

Furthermore, in humans, recent identification of polymorphisms in dectin-1 was associated with an enhanced colonization with Candida species (Plantinga et al., 2009), and patients with dectin-1 deficiency had defective production of IL-6, TNF-α and IL-17 in response to β-glucans and C. albicans (Ferwerda et al., 2009).

4.1.1. The aim of the study

In light of the preceding chapter of results which showed the β-glucan phosphate-mediated inhibition of fungi-mediated MR shedding, it was hypothesized that dectin-1 is responsible for the β-glucan-mediated MR shedding.

The aim of this chapter was to investigate if and how dectin-1, the major β-glucan receptor, was responsible for fungi-mediated MR-shedding. For this purpose, dectin-1-KO MΦ were treated with HK C. albicans or purified β-glucan particles (from C. albicans), and sMR production was examined by western blot. As dectin-1 has two murine isoforms with differences in structure and possibly in intracellular signalling, the effect of different isoform expression on sMR production was also examined by using MΦ isolated from C57 BL/6 and BALB/c mouse strains that display different dectin-1 isoform expression (Heinsbroek et al., 2006). The intracellular signalling responsible was investigated by using purified β-glucan particles as the fungi model, in the presence and absence of various signalling inhibitors.

4.2. Materials and Methods

4.2.1. Animals

The wt and MR-KO mice were bred and handled as described in section 2.2.1.

Dectin-1 knock-out (dectin-1-KO) mice and their controls were on a 129S6/SvEv genetic background. Animals were provided by Dr. Philip Taylor, and were maintained in accordance with institutional guidelines at Cardiff University, School of Medicine, U.K.

4.2.2. Cells

Thio-MΦ were obtained by following the protocol described in section 3.2.2.

4.2.3. Experimental conditions

Thio-MΦ were treated with particulate β-glucan (kindly contributed by David L. Williams, from East Tennessee State University, USA) curdlan (Wako), ionomycin (Sigma Aldrich), phorbol myristate acetate (PMA, Sigma Aldrich) or Pam_3CSK_4 (Invivogen) at the indicated concentrations in serum-free Opti-MEM with GlutaMAX (Invitrogen) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin for 3 hours at 37^oC, 5% CO_2.

For inhibition assays, thio-MΦ were pre-incubated with β-glucan phosphate (kindly contributed by David L. Williams, from East Tennessee State University, USA), mannan (Sigma Aldrich), Syk kinase inhibitor IV, wortmannin, Akt inhibitor VI, Raf-1 kinase inhibitor I, GM6001, GM6001 control, cytochalasin D, latrunculin A (all from Calbiochem), bafilomycin or chloroquine, (Sigma Aldrich) at indicated concentrations for 1 hour before treatment, and were present during the incubation with the stimuli.

The supernatants collected from MR-KO thio-MΦ treated with purified β-glucan particles were used to analyse the potential role of soluble components in the induction of MR shedding. After 3 hours of treatment, supernatants were collected and any β-glucan particle contaminant was removed by centrifugation at 13 000 rpm for 20 minutes, using a bench top centrifuge. The clarified supernatant was then used to incubate wt MΦ for 3 hours.

The cell lysates and culture supernatants were collected and protein concentration was determined by following the same protocol described in section 2.2.5.

4.2.4. Western blotting

The protocol was described in section 2.2.6. CD44 were visualised using rat anti-CD44 mAb (clone KM201, Abcam), in combination with HRP-conjugated anti-rat IgG (Chemicon).

4.2.5. Flow ctometry analysis

For the endocytosis assay, thio-MΦ were plated on non-tissue culture treated plastic as described in the previous chapter. Following a 3-hour treatment with β-glucan, the MΦ were then incubated for 30 minutes in serum-free media containing 5 μg/ml of SO_4-3-Gal-PAA-FITC (Lectinity), or Alexa-488 conjugated Ac-LDL (Invitrogen). The MΦ were collected by scraping using 1X trypsin-EDTA (Sigma Aldrich) and fixed in 1% (v/v) formaldehyde solution in PBS. The internalisation was then analysed using Beckman Coulter Epics Altra and Weasel software.

To examine MR expression on the cell surface, thio-MΦ plated on non-tissue culture treated plastic, were collected by scraping using non-enzymatic cell dissociation buffer (Sigma Aldrich), washed in FACS block (5% (v/v) heat-inactivated rabbit serum, 0.5% (w/v) BSA, 2 mM NaN_3, 5 mM EDTA in PBS) and incubated in FACS block containing 2.4G2 (10 μg/ml) for 30 min at 4^{o}C. After blocking MΦ were incubated with Alexa488-labelled anti-MR Ab (clone 5D3, Biolegend), for 60 min at 4^{o}C. After staining, cells were washed three times with FACS wash (0.5% (w/v) BSA, 2 mM NaN3, 5 mM EDTA in PBS) and fixed in 1% (v/v) paraformaldehyde in PBS. Isotype-matched Ab were used as controls. Labelling was analysed using a Beckman Coulter Epics Altra, and Weasel software.

4.2.6. Gelatin zymography

A precast SDS-PAGE gel (7.5%), containing 0.1% (w/v) gelatin (Invitrogen), was used to separate samples in 2X nonreducing sample buffer (0.125 M Tris-HCI, pH 6.8, 20% (v/v) glycerol, 4% (w/v) sodium dodecyl sulphate, 0.003% (w/v) bromphenol blue) at 120 V. SDS was removed by incubation with renaturing buffer (2.5% (v/v) Triton X-100 in distilled water) (Invitrogen) for 30 min at room temperature. The gels were incubated O/N at 37°C (Heraeus Incubator, Langenselbold) in developing buffer (20 mM Tris-HCl, pH 7.6, 10 mM $CaCl_2$ and 0.04% (w/v) NaN_3) (Invitrogen) and then stained with 0.1% (v/v) Coomassie blue in 40% (v/v) methanol and 10% (v/v) acetic acid and destained until clear proteolytic bands appeared on the contrasting blue background. Bands were visualized using the Gene Genius Bioimaging System (Cambridge).

4.2.7. Target opsonization

For Fc-mediated phagocytosis, 6 μm latex beads (Polysciences) were incubated O/N at 4°C in 10 mg/ml bovine serum albumin (BSA, Sigma Aldrich) in PBS, followed by three washes in PBS. The beads were then re-suspended in mouse-derived anti-BSA IgG antibody (Sigma Aldrich) for 1 hour at room temperature before being washed for three times and stored in PBS at 4°C (May et al., 2000).

4.2.8. Real-time quantitative PCR (qPCR)

The protocol was described in section 2.2.12.

Table 4. 1: Forward and reverse primer sequences used in qPCR experiments.

Gene	Forward oligonucleotide sequence (5'→ 3')	Reverse oligonucleotide sequence (5'→ 3')
HPRT	GTAATGATCAGTCAACGGGGGAC	CCAGCAAGCTTGCAACCTTAACCA
MMP-9	CAGAGGTAACCCACGTCAGC	GGGATCCACCTTCTGAGACTT
MMP-8	CTTTCAACCAGGCCAAGG	GAGCAGCCACGAGAAATAGG
MMP-2	ATAACCTGGATGCCGTCGT	TCACGCTCTTGAGACTTTGG
MMP-3	TTGTTCTTTGATGCAGTCAGC	GATTTGCGCCAAAAGTGC

4.2.9. Statistical analysis

The statistical analysis was performed as described in section 2.2.13.

4.3. Results

4.3.1. β-glucan particles induce MR shedding through dectin-1 engagement

Previous results indicated that β-glucan recognition is required for enhancing sMR production upon the recognition of fungi. The possible effect of purified β-glucan particles on sMR production was investigated to see if the recognition of particulate β-glucan was sufficient for inducing this effect. The data proved that particulate β-glucan enhanced MR shedding, when used at particle per cell ratio similar to that used in the case of C. albicans and A. fumigatus, and that it could be inhibited by pre-treatment with soluble glucan phosphate. This demonstrates that the observed effect is not mediated by any contaminant present in the preparation of particulate β-glucan (Figure 4.1A).

In order to establish the role of the major β-glucan receptor, dectin-1, in mediating MR shedding, wt and dectin-1-KO thio-MΦ were treated with particulate β-glucan. Dectin-1 expression was essential for the induction of MR shedding in response to particulate β-glucan (Figure 4.1A). The importance of dectin-1 was further confirmed by the significant reduction in sMR production observed in dectin-1-KO MΦ in response to HK C. albicans (Figure 4.1B).

