CHAPTER 4

Mycolic acids and macrophages

4.1 Introduction

4.1.1 Uptake of antigens

A primary challenge to the immune system is discrimination between self and non-self. Using a limited amount of receptors, the innate immune response then directs the adaptive immune response. The adaptive immune response has an extended range of highly specific antigen receptors distributed on T- and B lymphocytes to form a highly efficient effector network and longterm immunological memory. Phagocytosis of pathogens initiates the innate immune response of generic specificity. Cells of the innate immune system have acquired a number of strategies to internalize particles, including:

1. Endocytosis, occuring via
    (a) pinocytosis
    (b) receptor mediated endocytosis
2. Patocytosis
3. Phagocytosis

Most cells have phagocytic capacity, but only neutrophils, monocytes and macrophages of the innate immune system are known as professional phagocytes. Phagocytosis is extremely complex. Various receptor-types can bind, as well as internalize particles and can activate and inhibit each others' functions. Nevertheless, most of these receptors have the same function of internalizing particles after binding specific ligands on the surface (Kruth *et al.*, 1999). Macrophages and other haemocytes can bind pathogens directly by non-clonally distributed pattern-recognition receptors on their surfaces. Several soluble and membrane–bound mammalian proteins, including the mannose receptor, LPS receptor and collectins, exhibit this property.

4.1.2 Receptors involved in phagocytosis

4.1.2.1 Fc receptors

The functions of Fc receptors fall into two categories:

1. transport of immunoglobulins across epithelial borders
2. modulation (activation or inhibition) of effector functions

Receptors that mediate phagocytosis, fall in the category of effector activation. Fc receptors that mediate phagocytosis in humans are FcγRI, FcγRIIA and FcγRIII. Human-, as well as murine macrophages, have FcγRIIB, which is an inhibitory receptor and doesn't participate in phagocytosis. Fcγ receptors bind IgG in immune complexes, leading to the stimulation of polymerization of actin to induce phagocytosis. It seems that phosphatidylinositol kinase (PI-3 kinase), the Rho family of GTPases, protein kinase C and motor proteins are all involved in this process (Amigorena and Bonnerot, 1999). FcεRI (found on basophils and mast cells) and FcεRII CD23 (found on B cells and eosinophils) are also IgG binding receptors. CD23 are important in that they are found on alternatively activated macrophages, which are most common in lung and placental tissue (Goerdt *et al.*, 1999).

4.1.2.2 Complement receptors

Serum complement proteins opsonize pathogens and are then phagocytosed via C3b or C3bi receptors (CRs) on macrophages. Macrophages have several receptors that participate in complement–opsonized phagocytosis, including CR1, CR3, and CR4. In contrast to Fc receptors, that are constitutively directed towards phagocytosis, CRs can either bind, or bind and internalise particles; the latter depending on a second external stimulatory signal. This second signal can be TNFα, GM-CSF or phospho- kinase C activators such as PMA. Using the electron microscope, it was shown that although phagocytosis always require actin polymerisation at the site of ingestion, particles are internalised in different ways depending on whether they are opsonized with IgG or complement (Amigorena and Bonnerot, 1999).

FcγR, present on the macrophage, reacts with the IgG-opsonized particle. Phagocytosis is induced and a veil of membrane emanating from the surface tightly engulfs the particle and then draws it into the macrophage. Complement - induced phagocytosis is a more passive process. Complement-opsonized particles sink into the membrane mostly with a few small pseudopodia present on the surface of the macrophage. In complement-induced phagocytosis, the phagosome membrane is also more loosely wrapped around the particle, in contrast to Fc receptor induced phagocytosis. In complement-induced phagocytosis, point-like contact areas rich in a variety of cytoskeletal proteins are seen which are absent in phagosomes containing IgG-opsonized particles. It seems that the signal for internalisation and cytoskeletal protein arrangements depends on which phagocytic receptor is engaged. Besides this, Fc receptor-induced phagocytosis has the capacity to induce pro-inflammatory molecules (cytokines, reactive oxygen intermediates [ROI] and arachidonic acid metabolites), which is not seen in complement receptor-induced phagocytosis. For this reason, it is unlikely that macrophage-resident pathogens would utilise Fc receptors as means for entry (Amigorena and Bonnerot, 1999).

4.1.2.3 Mannose receptors

Non-specific receptor binding is used by phagocytic cells to recognize patterns, rather than specific ligands on foreign invaders. Several membrane bound and soluble proteins, including the mannose receptor, the collectins, LPS receptor CD14 and scavenger receptors, exhibit the properties of pattern recognizing molecules (Van Bergen *et al.*, 1999).

Mannose receptors (MR) on macrophages, recognise mannose and fucose on the surface of pathogens. This is a high affinity receptor with extensive pathogen specificity. During MR-induced phagocytosis some cytoskeletal proteins (paxillin and vinculin) are not recruited, in comparison to Fc- and complement receptor-induced phagocytosis. This reinforces the idea that different phagocytic receptors send different signals to the actin cytoskeleton, which activates different internalisation mechanisms. MR-induced phagocytosis also activates pro-

inflammatory signals. These include IL1β, IL6, GM-CSF, TNFα and IL12 (Underhill *et al.*, 1999).

4.1.2.4 Scavenger receptors

Scavenger receptors (SRs) exhibit broad ligand binding specificities and bind both gram positive and gram negative microbial surface molecules as well as whole bacteria. SRs are expressed on macrophages and other immune cells and can also exhibit activities typical of cell adhesion molecules. SRs are defined by their ability to bind low-density lipoproteins (LDL) that have been changed by oxidation and acetylation. Early studies on scavenger receptors were done to define their role in atherogenesis (Pearson, 1996). This will be discussed later.