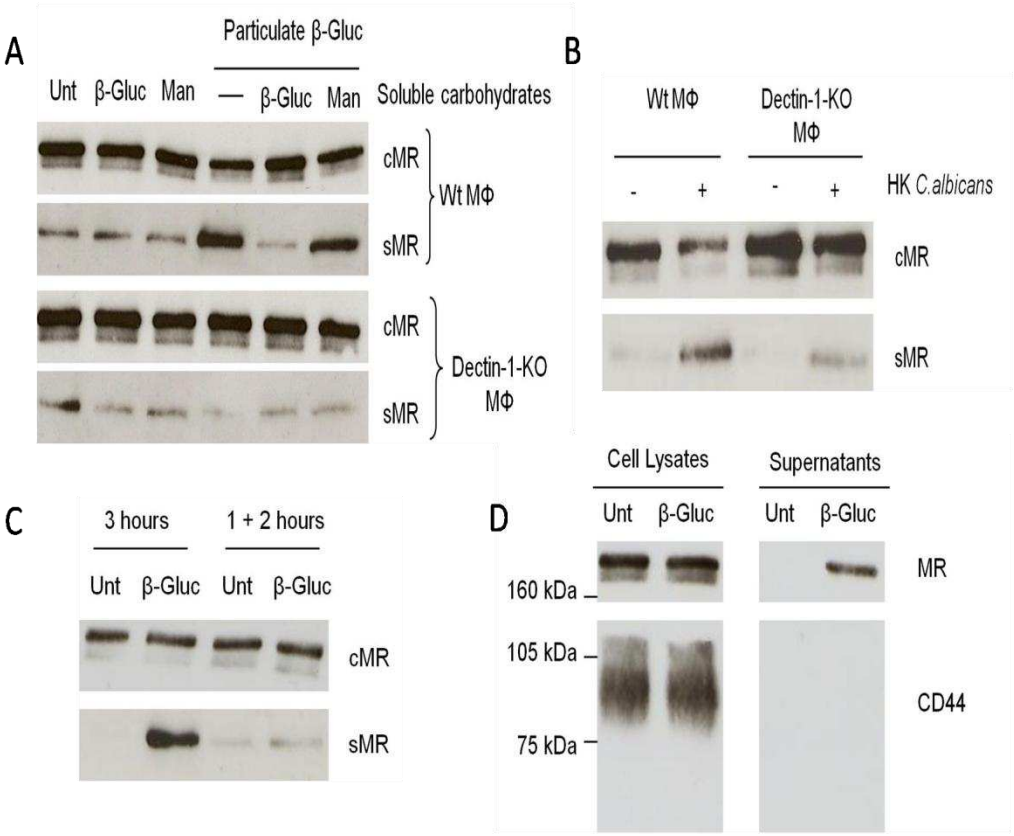

Figure 4.1: The role of dectin-1 in fungi-mediated MR shedding and its specificity to MR. The particulate β-glucan-induced sMR production was completely blocked by dectin-1 deficiency or pre-treatment with soluble β-glucan, but not by mannan (A). There was a significant inhibition of fungi-mediated shedding in dectin-1-KO MΦ (B). Continuous β-glucan presence is required for MR shedding during 3 hours of incubation, as the removal of β-glucan particles after 1 hour significantly reduced the ectodomain shedding (C). While cMR and sMR were detected in the cell lysate and supernatant, CD44 could only be detected in cell lysates collected from β-glucan treated wt MΦ (D). Data are representative of three independent experiments.

To examine if the initial dectin-1-induced signalling is enough for the enhanced MR ectodomain shedding observed after 3 hours of incubation, the β-glucan particles were removed after 1 hour and the MΦ were then left untreated at 37°C in serum-free media for two more hours (i.e. 3 hours in

total). As shown in the Figure 4.1C, continuous presence of β-glucan appears to be essential for MR shedding, as the intensity of the bands corresponding to sMR reduced significantly upon removal of β-glucan particles after 1 hour.

The selectivity of the signalling triggered was examined by studying the effect of particulate β-glucan treatment on the shedding of CD44. CD44 is a type I transmembrane glycoprotein with a broad range of functions (Nakamura et al., 2004) (Bazil and Horejsi, 1992). CD44 is shed off the cell surface as a result of multiple signalling pathways including protein kinase C (PKC), as well as the influx of intracellular Ca^{2+}, and is mediated by ADAM-10, ADAM-17 and MMP-14 activity (Kajita et al., 2001, Okamoto et al., 1999, Kawano et al., 2000). As revealed in Figure 4.1D, β-glucan treatment did not induce CD44 shedding, which indicates that particulate β-glucan recognition does not lead to a general release of receptors from the cell surface.

4.3.2. No difference in β-glucan-induced MR shedding between C57 BL/6 and BALB/c mice

Murine MΦ express two functional dectin-1 isoforms: dectin-1A and dectin-1B which are structurally distinct. Dectin-1A is composed of all structural domaina whereas dectin-1B lacks the stalk region.

According to the q-PCR data shown by Heinsbroek et al (2006), their expression is genetically determined such that both dectin-1 isoforms are expressed in equal amounts by BALB/c and other related mouse strains (e.g.

C3H/HeH and CBA/ca), while dectin-1B is the predominant isoform expressed by C57 BL/6, 129/SvEv and B10.BR (Heinsbroek et al., 2006). This differential expression appears to be the cause of the previously observed differences in the C. albicans-induced production of defensins, chemokines, and cytokines between C57 BL/6 and BALB/c mice (Schofield et al., 2005), since dectin-1B-expressing cells released significantly more TNF-α in response to zymosan than the dectin-1A expressing cells (Heinsbroek et al., 2006). Furthermore, differential expression of dectin-1 isoforms was shown to enhance susceptibility to coccidoides in mice (del Pilar Jimenez et al., 2008).

In an attempt to investigate if this differential dectin-1 isoform expression would also have an effect on sMR production following β-glucan treatment, the levels of ectodomain shedding and MR- and non-MR mediated endocytosis were compared between the two mouse strains by western blot and flow-cytometry respectively.

Flow-cytometry analysis revealed that both MΦ populations have a similar endocytic capacity before the treatment, and displayed similar levels of reductions in endocytosis of SO_4-3-Gal and Ac-LDL after being incubated with β-glucan particles (Figure 4.2A). Accordingly, both mouse strains showed the same level of sMR production in response to β-glucan particles (Figure 4.2B). In support of these data, thio-MΦ isolated from both mouse strains were previously shown to have similar level of cell-surface MR expression (Heinsbroek et al., 2006).