Scavenger receptors are divided into three classes: Class A (class AI, AII and MARCO), class B (B1 and CD36) and class C (CD68/microsialin receptor).
One of the principal functions of SRs is their removal of microbes and highly toxic microbial surface constituents from the body, using endo- and phagocytosis. LPS and other inflammatory stimuli up-regulate SR-AI/II, CD36 and microsialin /CD68 expression. In addition, some SRs also participate in recruitment of peripheral blood monocytes (PBMs) to infected tissues. Of relevance is that M-CSF, which induces monocyte to macrophage differentiation, can also up-regulate SR-AI and SR-AII expression. Class BI and CD36 primarily recognize lipids expressed by tissues and cells involved in host defense and /or lipid metabolism.
The relationship between host defence and lipid binding by macrophages is presently not well defined. Because anionic phospholipid vesicles are internalised, it is speculated that macrophages might be able to recognize non-self and damaged-self by surface expression of anionic phospholipids on target cells (Pearson, 1996). This pattern is not associated with normal host cells. Elomaa *et al.* (1998) demonstrated that MARCO, a recent identified scavenger receptor, is only present on a subset of macrophages in the peritoneum, spleen and lymph nodes, showing that different organ-derived macrophages might have distinctive roles in immunity.

4.1.2.5 Collectins

These are soluble effector proteins with collagen tails and globular lectin domains selectively recognizing carbohydrates on the surface of microorganisms. These molecules play an antecedent role in host defense before generation of specific immunity. Collectins include the serum mannose–binding protein (MBP), lung surfactant protein D (SP-D) and lung surfactant protein A (SP-A) (Malhotra *et al.,* 1994). Collectins aid the innate immune system to discern self from non-self. They are found in serum, nasal secretions, lungs, rheumatic joint fluid and inflamed sites. The role of collectins in the induction of cellular immune responses may be to bridge the gap between innate and adaptive immunity.

4.1.3 Receptors on macrophages that bind *Mycobacterium tuberculosis*

M. tuberculosis enters the macrophage by phagocytosis via cell surface molecules, including members of the integrin family CR1, CR3, mannose receptors (Schlesinger *et al.*, 1990) as well as CD14 (Fenton and Vermeulen, 1996). Phagocytosis via CR1 and CR3 avoids triggering of the oxidative burst (Wright *et al.,* 1983). The scavenger receptor type A was recently shown to play the most important role in the entry of *M. tuberculosis* into macrophages. (Zimmerli, *et al.,*1996).

4.1.4 Macrophages and TB

Monocytes arise from precursor cells in bone marrow and have a half-life of one to three days. By migration through the cell wall of capillaries, they leave the peripheral circulation and enter the tissues and organs to develop into macrophages. Macrophages may also arise from proliferation of macrophage precursor cells present in tissue. Common locations to find macrophages are lymph nodes, spleen, bone marrow, and serous cavities such as the peritoneum, pleura and synovium.

In broad terms, the macrophage acts in the expression of immune reactivity by mediating functions such as:

- The presentation of antigen

- Secretion of cytokines, chemokines and other secretory products to regulate immune reactions
- Phagocytosis of foreign and self particles via the various receptors

Few organisms can survive inside the phagocytic vacuoles of macrophages due to the vast amounts of hydrolytic enzymes, acid, and reactive oxygen and nitrogen intermediates that are released into this subcellular compartment.
Once the TB bacilli are inhaled and phagocytosed by the macrophage, it can either be killed, or survive to initiate an infection. Host macrophages are killed and mycobacteria are released to infect additional host cells. *M. tuberculosis* has the ability to establish infection and to replicate inside a variety of organs, but in infected tissues it is only found inside macrophages and polymorphonuclear leukocytes (Filley and Rook, 1991). The early exudate from an infection site contains chemotactic factors that attract circulating monocytes, lymphocytes and neutrophils. Granulomatous focal lesions, consisting of macrophage-derived epitheloid giant cells and lymphocytes, develop. The lymphocytes are not specific to tuberculosis antigens. Bacteria killed by heat or living bacteria are both equally effective as granuloma inducers.

4.1.5 Maturation of phagosomal vacuoles

All entries into the cell converge at endosomes. The first structures encountering endocytosed material are the early endosomes (EEs). EEs have a less acidic pH and have less proteolytic activity than late endocytic compartments. The EEs comprise a tubular network sometimes extending into the trans-Golgi network. Sorting endosomes are small vacuolar EEs in the cell periphery and part of the tubular network that sort soluble proteins and membrane proteins for transport down the endocytic pathway. Transferrin receptor, one of the recycling membrane proteins, is used as a marker for the EEs.

The first event after internalisation of mycobacteria into macrophages, is de-polymerisation of actin (F-actin) from the formed phagosome, leaving the actin

deprived membrane accessible to fusion with early endosomes. Internalized antigen that is not recycled to the cell surface, is transported to late endosomes (LEs) and lysosomes. Proteins that regulate vacuole maturation include annexins. Whereas annexin I, II and III associate with endosomes, annexin IV only binds to more mature vacuoles. Their significance in the endosomes is not known. Small molecular mass GTPases rab5, rab7 and rab1 sequentially associate with the endosome as it matures. Via *et al.* (1998) showed that the pH sensitive LysoTracker can be used as a fluorescent marker for vacuoles containing lysosome associated membrane protein (LAMP1) and rab7, which are characteristically found in late stage endosomes. LysoTracker is therefore useful to evaluate endosome maturation, although a narrow window of overlap exists in endosomes that express rab5, a less mature type. Late endosomes (LEs) contain a full set of acid hydrolases, have a low pH and are degradative. LEs gradually change into lysosomes and no sharp distinction is made between the two compartments (Claus *et al.,* 1998). Formation of a phagolysosome is not a terminal event as more lysosomes can continue to fuse with the organelle.