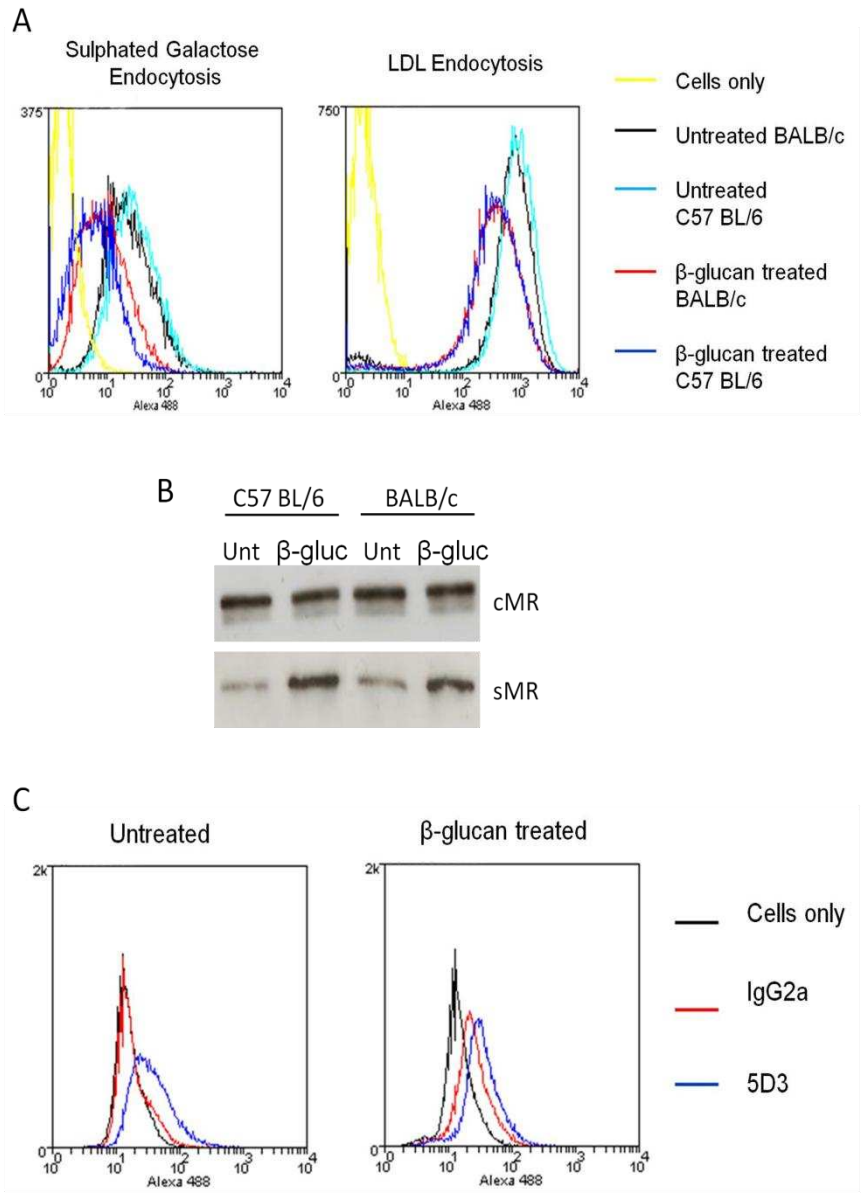

Figure 4.2: There is no difference between C57 BL/6 and BALB/c mice strains in dectin-1-mediated reduction of endocytic internalization and MR shedding. Flow cytometry analysis of sulphated galactose and ac-LDL internalization by thio-MΦ, left untreated or treated with β-glucan particles (A). The endocytosis level was identical between the mouse strains in both untreated and treated samples, and they showed a similar rate of reduction in their endocytic capacity upon treatment with β-glucan particles. This is in support of the similar amount of MR shedding as detected in the supernatants collected from the same cell populations used in the endocytosis assay (B). The enhanced IgG2a binding observed in the β-glucan treated samples did not allow drawing a reliable conclusion on the cell surface MR expression by using flow-cytometry (C). Data are representative of three independent experiments.

As discussed in the previous section, zymosan-induced reduction of MR endocytosis was not reversible by the presence of GM6001, and therefore apart from mediating MR shedding, zymosan was thought to decrease surface MR expression by another GM6001-insensitive pathway, which may include the enhanced internalization of the receptor. That led us to examine the levels of surface MR expression after β-glucan treatment in the presence of GM6001. However, the attempts failed to provide reliable data as MΦ displayed enhanced non-specific binding to the IgG2a isotype control antibody upon treatment with β-glucan particles, creating doubts about the specificity of 5D3 binding (Figure 4.2C).

The underlying reason for this enhanced non-specific binding was thought to be because of enhanced FcR expression on the MΦ surface upon dectin-1 signalling. However, the non-specific binding was not blocked even after the addition of 100 μg/ml of rat IgG to the blocking buffer (data not shown). A similar type of enhanced binding was also observed in the case mouse IgG1 isotype control antibody (data not shown).

4.3.3. β-Glucan mediated MR shedding depends on Syk and, partially on Raf-1 kinases

Upon ligand binding, dectin-1 triggers intracellular signalling through Syk-dependent and Syk-independent Raf-1-mediated pathways. Syk kinase

was observed to be indispensible for the β-glucan-induced sMR production, since MR shedding was completely blocked by Syk kinase inhibition (Figure 4.3A). However, Raf-1 kinase seemed to be partially responsible for the phenomenon, as reduced sMR production was observed only at high concentrations of Raf-1 kinase inhibitor (Figure 4.3B).

Syk-kinase was previously shown to be required for collaborative signalling between dectin-1 and TLR-mediated pathways that sustains degradation of IκB and enhances nuclear translocation of NF-κB (Dennehy et al., 2008). The high Syk-dependence of dectin-1-mediated MR shedding raised the question of whether sMR production could be enhanced by the activation of TLR signalling. By using a TLR2 agonist (Pam_3CSK_4), it was shown that TLR2 engagement does not affect MR shedding in isolation or in combination with β-glucan (Figure 4.3C).

The observed crucial effect of Syk in the enhancement of MR-shedding in response to β-glucan particles, led us to examine if the sMR production can also be triggered by other ITAM-associated receptors. In an attempt to investigate this, latex beads coated with mouse IgG were used. To make sure that the antibody Fc fragments are free to engage with FcR, latex beads were incubated with BSA and then with mouse anti-BSA antibody.

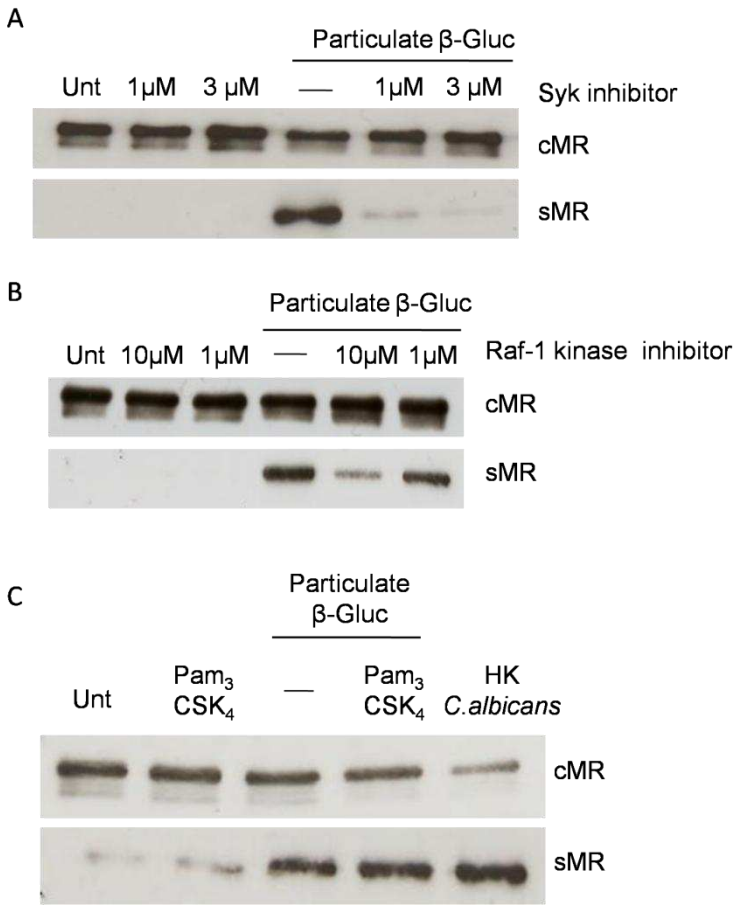

Figure 4.3: Dectin-1-mediated MR shedding is dependent on Syk, and partially on Raf-1. Western blot analysis of MR expression by MΦ treated with β-glucan particles in the presence and absence of Syk kinase (A) or Raf-1 kinase inhibitors (B). While Syk inhibition completely abrogates MR shedding in response to β-glucan, Raf-1 inhibition has a minor effect at high concentrations. The β-glucan effect could not be enhanced by the addition of Pam_3CSK_4 which indicates that dectin-1-mediated MR-shedding is independent of TLR-signalling (C). Data are representative of three independent experiments.

As can be seen in Figure 4.4, there was not any enhanced sMR production in response to IgG-coated latex beads. However, this could also be explained by the lack of efficient intracellular signalling, since only approximately two particles were successfully internalized by each MΦ (data

not shown). Lack of efficient phagocytosis implies the lack of receptor cross-linking, which is also required for FcR-mediated signalling (Garcia-Garcia and Rosales, 2002).

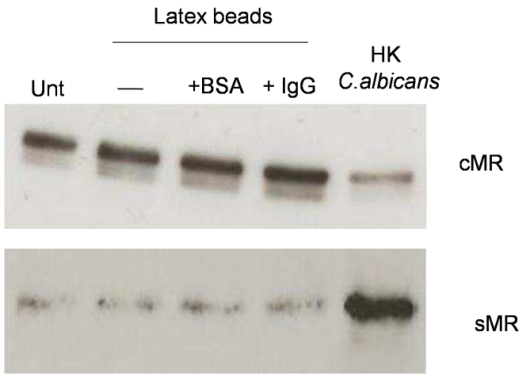

Figure 4.4: IgG-coated latex beads cannot induce sMR production. Western blot analysis of MR expression by thio-MΦ treated with HK C. albicans or latex beads with/without BSA or mouse IgG coat. Data are representative of three independent experiments.

4.3.4. β-glucan-induced MR shedding is PI3K, but not Akt, dependent

Phosphoinositides are crucial second messengers for intracellular signalling pathways including the one initiated by dectin-1-mediated Syk-kinase activation (Hiller et al., 2000), (Olsson and Sundler, 2007), (Olsson and Sundler, 2006), (Shah et al., 2009), (Lee et al., 2008), (Crowley et al., 1997). Among several other downstream elements that phosphoinositide-3 kinase (PI3K) activates, Akt (aka Protein kinase B) plays a central role in innate immunity, since the PI3K/Akt pathway was shown to be required for the production of cytokines such as IL-12, IL-10, as well as for controlling cell

proliferation and survival (Weichhart and Saemann, 2008, Martin et al., 2003, Polumuri et al., 2007, Koyasu, 2003).