Depending on the nature of the ingested particle and the type of receptor activated, the rate of phagolysosome fusion varies. In FcR- and MR-mediated phagocytosis, fusion of lysosomes occurs within 30 minutes after internalisation. Latex-coated inert particles may take up to several hours to complete the same process. It is speculated that the membrane surrounding the particle or pathogen, can be modified to change the maturation process, or that the rate of maturation depends on the interaction of the particle surface and the membrane surrounding the particle. An example is the close apposition of the phagocytic membrane to hydrophobic inert particles as well as mycobacterium. Storrie and Desjardins (1996) suggested a "kiss and run" theory, where there is a transient and incomplete fusion between compartments. It is currently unknown exactly how the luminal contents of phagosomes are sorted.

Effective defense requires that the macrophage discriminate between different pathogens. Underhill *et al.* (1999) demonstrated that two classes of innate immune receptors are involved in phagocytosed particles. These receptors coordinate to internalize (mannose and other receptors) and then recognize (Toll-like receptors) the phagocytosed particles. Toll-like receptors sample the internalized particle or organism and then trigger an appropriate specific inflammatory reaction.

The killing of *M. tuberculosis* after ingestion by macrophages is evaded by modification of the phagosome during maturation. Russell and co–workers (1996) demonstrated that vacuoles containing *Mycobacterium avium* did not acidify below pH 6.5, which seemed to occur because of the specific exclusion of the vacuolar proton pump. Clemens and Horwitz (1995) showed that *M. tuberculosis* retards the maturation of its phagosome along the endosome-lysosome pathway and ends in a compartment with endosomal rather than lysosomal characteristics. Electronmicroscopy was applied to show that *M. tuberculosis* containing phagosomes do not mature. Thus, *M. tuberculosis* escapes killing within the macrophage phagosome. Macrophages activated with interferon-γ and bacterial lipopolysaccharide (LPS) prior to ingestion with mycobacteria kill the internalized pathogen by being able to acidify the phagosome to below 5.3. Macrophage activation is accompanied by coalescence of vacuoles containing a single mycobacterium into larger vacuoles containing many bacteria. This reduces the bacterial survival in the macrophage.

4.1.6 Macrophages and foam cell formation

4.1.6.1 Definition:

Monocyte/macrophage-derived foam cells are activated cells that take up seemingly unlimited amounts of cholesterol and cholesterol esters via scavenger receptors.

4.1.6.2 Introduction to foam cells

The study of receptor-mediated endocytosis of LDL, and the subsequent formation of foam cells, leading to the development of atherosclerosis was pioneered by Brown and Goldstein (1984). In their studies they showed that foam cell formation is not dependent on the LDL receptor. It was also confirmed that cholesterol accumulation associated with foam cell formation, is not possible in mouse peritoneal cells even after incubation with high levels of LDL. Only when the LDL was modified to acylated-LDL could foam cell formation be induced *in vitro*. The cholesterol uptake could be attributed to the acylated-LDL-receptor that does not take up native LDL and is insensitive to increasing intracellular cholesterol concentration. The acylated-LDL receptor recognizes negatively charged lipoproteins after chemical modification of positively charged lysine residues. Other chemical modifications of LDL such as acetoacetylation (Mahley *et al.*, 1979) and conjugation with malondialdehyde (Fogelman *et al.*, 1980) also resulted in increased affinity of the acylated-LDL receptor leading to foam cell formation. The acylated-LDL receptor is currently known as a scavenger receptor due to its broad binding capacity. It is found on monocytes/macrophages, Kupffer cells, and endothelial cells. Macrophages do not readily take up lipoproteins and only turn into foam cells when exposed to oxidized, acylated, or otherwise covalently modified lipoprotein. Elucidation of lipid-modification processes is important in understanding atherogenesis and the role of foam cells.

4.1.6.3 Macrophage scavenger receptors involved in atherogenesis/foam cells.

It is well known that LDL receptor-deficient macrophages can develop into foam cells indicating that other distinct receptor/s are involved for uptake of modified lipids.

At present, all known scavenger receptors are transmembrane molecules that bind to negatively charged macromolecules. With regard to atherogenesis, the most important scavenger receptors are at present class A (SR-A), types I and II,

and the class B (SR-BI and CD36). Table 4.1 shows important macrophage scavenger receptors involved in atherogenesis / foam cell formation.

Table 4.1. Scavenger receptors in atherogenesis and foam cell formation (Huh *et al.*, 1996).

Receptors	Major ligands	Pro/anti-atherogenic
SR-A	acetyl LDL, oxidized LDL	Pro/anti
CD36	oxidized LDL	pro
SR-B1	HDL	anti
CD68	oxidized LDL	No data

The three classes of scavenger receptors namely

- class A,
- class B (includes CD36),
- and the macrosialin/CD68 receptors

are all implicated in accumulation of cholesterol esters (CE) in atherosclerotic lesions.

Class A scavenger receptors consist of type I and II (SR-I, SR-II) and are found predominantly on macrophages and activated smooth muscle cells. Ligands for class A scavenger receptors include acylated-LDL, oxidized-LDL, fucoidan, and carrageenan. CD36 is an 88 kD integral membrane glycoprotein expressed on monocytes, platelets and some endothelium cells found in adipose tissue, lung and liver. CD36 is also known as fatty acid translocase (FAT), which was cloned as a receptor taking up oxidized modified lipoprotein. This receptor is currently the best candidate for mediating foam cell formation because of it:

a) being an adhesive receptor for
- thrombospondin
- collagen
- *Plasmodium falciparum* infected erythrocytes

b) being a scavenger receptor for
- oxidized LDL
- apoptotic neutrophils.

Steady-state mRNA levels of CD36 increase eightfold during the first 4 days of monocyte to macrophage differentiation and then decrease to monocyte baseline in another four days. Macrophage colony stimulating factor (M-CSF), a known transcriptional regulator of CD36, stimulate macrophages towards foam cell formation, but not in the presence of antibodies directed against CD36. This implies that CD36 expression correlates with foam cell formation (Huh *et al.*, 1996).