The potential role of the PI3K/Akt pathway in β-glucan-induced MR shedding was investigated by using a PI3K and Akt inhibitors. Enhanced MR shedding was completely blocked upon PI3K inhibition by the PI3K inhibitor wortmannin (Figure 4.5A), but Akt inhibition had no effect (Figure 4.5B). Therefore, these results suggest that β-glucan induces MR shedding through an Akt-independent, PI3K-mediated signalling pathway.

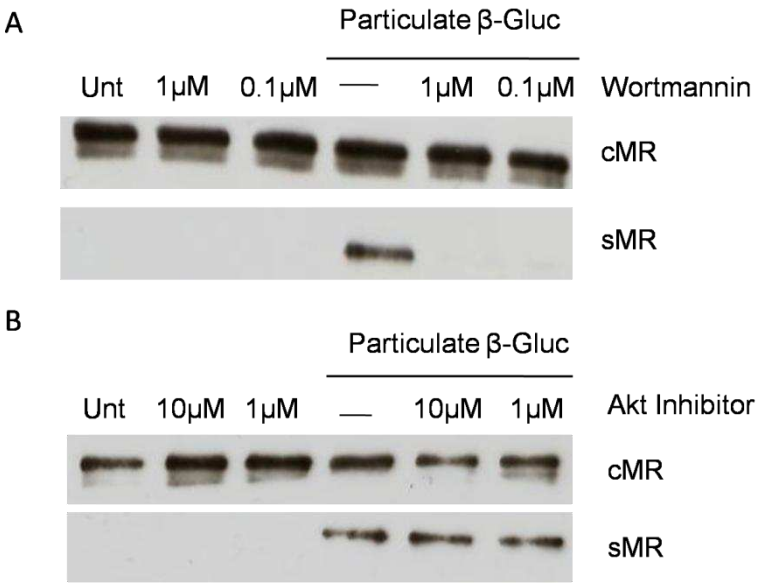

Figure 4.5: Dectin-1-mediated MR shedding utilizes Akt-dependent PI3K pathway. The cMR and sMR expression levels by β-glucan-treated MΦ in the presence and absence of wortmannin (A), or Akt inhibitor I (B). While wortmannin completely blocks MR shedding in response to β-glucan, Akt inhibition had no effect. Data are representative of three independent experiments.

4.3.5. β-glucan-induced MR shedding is phagocytosis-independent but requires actin-polymerisation

Even though dectin-1 is a phagocytic receptor (Herre et al., 2004) (Underhill et al., 2005), it does not require phagocytosis to induce inflammatory responses (McCann et al., 2005). In fact, dectin-1-mediated intracellular signalling is enhanced by frustrated phagocytosis (Hernanz-Falcon et al., 2009, Rosas et al., 2008). In order to investigate if phagocytosis is required for dectin-1-mediated MR shedding three different approaches were used: (i) investigating MR shedding in response to the non-phagocytosable β-glucan particle curdlan, (ii) inhibiting actin polymerisation with cytochalasin D or latrunculin A and (iii) inhibiting phagosome acidification.

As shown in Figure 4.6A, incubation of thio-MΦ with curdlan particles enhanced MR shedding, which suggests that dectin-1-mediated MR shedding is independent from particle internalisation. To eliminate the possibility of small curdlan fragments being responsible for its effect on MR shedding, only curdlan particles repeatedly (three times) retained in a 100 μM cut off filter were used for these experiments. Microscopical examination of these preparations demonstrated that all small fragments had been eliminated.

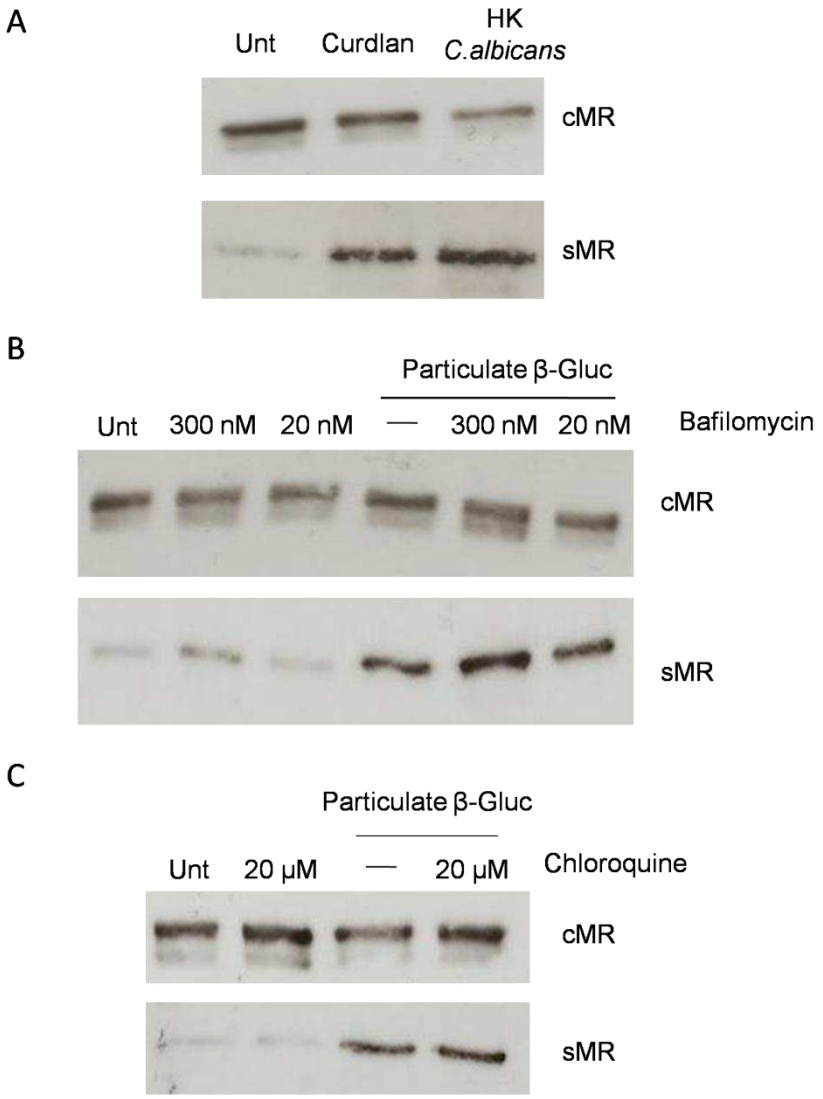

Figure 4.6: Dectin-1-induced sMR production can be induced by curdlan but cannot be blocked by bafilomycin or chloroquine. Western blot analysis of MR expression by thio-MΦ treated with curdlan and HK C.albicans (A) at concentrations of four particles/cell (10 μg of curdlan would contain ~550 particles (Rosas et al., 2008)) and 50 particles/cell, respectively. Both particles were found to enhance MR shedding (A). Inhibition of phagosome acidification using bafilomycin (B) or chloroquine (C) did not change the level of sMR produced in response to β-glucan particles. Data are representative of three independent experiments.

In support to phagocytosis not being required for MR shedding in response to β-glucan particles, inhibition of phagosome acidification using

bafilomycin (Figure 4.6B) or chloroquine (Figure 4.6C) did not alter sMR production in response to β-glucan.

Nevertheless, it was observed that inhibitors of actin-polymerisation, cytochalasin D (used at 0.5-1 μM) and latrunculin A (used at 0.5-5 μM), blocked sMR production in response to β-glucan (Figure 4.7A-B), in spite of the ability of the non-phagocytable β-glucan particle, curdlan, to promote MR shedding. Therefore, two conditions under which dectin-1-mediated signalling is enhanced because of the increased receptor engagement have opposite effects on MR shedding.

This could be due to actin polymerisation being required for MR stability/recycling, since high concentrations of actin-polymerisation inhibitors (5-10 μM for cytochalasin D, and 10 μM for latrunculin A) reduced the cMR expression level in the absence of β-glucan particles (Figure 4.7C).