4.1.6.4 Regulation of LDL- and scavenger receptors and their biological impact

Regulation of the major cell surface receptor for LDL (LDL-R) occurs primarily at the transcription level and is controlled by free cholesterol in the cell. Inflammatory mediators such as growth factors and cytokines, can promote the binding and uptake of LDL. In Table 4.2, the effect of cytokines on lipoprotein activities and intracellular cholesterol metabolism are shown.

Table 4.2 Effect of cytokines on lipoprotein (Meiner *et al.*, 1996).

Cytokines and growth factors	Receptor/ enzyme	Response
TGF-β, TNF-α, IFN-γ	SR-A	Decrease
GM-CSF	LDL-R, SR-A	Increase
M-CSF	ACEH, NCEH, CD36, SR-A	Increase

ACEH: acid cholesterol ester hydrolase; NCEH: neutral cholesterol ester hydrolase

Some of these mediators, such as TNFα and IL1, affect transcriptional regulation of the LDL-R gene at the level of the promoter. It is unlikely that cytokine activation of the LDL-R can result in cellular cholesterol accumulation since TNFα and IL1 cannot override the inhibition of LDL-R activity by exogenous cholesterol.

4.1.6.5 Metabolism of cholesterol

Over the last decade, a crucial role has emerged for acyl-coenzyme A cholesterol-acyltransferase (ACAT) at several stages in the development of

atherosclerosis. This enzyme participates directly in promoting storage of lipid in arterial macrophages, playing a key role in promoting formation of foam cells. Meiner *et al.* (1996) reported the phenotype of ACAT knockout mice, but it was shown that cholesterol esterification still existed in the liver, indicative of another type of ACAT enzyme activity. ACAT2 was subsequently cloned by three different groups in 1998 and showed 60 % similarity to ACAT1. The tissue distribution of the two enzymes are of interest as ACAT1 is found in a wide variety of cells including macrophages, compared to ACAT2, which is found primarily in the intestines and liver. A working model for lipoprotein metabolism is illustrated in Figure 4.1.

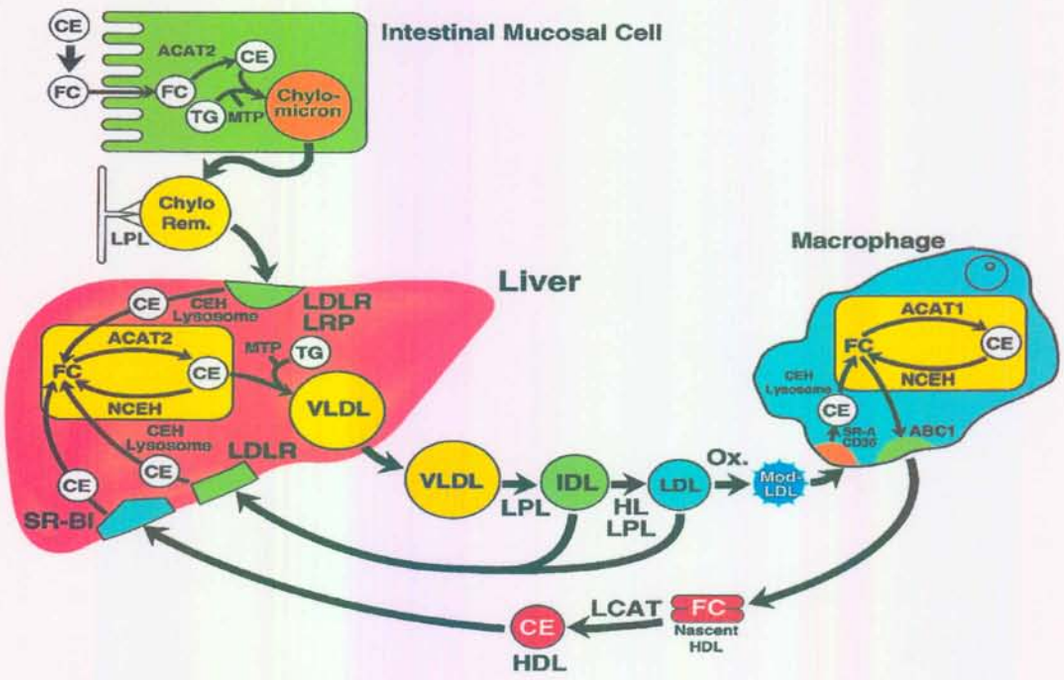


Figure 4.1. Schematic overview of cholesterol metabolism. Details are given below in text. FC–free cholesterol, CE-cholesterol ester, TGs – triglycerides, MTP-microsomal transfer protein, LDL-low density lipoprotein, VLDL-very low-density lipoprotein, CEH-cholesteryl ester hydrolase, NCEH-neutral cholesterol ester hydrolase, LPL- lipoprotein lipase, LRP – LDL receptor-related protein, IDL-intermediate density lipoprotein, LCAT-lecitin cholesterol acyltransferase, ABC1-transporter for FC. (Taken from Brewer, 2000).

In the model, ACAT 2 plays a role in absorption from the intestine. Enterocytes take up FC hydrolysed by pancreatic lipase from dietary CE. Free cholesterol (FC) is re-esterified to CEs by ACAT2 and the resulting CE, together with triglycerides, are formed into chylomicrons and secreted by the cell. In the plasma, the triglycerides are hydrolysed by lipoprotein lipase (LPL). Chylomicron remnants containing CE derived from enterocytes, are transported to the liver and removed from plasma by the hepatic LDL receptor and related LDL-receptor-related protein (LRP). CEs derived from chylomicrons are hydrolysed to FC in lysosomes, re-esterified by hepatic ACAT2 and repacked together with triglycerides, to form VLDLs and secreted into plasma. Triglycerides in very low-density lipoproteins (VLDLs) are hydrolysed by LPL to form intermediary low-density lipoproteins (ILDLs) and then cholesterol rich LDL. LDL may be removed by the LDL receptor, or undergo modification to form oxidized LDL, which in turn are taken up by scavenger receptors (CD36, SR-A). In this model, the ACAT2 enzyme present in the intestines and the liver, synthesizes CE, which is incorporated into lipoprotein particles and released into the plasma. ACAT1, on the other hand, plays a role in macrophages and most other tissues. ACAT1–catalyzed CE synthesis in macrophages is important for the differentiation/conversion into foam cells. When appropriately modified LDL accumulates in the plasma, increased uptake of cholesterol by macrophages promotes foam cell formation.

4.1.6.6 Pathogenesis of foam cell formation

Uptake of native LDL alone does not lead to foam cell formation. Macrophages can internalize large amounts of LDL, but the cholesterol is unable to stimulate esterification (Nancy *et al.*, 1999).

The generally accepted scene for foam cell formation, starts with modified lipoproteins bound to receptors and internalized by coated–pit endocytosis. One group of such receptors is scavenger receptors. The lipoproteins are delivered to endosomes and the CE hydrolyzed to FC, transported to the membrane and, via ACAT, re-esterified. This product is stored as intracellular lipid droplets until needed. Foam cells accumulate unregulated amounts of cholesterol through this

route and are formed when there is an imbalance in the amount of cholesterol stored, compared to the amount of cholesterol released.

4.1.6.7 Lipoprotein modification

Despite the fact that a plethora of *in vitro* studies have been performed, the mechanism of lipoprotein modification occurring *in vivo* is poorly understood. One of the problems is the wide range of modified LDL that is taken up. In Figure 4.2, the action of a wide range of enzymes that can modify LDL is shown. These enzymes include sphingomyelinase (SMase) and phospholipase A2 (sPLA2). The above-mentioned enzymes change phospholipids into more biologically active types. Other LDL modifying enzymes not working on phospholipids include lipo-oxygenase (LO), nitric oxide synthetase and myeloperoxidase (MPO). These enzymes change LDL into more atherogenic structures/entities that can induce foam cell formation.

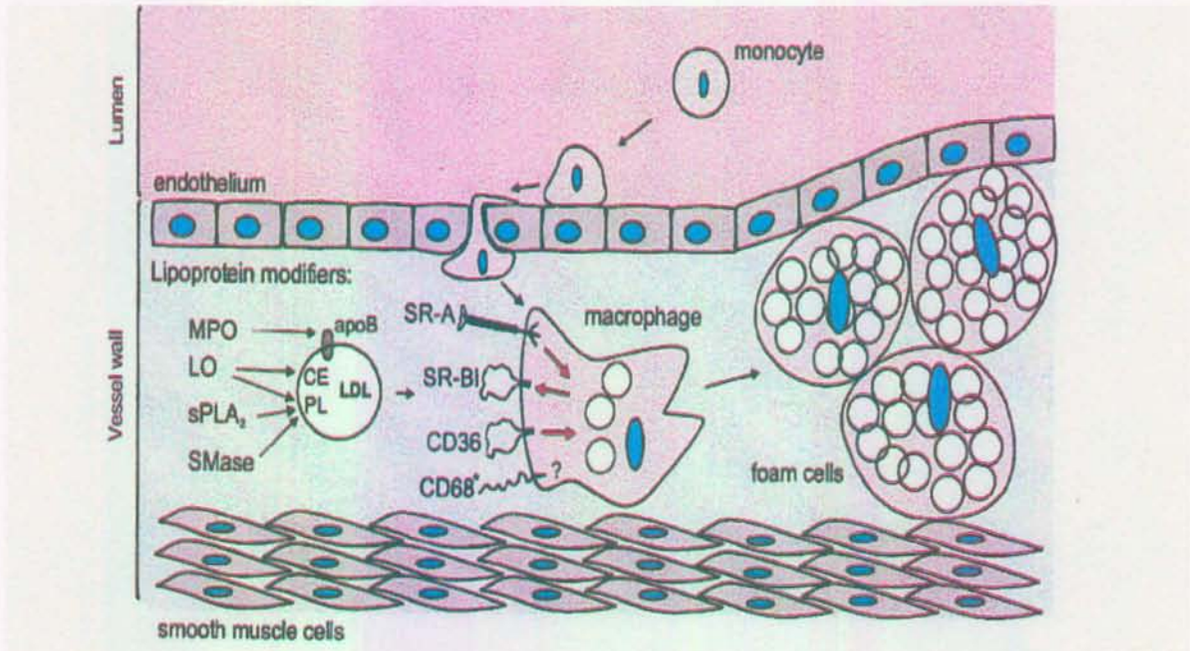


Figure 4.2 Lipid-modifying enzymes and scavenger receptors from macrophages which play a role in foam cell formation. Arrows from the LDL-modifying enzymes indicate on which component it is working. CD68 is included as it binds oxLDL but its role is still not clear. SMase-sphingomyelinase, sPLA2-phospholipase A_2, LO- lipo-oxygenase, MPO-myeloperoxidase.(De Winther and Hofker, 2000)

The type of LDL modulation that results in take-up, consists of acetylation and oxidation. Liposomes formed by using acidic phosphatidyl choline and cholesterol can also lead to accumulation of neutral lipids (foam cell formation). Liposomes that do not contain negatively charged PC (acidic PC), do not induce accumulation of fats in cells (De Winther and Hofker, 2000)

4.1.6.7 Oxidation of lipoproteins by monocytes-macrophages

Steinbrecher *et al.* (1984), proposed that endothelial cells could oxidize LDL and that macrophages recognize the modified LDL. They proposed a concept of reactive oxygen species causing transformation of LDL into atherogenic entities. Although it is well known that free ferrous or cupric ions catalyse lipid peroxidation reactions *in vitro* leading to oxidation of LDL, most *in vivo* systems bind any free transitional ions and render them inactive.