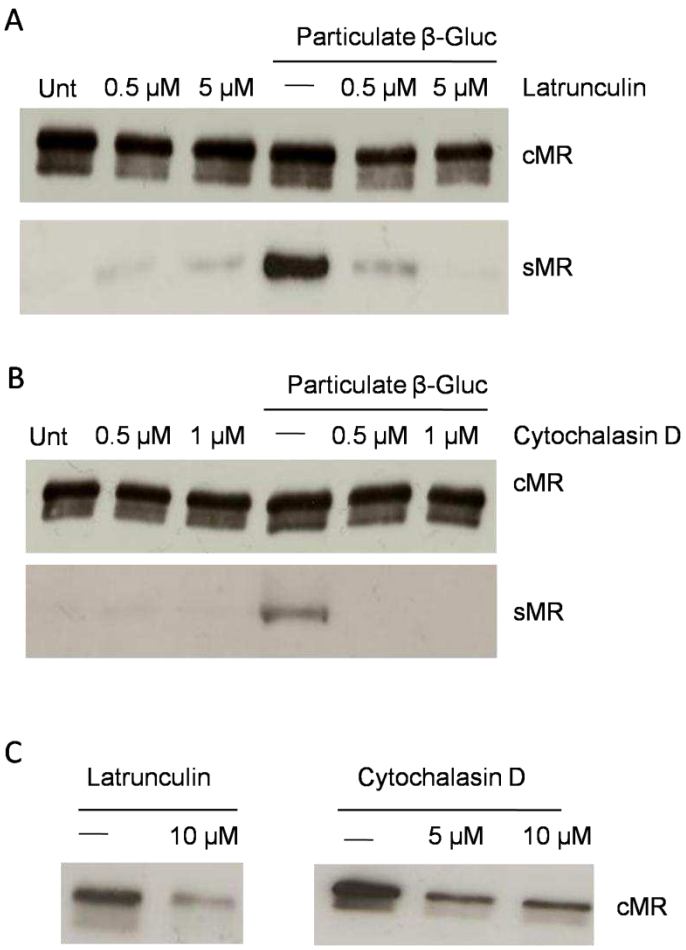

Figure 4.7: β-glucan-induced MR shedding can be blocked by actin-polmerisation inhibitors. Both latrunculin A (A) and cytochalasin D (B) inhibited the production of sMR in response to β-glucan treatment. This was thought to be because of the effect of actin-polymerisation inhibitors on MR expression, as when used at high concentrations cMR expression was decreased in the absence of β-glucan particles (C). Data are representative of three independent experiments.

4.3.6. β-glucan-induced MR shedding is mediated by a non-secreted metalloprotease

Previously, fungi-mediated ectdomain shedding through β-glucan recognition was shown to be mediated by MMP/ADAM activity (Figure 3.3).

To investigate if dectin-1 engagement on its own also utilises the same mechanism to promote MR shedding, the β-glucan treatment was repeated in the presence and absence of GM6001, a wide spectrum MMP/ADAM inhibitor. The pre-treatment of MΦ with GM6001 inhibited sMR production, which suggests that dectin-1-induced MR shedding, like in the case of steady-state and fungi-induced sMR production, is MMP or ADAM dependent (Figure 4.8A).

In an attempt to discern if the metalloprotease responsible for MR shedding was secreted into the medium, wt thio-MΦ were incubated with culture supernatants collected from β-glucan-treated MR-deficient MΦ. It was reasoned that this would be a suitable approach, because β-glucan recognition is not affected by MR deficiency (Heinsbroek et al., 2008). As demonstrated by the results shown in Figure 4.8B, MR shedding was not altered upon treatment with culture supernatants collected from MR-KO MΦ treated with β-glucan. These results indicate that the protease responsible for MR shedding is probably membrane-anchored.

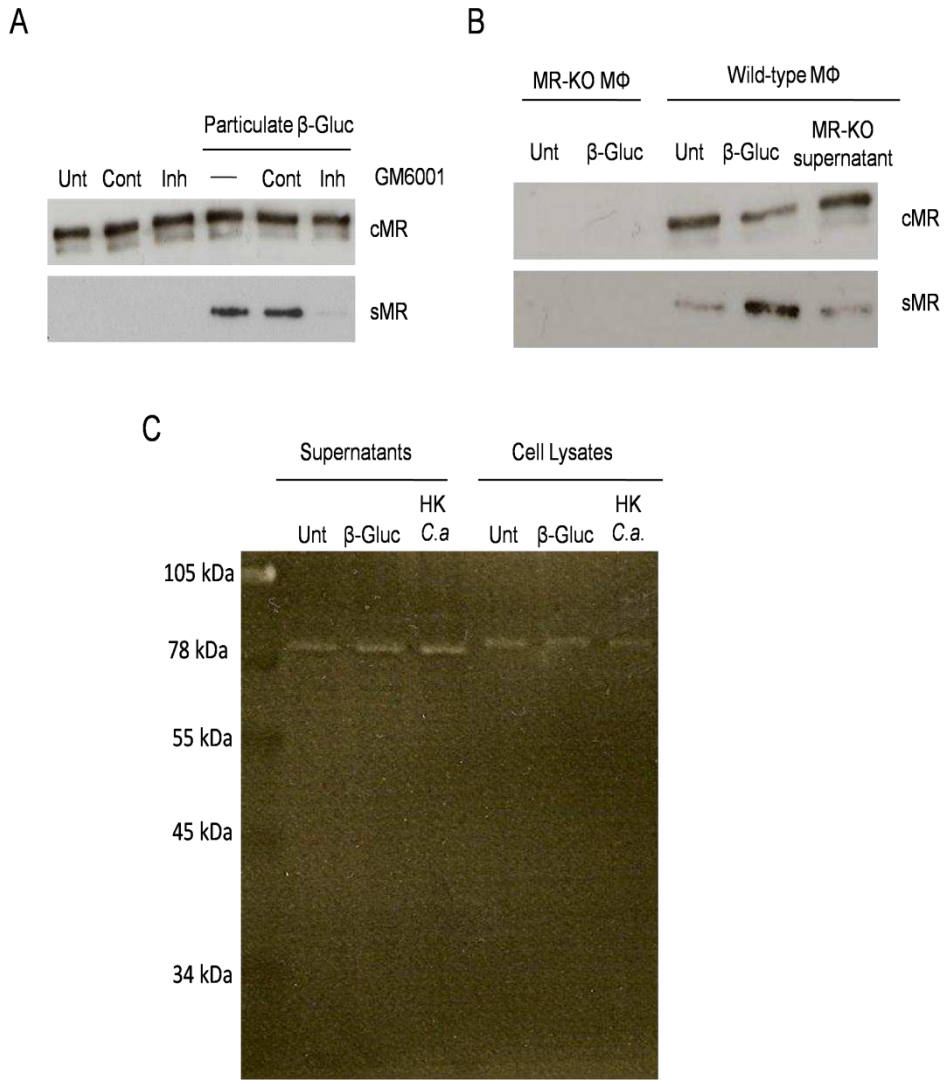

Figure 4.8: Dectin-1-induced MR shedding is mediated by a non-secreted metalloprotease. GM6001 treatment inhibits MR shedding in response to β-glucan particles (A). No enhanced MR shedding by wt MΦ was observed upon treatment with supernatants collected from MR-KO MΦ incubated with β-glucan particles for three hours (B). Analysis of cell lysates and supernatants from MR-KO cells demonstrated that detection of MR in samples from wt cells was specific. Analysis of metalloprotease activity by gelatin zymography in the supernatants and cells lysates from β-glucan and HK C. albicans (C.a.)-treated thio-MΦ (C). Data are representative of three independent experiments.

In agreement with these observations, gelatine zymography data does not suggest a role for MMP-2 or MMP-9 in MR shedding in response to β-glucan. Only a band corresponding to MMP-9 was visualised in cell lysates and supernatants from untreated and β-glucan or HK C. albicans-treated thio-MΦ, and this band was unaltered by β-glucan or HK C. albicans-treatment (Figure 4.8C). These observations are in correlation with qPCR results demonstrating the lack of MMP-2 and -3-specific mRNA (data not shown) and the presence of MMP-9-specific mRNA in thio-MΦ. Additionally, levels of MMP-8- and MMP-9-specific mRNA were not affected by the presence of β-glucan even after O/N treatment (Figure 4.9).

To examine possible ADAM participation in enhanced MR shedding, MΦ were treated with two pharmacological agents known to activate ADAM proteins: ionomycin, and PMA. Ionomycin, acting as a Ca^{2+} ionophore, stimulates ADAM-10 activity whereas treatment with PMA, a potent PKC activator, results in ADAM-17 induction (Nagano et al., 2004). Through ADAM-mediated processes, both ionomycin and PMA were previously shown to mediate ectodomain shedding of various receptors including CD14, TNF-α, CD44 and L1 (Stoeck et al., 2006) (Nakamura et al., 2004) (Liu et al., 2006) (Bazil and Strominger, 1991). Among those, CD44 was used as a positive control in this study.