Chisolm *et al.* (1999) proposed that monocytes/macrophages themselves are the most likely producers of oxidized LDL, as they are:

- the most prominent in arterial lesions,
- known to generate activation-dependent reactive oxygen species, and
- unlike smooth muscle and endothelial cells, they can induce *in vitro* oxidation of LDL in medium free of metal ions.

There are a few possible ways in which macrophages may promote extra-cellular oxidation of LDL through enzymatic and non-enzymatic systems:

- **Oxidation through the working of ceruloplasmin:** The copper-containing acute phase protein in plasma, ceruloplasmin (Cp), previously studied for its role as anti-oxidant, could act instead as a potent oxidant of LDL. Cp is over-expressed in macrophages and a possible role of LDL oxidation by Cp was demonstrated by Chisolm *et al.* (1999), who also showed that other cell-derived factors are needed for optimal working. Superoxide and lipo-oxygenase are two factors that may be involved to reduce the Cp-bound copper.

- **Superoxide (O_2^-):** The role of superoxide in LDL oxidation has been debated for many years. Chisolm *et al.* (1999) stated that superoxide and the dismutation product hydrogen peroxide was essential for LDL oxidation *in vitro,* but, the macrophages must be activated by either opsonized zymosan, or LPS. Recent data suggests that the enzymatic source of the superoxide is from NADPH oxidase (Chisolm *et al.*, 1999).
- **The role of 15-lipo-oxygenase (LO):** These non-heme iron-containing enzymes are found in various reticulocytes, macrophages and some endothelial cells. They catalyse the direct insertion of molecular oxygen into polyenoic fatty acids, which leads to hydrogenperoxide formation. LO oxidises cellular fatty acids, cholesterol or phospholipid substrates and the hydroperoxide products could transfer to LDL making it more susceptible for oxidation. Peritoneal macrophages from animals without LO, showed impaired LDL oxidation. Sparrow and Olszewski (1992) demonstrated that LDL incubated with LO and phospholipase A_2, lead to oxLDL in a cell free system. Moreover, LO inhibitors are able to inhibit this cell mediated oxidation process.
- **Myeloperoxidase:** This abundant heme protein is released by activated neutrophils and monocyte/macrophages, especially those found in vascular lesions. MPO can amplify the oxidizing potential of H_2O_2, the dismutation product of superoxide, by using it as a co-substrate to generate oxidants as radical species, reactive halogens, aldehydes, and nitrating agents (Chisolm *et al.*, 1999). The heme group of MPO is buried in a deep hydrophobic pocket and catalyses the oxidation of small molecules that easily diffuse and damage cellular targets. MPO catalyse the oxidation of chloride and forms the powerful oxidant hypochlorous acid (HOCl), which modifies LDL into a high-uptake form for macrophages. Hypochlorous acid oxidizes α-amino acids turning them into aldehydes. MPO generated aldehydes can modify nucleophilic targets on LDL protein and lipids. Generated aldehydes can also induce the tyrosyl radical and initiate LDL lipid peroxidation and dityrosine cross-linking of proteins. Another potential MPO-dependent pathway that

monocytes/macrophages may employ for LDL oxidation, involves formation of nitrogen species forming a reactive intermediate capable of nitrating aromatic compounds. This can lead to lipid peroxidation and protein nitration. LDL, modified by MPO-generated nitrate intermediates, are rendered ligands for high affinity binding and uptake by macrophages.

Thus, there are multiple pathways in which macrophages can promote oxLDL. In vascular lesions LO, Cp and MPO are abundant and all may play a role in foam cell formation.

The presence of activated macrophages is a general feature of inflammation. Macrophage-derived foam cells are not as common, but are found in more diseases than only atherosclerosis, namely:

- Lepromatous leprosy (Mochizuki *et al.*, 1996)
- *Chlamydia pneumonia* infection: Although infection is associated by atherosclerotic heart and vessel disease, a causal relationship had not been established until it was shown that foam cells can be formed by infection with this pathogen. Moreover, it seems that chlamydial lipopolysaccharide might be the factor inducing foam cell formation in macrophages (Kalayoglu and Byrne, 1998).
- Repeated platelet transfusions (Ishihara *et al.*, 1986).
- Acquired immunity deficiency syndrome cholangiopathy with *Encephalitozoon intestinalis* (Liberman and Yen, 1997)

4.1.6.8 CD1, foam cells and atherosclerosis

Atherosclerotic plaques are chronic inflammatory lesions composed of dysfunctional endothelium, smooth muscle cells and lipid-laden macrophages. CD1 presenting molecules are not seen in normal arterial cells, but are expressed in $CD68^+$ lipid-laden foam cells. Foam cells generated by either oxidized or acetylated LDL, were capable of inducing antigen-dependent proliferation of a mycolic acid-specific CD1b-restricted T cell line (Melian *et al.*, 1999).

4.1.6.9 Foam cells and IFNγ

Interferon gamma is present in atherosclerotic lesions, and most likely contributes to the progression of the lesion by classical activation of the macrophages. The role of IFNγ in foam cell development appears to be the alteration of the pathway of intracellular cholesterol trafficking. Addition of IFNγ leads to a 2-fold increase in ACAT1, leading to an increase in intracellular cholesterol and cholesterol-esters (Panousis and Zuckerman, 2000).

4.1.6.10 Surface phenotype of foam cells

In Table 4.3, the phenotype of human foam cells in atherosclerotic plaques and *in vitro* cultured monocyte/macrophage derived foam cells, is summarized.

Table 4.3 Phenotypes of foam cells. Definite staining: +, intense staining: ++, no staining and not present. Taken from Melian *et al.*, 1999.

		Foam cells induced by :		
	Macrophages	**AcLDL**	**oxLDL**	**Atherosclerotic**
CD1a	-	++	+	++
CD1b	-	++	+	++
CD1c	-	++	+	++
CD1d	+	+	+	++
MHCII	+	++	+	++
CD11b	++	++	++	++
CD14	++	+	+	+

From the above, it is seen that CD1a-c expression is not usually present on the surface of macrophages. Once turned into foam cells CD1 a-c are expressed on these cells. In comparison, CD1d is present on macrophages and doesn't change if oxLDL or AcLDL foam cells are induced, but in atherosclerotic foam cells CD1d is highly expressed (Melian *et al.*, 1999).

4.1.7 Hypothesis

This study addressed the question whether mycolic acids (MAs) induce foam cell formation in mouse peritoneal macrophages.

The experimental evidence to test this hypothesis was obtained by labelling MAs fluorescent and using them as a tool to show that MAs on carrier systems as discussed in Chapter 3, are phagocytosed by macrophages. Macrophage phagosomes containing MAs are shown to be processed to late stage phagosomes. Finally it is shown that MAs induce the macrophages to undergo morphological and immunological changes to resemble macrophage-derived foam cells.

4.2 Materials

4.2.1 Macrophage cultures

Macrophage cultures from C57BL/J6 (4.4) and BALB/C (2C11/12) mice were gifts from Professors Johan Grooten and Patrick de Baetselier respectively. Cultures were maintained in full medium: RPMI 1640 (Life Technologies, Paisley, U.K.), supplemented with 10% foetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100U/ml), streptomycin (100U/ml), sodium pyrovate (1 mM) and β-mercaptoethanol ($5x10^{-5}$ M). Washing medium for cells consisted of RPMI 1640 medium without any additives.

4.2.2 Animals

Female C57BL/J6 (H-2^b) and BALB/C (H-2^d) were purchased from the Broekman Institute (Eindhoven, The Netherlands). Mice were 9-14 weeks old at start of experiments.

4.2.3 5-Bromomethyl fluorescein (5-BMF) derivatisation of mycolic acids

5-Bromofluorescein (5-BMF) was purchased from Molecular Probes (The Netherlands). Potassium carbonate solution (2% in 1:1 water: methanol) and 18-crown-6-ether were obtained from Sigma Chemical Co (St Louis, MO). Silica gel GHL plates, mobile phase 1: chloroform: methanol: water (60: 25: 4) and mobile phase 2: methanol 100%, were obtained from Merck (Germany).

4.2.4 Antibodies

Purified monoclonal rat anti-mouse CD16/CD32 (anti-Fc receptor antibody), B7-1, -CD40, -MHC class II, -CD1d, -ICAM1, -CD11b, and -CD23. were obtained from Pharmingen (San Diego, USA).

4.2.5. Lysis buffer

Red blood cells were lysed using ammonium chloride lysing buffer (8.029 g NH_4Cl, 1.0 g $KHCO_3$ and 0.0372 g EDTA / litre, pH 7,4).

4.2.6 DNA stain for flow cytometric determination of DNA synthesis

Each 1ml solution contained: 250µl propidium iodide (PI) Sigma Chemicals Co St Louis, MO), 5 mg RNAse (EC 3.1.27.5; Sigma) and 1% TritonX100 in distilled water.

4.2.7 Carrier systems

Liposomes, liposomes-MA beads and beads-MA were prepared as described in chapter 3.4.

4.2.8 LPS testings

Mycolic acids, lecithin, cholesterol and PBS used in liposome productions, were individually tested for the presence of LPS, using the kinetic-QCL limulus amebocyte lysate test kit. Kinetic-QCL limulus amebocyte lysate test kit was obtained from Sigma, St Louis, MO.

4.2.9 LysoTracker

LysoTracker was purchased from Molecular Probes (The Netherlands).

4.2.10 Microscopy

Glutaraldehyde (25%) and osmium-tetraoxide were purchased from Poloron, Biorad, UK. Quetol embedding resin was prepared by adding quetol-3.9g, nadic methyl anhydrate-4,6g, dodecyl succinic anhydrate-1.6g, araldite Cy212 resin-

0,2g and di-ethyl amino ethanol-0.1g together. All reagents were purchased from Sigma, St Louis, MO. Haematoxillin- , Giemsa-, Eosin- as well as Oil red O stain were purchased from Sigma, St Louis, MO.

4.2.11 Lyophilised *Mycobacterium tuberculosis*

Mycobacterium tuberculosis H37Rv ATCC 27294 strain bacteria was obtained from American Type culture Collection, Maryland, USA.

4.2.12 Reagents for cholesterol determination

Butylated hydroxytoluene, cholesterol and cholesterol esters (linoleate, arachidonate, oleate heptadecanoate) were purchased from from Sigma, St Louis, MO. Methanol, hexane, acetonitrile and isopropanol were analytical grade and obtained from Merck.

4.3 Methods

4.3.1 Bromomethyl fluorescein (5-BMF) derivatisation of mycolic acids

Freshly saponified MA (2mg) in chloroform was dried by heat (80°C, heating block) and nitrogen gas, in a sterile new amber glass vial. A quantity of 100 µl potassium carbonate solution (2% in 1:1 water: methanol) was added and the mixture evaporated to dryness at 100 °C, using nitrogen gas. The vial was cooled down and 1 mg 5–BMF dissolved in 200 µl DMF and 2.4 mg 18-crown-6 ether dissolved in 500 µl chloroform, was added. The vial was sealed using a Teflon-lined cap and heated at 90 °C for one hour. After the vial was cooled down, chloroform (1ml) was added, and it was left for 1 hour at room temperature. The product was washed 5 times with chloroform saturated hydrochloric acid water (1:1), and 5 times with chloroform saturated water. A sample of the MA–5-BMF product was spotted onto a silica gel GHL thin layer plate together with 5-BMF alone and MA. Mobile phase 1 and 2 were used in succession in two dimensions. Iodine vapor was used to detect the MA sample.