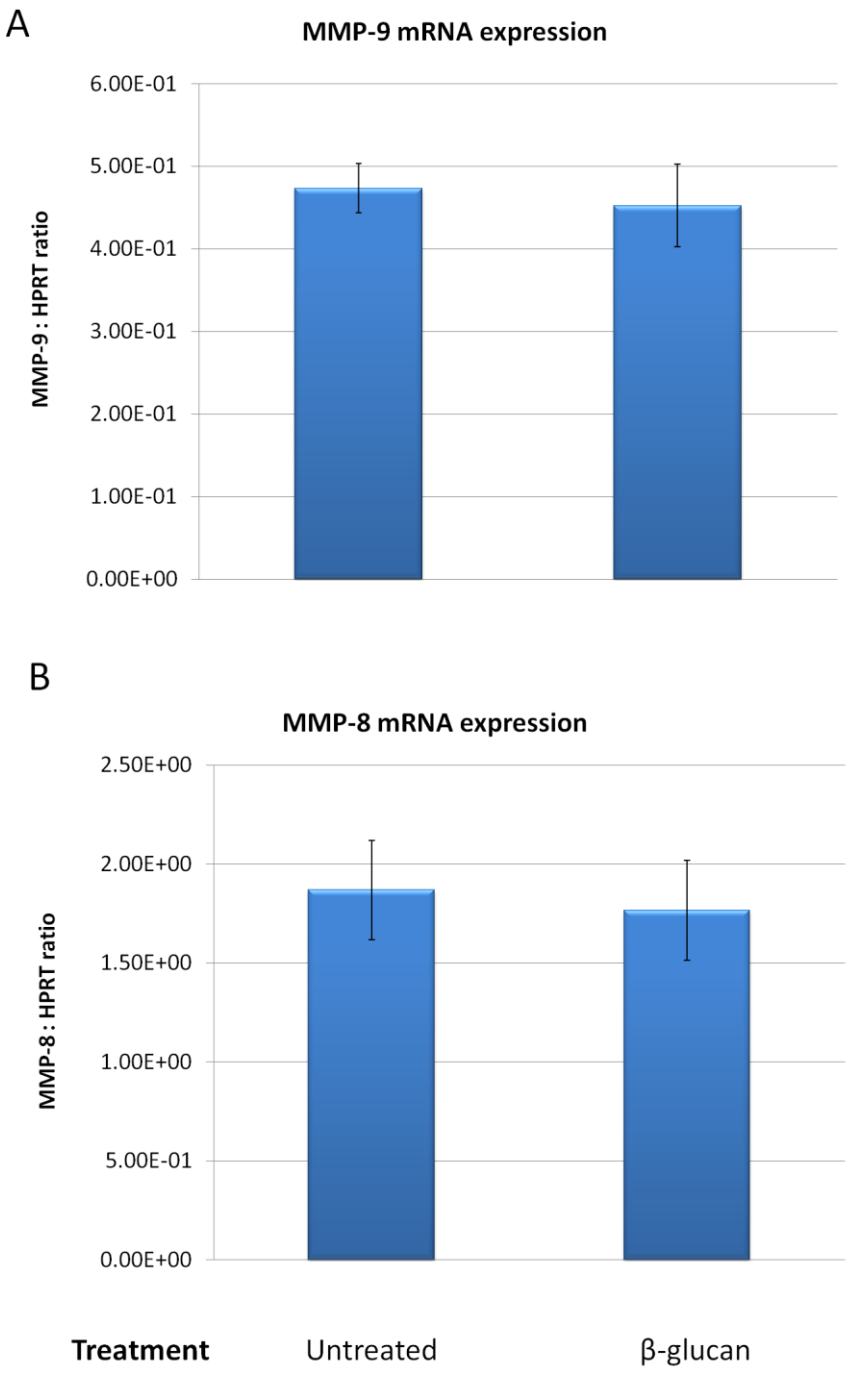

Figure 4.9: Quantification of MMP-8 and MMP9-specific mRNA in thio-MΦ treated with particulate β-glucan. QPCR analysis of MMP-9 (A) and MMP-8 (B) mRNA expression by MΦ treated with β-glucan particles for 3, 6 hours or O/N (B). The data represent the mean ratio of mRNA expression of MMP-9 or MMP-8 to HPRT ± SEM obtained from four independent experiments.

As demonstrated in Figure 4.10, only ionomycin was able to induce MR shedding under the conditions tested, while sMR production by PMA-induced MΦ was not significantly altered. The effect of ionomycin is very dramatic such that the sMR produced is more than that induced by β-glucan treatment. However, the observed effect seemed to be a consequence of extensive cell death, as revealed by microscopical examination and BCA data showing reduced protein concentrations in ionomycin-treated MΦ. This was further supported by the lack of change in the level of sMR produced when treatment was done in the presence of 10% (v/v) FBS in order to increase the cell survival. The sMR band detected in untreated samples was found to be derived from the FBS used, which is in correlation with the previous data that reported the presence of sMR in the mouse serum under steady-state conditions (data not shown) (Martinez-Pomares et al., 1998).

The other explanation for the lack of enhanced MR-shedding in FBS-containing conditions is the presence of bovine serum albumin (BSA), which was previously reported to counteract the ionomycin-mediated synoptosomal hydrogen peroxide production and Ca^{2+} movement through binding the ionophore. The inhibition was overcome by the addition of excess ionomycin (Zoccarato et al., 1989). Accordingly, besides sMR, enhanced soluble CD44 (~90kDa (Katoh et al., 1994)) production in response to ionomycin was also blocked by the addition of FBS.

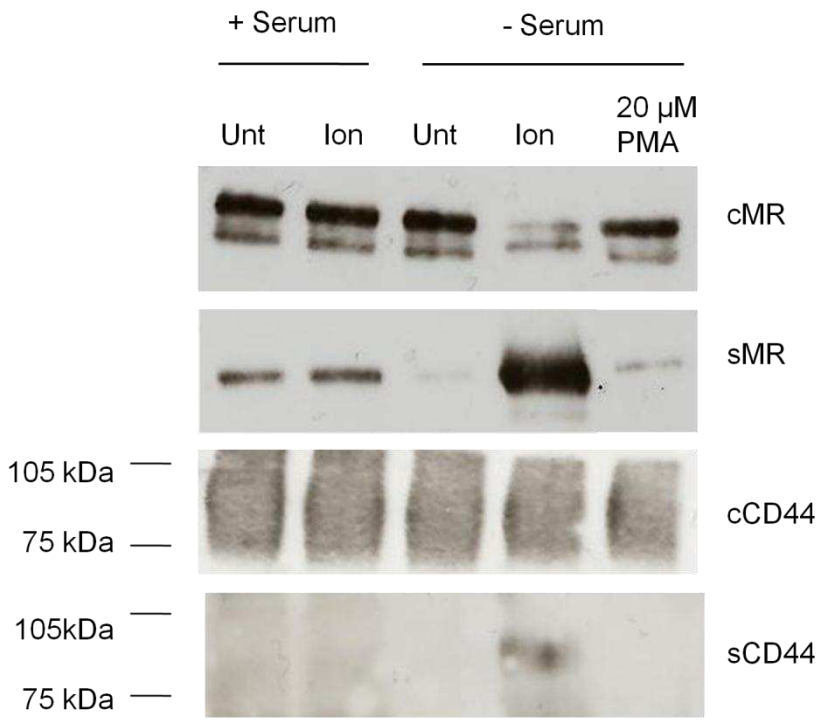

Figure 4.10: CD44 and MR expression upon treatment with ionomycin or PMA. Westen blot analysis of cMR, sMR, cCD44 and sCD44 expression by MΦ treated with 2.5 µM (not shown) or 5 µM ionomycin (Ion) in the presence or absence of 10 % (v/v) serum, or PMA at concentrations of 10 µM (not shown) and 20 µM in serum-free media. Data are representative of three independent experiments.

In contrast to ionomycin data, strikingly there was not any detectable enhanced CD44 cleavage in response to PMA even at concentrations as high as 20µM. The underlying reason may be because of the differences in the cells being used, as most of the studies that showed CD44 shedding in response to PMA were done using tumour cells (Gasbarri et al., 2003) (Nagano et al., 2004) (Murai et al., 2004).

4.4. Discussion

In this study it was shown that: (i) purified β-glucan particles can induce MR-shedding on their own and this is not enhanced by the addition of the TLR2 ligand Pam_3CSK_4, (ii) the β-glucan-mediated MR shedding depends on dectin-1, Syk kinase, PI3K and membrane-anchored MMP/ADAM activity, and partially on Raf-1 kinase, and is independent of phagocytosis and Akt, and (iii) there is no difference between C57BL/6 and BALB/c mouse strains in terms of β-glucan-mediated MR shedding, and reduction in the cellular endocytosis capacity.

In correlation with the previous chapter showing the β-glucan phosphate-mediated inhibition of fungi-mediated MR shedding, by using wt and dectin-1-KO MΦ, it was demonstrated that the ectodomain shedding is mostly induced by dectin-1, the main β-glucan receptor expressed by myeloid cells. The exclusive role played by dectin-1 was confirmed using dectin-1-KO MΦ in which MR shedding, in response to purified β-glucan particles isolated from C. albicans, was not observed. Overall these data suggest that the β-glucan recognition required for MR shedding is largely mediated by dectin-1.

Murine MΦ express two functional dectin-1 isoforms, dectin-1A and dectin-1B, which are structurally distinct: dectin-1A is composed of all structural domains whereas dectin-1B lacks the stalk region. It was quite recently shown that they also differ in intracellular signalling, as dectin-1B expressing cells were reported to produce more TNF-α than the dectin-1A

expressing cells (Heinsbroek et al., 2006). As both isoforms were equally efficient in zymosan recognition, the stalk region was thought to influence the ability of the receptor to interact with other molecules (such as TLR2, CD63), as it is often used by other C-type lectins related to dectin-1 to form dimers (Heinsbroek et al., 2006) (Marshall and Gordon, 2004).

This difference in intracellular signalling was proposed to be the cause of the observed differences in chemokines, cytokine and β-defensin responses to candidiasis between C57 BL/6 and BALB/c mouse strains (Schofield et al., 2005), as they are known to differ in dectin-1 isoform expression: BALB/c mice express both isoforms in equal amounts while C57 BL/6 mice predominantly express dectin-1B (Heinsbroek et al., 2006). However, as shown in Figure 4.2, MΦ from both mouse strains responded equally to the β-glucan particles in terms of MR-shedding, and the reduction in MR- and non-MR-mediated endocytosis.

During the induction of MR shedding, there is no requirement for dectin-1 to cross-talk with TLR as treatment with the TLR2 agonist, Pam_3CSK_4, on its own or in combination with particulate β-glucan, even after prolonged incubation periods (e.g. overnight), does not influence sMR production. This further confirms S. aureus data shown in Figure 3.7, which is known to activate TLR1/TLR2 and TLR2/TLR6 heterodimers (Issa et al., 2008) (Kurokawa et al., 2009). Together, these results imply that the sMR production is independent of TLR-mediated signalling.

Among the two signalling pathways induced by dectin-1, the Syk-dependent pathway is the most characterised, thanks to the common elements shared with other ITAM-mediated pathways, while the Syk-independent signalling pathway is still poorly understood, but has been shown to involve a Raf-1 kinase activity (Gringhuis et al., 2009). In this study, it was shown that, the effect of the Syk-independent pathway on sMR production was significantly weaker than that of the Syk-dependent pathway whose inhibition completely stopped the enhanced MR shedding.

The attempt to examine whether other ITAM-associated receptors can also induce MR-shedding failed to give reliable results, due to the doubts concerning the intracellular signalling. Since only approximately one or two particles (out of 50) were internalized by each MΦ, it was suggested that the intracellular signalling triggered by FcR is not sufficient to initiate any cellular activity (Garcia-Garcia and Rosales, 2002). Similar levels of latex bead uptake by MΦ were also reported by Chavele et al (Chavele et al., published online in 2010) implying that in future studies on the effect of FcR on MR-shedding, different approaches should be used.

The partial dependence of the phenomenon on Raf-1 kinase suggests the requirement of a possible cross-talk between Syk kinase- dependent and – independent pathways. This might occur via PI3K, since PI3K and Raf-1 kinase were previously shown to induce activation of each other in response to various growth factors (Wang et al., 2009, Sutor et al., 1999, Wennstrom and Downward, 1999, Scheid and Woodgett, 2000). Apart from PI3K, Raf-1 also

stimulates downstream kinase MAP kinase/ERK kinase (MEK), which in turn activates ERK 1/2 (Yart et al., 2002) (Hagemann and Rapp, 1999) (Wang et al., 2009). Future work will need to address the question whether this pathway is required for dectin-1-mediated sMR production.

The requirement for actin polymerisation in this model is intriguing since this does not appear to be caused by a requirement for phagosome formation. A possibility that could explain these observations is the requirement of actin polymerisation for MR recycling, as suggested by Deslee et al. In this study, MR-mediated endocytosis of dextran was shown to be reduced significantly in the presence of cytochalasin D (Deslee et al., 2002). This would also explain the drastic reduction in cMR levels observed when actin-polymerisation inhibitors were used at higher concentrations (5-10 μM for cytochalasin D and 10 μM for latrunculin A), as non-recycled MR could be targeted for degradation.

The data presented suggest the requirement for a cell-associated protease, sensitive to the broad metalloprotease inhibitor GM6001, known to inhibit both MMPs and ADAMs. In contrast to ADAMs, not all MMPs are transmembrane proteins. Membrane-anchored MMPs include MMP-7, MMP-12, MMP-14, MMP-15, MMP-16, MMP-17, MM-19, MMP-20, MMP-23, MMP-24, MMP-25, MMP-26, MMP-28 (Webster and Crowe, 2006).

The possible ADAM participation was further investigated by treating thio-MΦ with PMA or ionomycin; conditions known to promote ADAM-10 and ADAM-17 activity respectively (Huovila et al., 2005). Treatment with

PMA for 3 hours did not enhance MR ectodomain shedding. Surprisingly, there was no detectable amount of soluble CD44 in PMA-treated samples either. However, differences in the cell types and the incubation times used could explain the lack of PMA-induced CD44 shedding. Treatment of thio-MΦ with ionomycin had no effect on both CD44 and MR cleavage in the presence of serum. On the other hand, when ionomycin was added in the absence of the serum, sCD44 could be detected and there was a major increase in sMR production. This could be a consequence of increased cell death observed under these conditions, or of the serum BSA-mediated inhibitory effect as previously reported by Zoccarato et al (Zoccarato et al., 1989). The observed enhanced levels of MR ectodomain shedding in conditions of increased cell death suggests the possible involvement of mincle, an ITAM-coupled receptor known in the recognition of dead cells as well as fungi species including C. albicans (Yamasaki et al., 2008) (Yamasaki et al., 2009, Wells et al., 2008). Nevertheless, the preliminary data showed that the uptake of dead cells did not enhance MR ectodomain shedding (LMP, personal communications).

MR shedding promoted by dectin-1 could be regarded as a way of modulating MR involvement during fungal uptake. For instance, it could explain the surprising redundancy observed for MR in murine models of fungal infection (Lee et al., 2003, Swain et al., 2003) that does not correlate with observations in human models involving alveolar macrophages and cultured peripheral blood mononuclear cells (Netea et al., 2008, Netea et al., 2006, Zhang et al., 2005b, Zhang et al., 2004). In this regard, the major effect

that cellular differentiation has on dectin-1-mediated signalling should be considered. While the cells used during the course of these studies (thio-MΦ) do support robust sMR production upon dectin-1 engagement, this does not occur in the case of bone marrow-derived MΦ (Jia Wang and LMP, personal communications). An intriguing possibility is that in human tissue culture-based studies the relevance of cMR becomes apparent because MR shedding might not occur. Indeed, it is possible that the balance between sMR and cMR could set the fate between escape and protection; something that cannot be observed under conditions where there is a complete lack of MR expression (i.e. MR-KO animals).

Therefore, further studies on fungi- and non-fungi- induced MR shedding may enlighten the mechanism of how pathogens use or abuse MR to modulate the immune response, and can also prove to be useful in developing new drug strategies.

5. GENERAL DISCUSSION AND FUTURE STUDIES

MR is a type-I membrane protein with a single transmembrane region, and a cytoplasmic domain that mediates receptor internalization and recycling. Through its extracellular region, MR is involved in the recognition of a wide-range of ligands, including sugars terminated in SO_4-3-Gal or SO_4-3/4-GalNAc by the CR domain, and sugars terminated in D-mannose, L-fucose or GlcNAc by the eight tandemly arranged CTLD. In contrast to the CR domain, the CTLD are involved in the recognition of both self- (e.g. myeloperoxidase, and lysosomal hydrolases) and non-self antigens (e.g. C. albicans, Leishmania, M. tuberculosis, HIV and P. carinii) (Gazi and Martinez-Pomares, 2009).

In addition to these, MR was also shown to recognise collagen molecules through its FNII domain. As collagen is the most-abundant protein in animals, it was suggested that MR might also serve as an ECM adhesion receptor, which in turn may influence its activity as an endocytic receptor. The initial studies presented by this thesis focused on the possible effect of MΦ adhesion to collagen on MR function.

The data showed that the cellular adhesion to collagen I or IV does not influence MR-mediated endocytosis. This was not because of the changes in the MR expression as revealed by western blot and qPCR data. Therefore, MR may not to participate in ECM adhesion and its interaction with collagen may simply be crucial for removing collagen from the microenvironment during

tissue remodelling and wound healing. It was also shown that in addition to collagen and chondroitin sulphates, MR can also recognise another ECM component, laminin, in a mannose-dependent manner via its CTLD4-7 domain.

Furthermore, the immunocytochemistry data showed no obvious changes in the pattern of MR expression by MΦ upon adhesion to the ECM proteins. However, this does not imply an unaltered MR-expression on the MΦ surface in contact with the adhesive surface. This can be investigated by using confocal microscopy, which in contrast to fluorescence microscopy, can detect light emitted only from the focused points.

A recent study by Sturge et al. showed enhanced random migration of MR $^{-/-}$ BM-MΦ (Sturge et al., 2007). This can be explained by the possibility that MR expression at the cell surface may be enough to mediate both the cell-adhesion and antigen internalization through endocytosis. Thereby participation of MR in cellular adhesion to ECM protein-coated wells will not affect its function as an endocytic receptor. Comparing the cell migration of both wt and MR-KO MΦ on ECM protein-coated plates may further help to enlighten this area of research.

As well as a cell-associated form (cMR), MR is also expressed in a soluble form (sMR), which is comprised of only an extracellular region of intact MR (Taylor et al., 2005a, Martinez-Pomares et al., 2006, Martinez-Pomares et al., 1998, Jordens et al., 1999). Even though it was initially suggested to have an important role in transferring mannosylated antigens to a

subset of MΦ population in secondary lymphoid organs (Martinez-Pomares et al., 1999), enhanced sMR production was later shown to assist pathogens to evade the immune response by the formation of sMR-coated fungi that cannot be phagocytosed by MΦ (Fraser et al., 2000).

In this study it was shown that this way of escaping the immune response may also be used by other fungal species, since A. fumigatus, C. albicans, and the yeast-derived zymosan particles were able to induce enhanced sMR production after an incubation period as short as 3 hours. By using β-glucan phosphate and soluble mannan as inhibitors, and dectin-1-KO mice, MR ectodomain shedding was shown to be mainly triggered by the recognition of the β-glucan component of the fungi cell wall by dectin-1. Differential expression of dectin-1 isoforms does not appear to have an effect on MR-shedding, since the level of β-glucan-induced sMR production did not differ between the C57 BL/6 and BALB/c mouse strains.

This enhanced sMR production may also explain the previous observation by Heinsbroek et al which reported the absence of MR in the early phases of phagosome formation (Heinsbroek et al., 2008). According to this study, dectin-1 and CR3 accumulates at the phagocytic cup and as the phagosome matures, both of these receptors disappear while MR becomes recruited to the phagosomes.

In agreement with the previous studies by Martinez-Pomares et al (1998 and 2006) (Martinez-Pomares et al., 2006) (Martinez-Pomares et al., 1998), it was shown that the dectin-1-mediated signalling induces MR-

shedding through membrane-anchored ADAM/MMP activity. In an attempt to characterise the protease responsible for the shedding, MΦ were treated with PMA or ionomycin. However, the results are not conclusive as PMA surprisingly was not able to induce CD44 shedding, and ionomycin-induced sCD44 production was inhibited by the addition of FBS.

The FBS-mediated inhibition was initially thought to be due to the inhibition of the extensive cell death that was observed upon ionomycin treatment in serum-free conditions. However, preliminary data showed that the uptake of dead cells did not enhance MR ectodomain shedding (LMP, personal communications). The alternative reason for the observed FBS-mediated inhibition is the presence of BSA in the serum, which was previously demonstrated to block ionomycin-mediated synoptosomal hydrogen peroxide production and Ca^{2+} movement through binding the ionophore. Therefore, using specific ADAM or MMP knock-out mice appears to be a better way to identify the MMP/ADAM responsible for the ectodomain shedding.

It also appears that the signalling responsible for sMR production does not require cross-talk between dectin-1 and TLR signalling, and by using a wide-range of signalling inhibitors, it was shown that dectin-1 utilizes both Syk-dependent and –independent pathways to trigger sMR production. The dectin-1-mediated sMR production was highly dependent on Syk-kinase, as its inhibition completely blocked the ectodomain shedding. The cross-talk between the Syk-dependent and –independent pathways may occur via the

Akt-independent PI3K-pathway whose inhibition also completely blocked the ectodomain shedding.

In an attempt to investigate the effect of other ITAM-associated receptors on MR-shedding, mouse IgG-coated latex beads were used in place of fungal particles. As was shown in Figure 4.4, there was no enhanced sMR production in response to coated or uncoated latex beads. However, this may be due to the lack of efficient intracellular signalling, since there was not sufficient uptake of latex beads. Therefore, the possibility of other ITAM-associated receptors (such as Fc-receptors) being able to induce sMR production is still open to debate.

Phagocytosis is not important for dectin-1-mediated MR-shedding as curdlan particles are able to trigger sMR production, and the inhibition of phagosome maturation did not alter the level of ectodomain shedding. However, actin-polymerisation has an important role in the induction of MR-shedding since the inclusion of cytochalasin D or latrunculin during the treatment with β-glucan particles reduced the level of sMR released. As suggested previously by Deslee et al (Deslee et al., 2002), actin-polymerisation may be required for MR recycling between the plasma membrane and the endocytic vesicles. Further studies with inhibitors specific to endocytosis (e.g. dynasore (Newton et al., 2006)) and exocytosis (e.g. botulinum toxin type A (Kanno et al., 2009)) are required to clarify the role of actin-polymerisation.

As sMR production was previously suggested to block phagocytosis of fungal particles (Fraser et al., 2000), it was hypothesized that it may also affect

the cytokine levels released by MΦ. For this purpose, the levels of released KC, MCP-1, IL-6, and IL-1β were screened, since cMR was previously shown to facilitate their production (except for KC) in response to C.albicans (Yamamoto et al., 1997) (Heinsbroek et al., 2008).

The capture-ELISA studies suggested that the inhibition of ectodomain shedding by GM6001 does not change the KC and MCP-1 levels in the supernatants collected after 3 hours of treatment with HK C. albicans, while IL-6 and IL-1β production were below the detection level. Although these data imply that sMR production does not have a role in fungi-mediated cytokine release, its possible role in the later phases of infection cannot be excluded. As was observed by Fraset et al, after a prolonged incubation period, sMR may form a protective coat around the fungal pathogen that would disrupt its interaction with MΦ (Fraser et al., 2000).

Following the treatment of MΦ with fungal particles, MR-mediated endocytosis was significantly reduced. However this decrease was surprisingly not as a result of enhanced MR-shedding, since the inclusion of GM6001 was not able to restore the level of endocytosis. This may be due to possible lack of membrane availability due to the phagosome formation which can be confirmed by repeating the same study with curdlan particles which cannot be phagocytosed because of their large sizes.

In addition to fungal particles, it was shown that particulate HDM can also trigger MR-shedding, while it is not evident upon treatment with S. aureus. This further emphasizes the importance of β-glucan-dectin-1

interaction in the induction of sMR production, since in contrast to bacteria, HDM was previously shown to induce the immune response through β-glucan recognition (Nathan et al., 2009).

However the process of MR-shedding appears to differ from the one triggered by fungal particles since instead of one, two sMR populations were detected in the supernatants collected from HDM-treated samples. Since there is not enough information about the second sMR population which could lack the CR domain, as the cMR detected in the small intestine, ELISA based binding analysis would help to reveal more about its structure and ligands.

The cross-talk between MR and dectin-1 encourages the re-examination of the data on the roles of the two PRRs in anti-fungal immune response. For instance, previously MR deficient mice did not display any changes in susceptibility to fungal infections and showed minor changes in lung pathology and fungal burdens upon infection with P. carinii and C. albicans, respectively (Swain et al., 2003) (Steele et al., 2003) (Willment and Brown, 2008). This lack of significant difference between wt and MR-KO mice may be because of the dectin-1-mediated sMR production that may reduce the level of cMR expression on the wt MΦ surface. In fact, MR-deficient MΦ were reported to become less efficient in clearing P. carinii (Swain et al., 2003). Accordingly, studies with the inhibition of dectin-1 expression and/or function also require to be readdressed, since the sMR production would not be induced in response to fungal particles.

Therefore inhibition of MR-shedding is essential to identify the precise roles of dectin-1 and MR during fungal infections. Inclusion of GM6001 is not a reliable approach, as it can inhibit a wide-range of MMP/ADAM, and therefore can influence the progress of inflammation independent of MR-shedding (e.g. TNF-α release). Instead, point-mutation studies would help to identify the sequence/region of MR targeted during the ectodomain shedding, and facilitate the generation of mice unable to produce sMR. By knocking down dectin-1 or MR expression, the sMR-deficient mice can be used to identify the precise roles of sMR, cMR and dectin-1 in triggering the immune response against fungi.

As revealed by Fraser et al, MR-shedding may be another way of pathogens to evade the immune response (Fraser et al., 2000). This can be i) either through the reduction of cMR expression level on the cell surface, which was shown to facilitate phagocytosis, cytokine production, and antigen processing and presentation (Gazi and Martinez-Pomares, 2009), ii) or the enhancement in sMR production that may form a protective-coat around the invading pathogen (Fraser et al., 2000). However like cMR, the sMR role in immune response requires further confirmation since, alternatively, it may also play an important role in transferring mannosylated antigens to a subset of macrophages present in secondary lymphoid organs (Martinez-Pomares et al., 1999). Therefore, it is possible to say that the balance between cMR and sMR expression may have a decisive role in the destruction of the pathogen.

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