In a clear vial, DMF was added to an equal amount of 5-BMF-MA sample in chloroform, the vial vortexed and the two layers allowed to separate.

In another clear vial, 10 volumes of cold acetone were added to the 5-BMF-MA product and left for 1 hour to precipitate the labelled and unlabelled MA.

4.3.2 Collection of peritoneal exudate macrophage cells

Mice were sacrificed using CO_2. Into the peritoneal cavity, 8 ml sterile sucrose (10 g/100 ml water) at 4°C were introduced, using a 25-gauge needle. After 5 minutes, the sucrose was drained using a 17-gauge needle, by piercing an area caudal from the liver, taking care to exclude contamination with blood. The peritoneal exudate cells (PECs) thus obtained were washed in full macrophages culture medium after centrifugation at 200xg for 10 minutes.

4.3.3 Collection of alveolar macrophages

Mice were sacrificed with an injection overdose of sodium penta-phenobarbitone. The anterior neck area was dissected to reach the trachea. A 21-gauge jelco™ was placed in the trachea and the teflon canula secured with string. A lung lavage was done, using 1ml RPMI 1640 medium. Cells were counted and put into culture flasks using macrophage full culture medium.

4.3.4 Collection of cells from the spleen

Mice were sacrificed by cervical dislocation. The spleen was removed in a sterile procedure, and put in full RPMI 1640 medium. The plunger of a 10ml syringe was used to release the cells from the capsule of the spleen. Erythrocytes were removed by addition of erythrocyte lysis buffer. Cells were washed twice in full medium and the number of macrophages counted.

4.3.5 Collection of blood samples and preparation of mouse serum

Mice were bled from the tail vein and the blood collected into Eppendorf tubes. The collected blood was left at 4°C overnight for the clot to retract. The serum was recovered by centrifugation (Beckman J-6 centrifuge, at 1000 g for 15 min), filter sterilised (0.22µm) and stored at –20°C. Before use, serum was heated at 56°C for 1 hour to inactivate complement.

4.3.6 Preparation of mycolic acid-mouse serum conjugates

A volume of dissolved mycolic acids in chloroform was combined with a volume of mouse serum, such that the MA-chloroform solution constituted 2% of the volume of mouse serum. Mycolic acids, (2,5 mg), dissolved in 200 µl chloroform, were added to 10,0 ml of mouse serum, previously filtered through a 0,22 µm filter.

The mixture was sonicated using a Branson Sonifier B 30 Cell Disruptor at 20% duty cycle, at optimal energy output, for 50 pulses of 2 seconds each, at room temperature over 5 minutes. Air bubbles formed during sonication were allowed to escape at room temperature for 1 hour. In order to remove chloroform, nitrogen was bubbled through the conjugate until the chloroform odour was removed. The conjugate was prepared immediately before administration to the experimental animals or cultures. Control serum without MA was prepared similarly.

4.3.7 MA uptake and LysoTracker co-localization studies in macrophages

Mycolic acids were fluorescently labelled as described before, and incorporated with the following carriers:

Serum: Fluorescently labelled mycolic acids (250 µg) were solubilized in 1 ml serum as described before (4.3.6). *Ex vivo* loading of macrophages was done by adding 50 µl of labelled mycolic acids in a 6 well plate containing 5 ml full medium and $2x10^6$ PECs per well and incubating for 2 hours at 37°C in a 5% CO_2 incubator. *In vivo* loading of macrophages with serum and serum-MA was done by injecting 100 µl of serum-MA or serum solution into the peritoneal cavity. After 2 hours, the PECs were removed as described (4.3.2) and cells put into culture.

Beads: Fluorescently labelled mycolic acids were melted onto the beads as described before (3.5.5). Macrophages, either as the cell line culture or from the peritoneum, were cultured in 6-well plates at $2x10^6$ cells per well. For *ex vivo* loading, $1x10^7$ beads or beads-MA were added to each well to a total volume of 5 ml. *In vivo* loading of beads was done by injecting $1x10^7$ beads, or beads-MA

suspended in 100 µl PBS, into the peritoneal cavity. After 2 hours, the PECs were removed as described (4.3.2) and cells put into culture.

Micelles: Fluorescently labelled mycolic acids (250 µg) micelles were made in 1 ml 10% (v/v) Solutol/PBS as described before (3.4.6). *Ex vivo* loading of macrophages was done by adding 50 µl of labelled mycolic acid micelles to a 6-well plate containing 5 ml full medium and $2x10^6$ (C57BL/J6) PECs. Incubation was done for 2 hours in a 5% CO_2 incubator at 37°C. *In vivo* loading of Solutol-MA micelles was done by injecting 100 µl micelle suspension into the peritoneal cavity. After 2 hours, the PECs were removed as described (4.3.2) and cells put into culture.

Liposomes: Fluorescently labelled mycolic acid (250 µg) liposomes were made in 1 ml PBS as described before (4.3.1). *In vivo* loading was done by injecting 100 µl of labelled mycolic acid liposomes i.p. into a mouse. After 2 hours, the PECs were removed as described (4.3.2), and cells put into culture.

To the above cultures, the acidotropic dye LysoTracker Red DND-99 was added to culture medium to a final dilution of 1:10 000 for co-localization experiments. Cells were incubated for 3-5 hours before examination under the confocal microscope (LSM 410 invert; Zeiss, Germany).

Labelled mycolic acids were either used on serum, beads, micelles or liposomes to evaluate uptake into macrophages by confocal microscopy and flow cytometry (FACS). Solutol micelles and liposomes were loaded by peritoneal injection *in vivo* only, whereas beads and serum were loaded *ex vivo* as well.

4.3.8 Phagocytosis of liposomes by PECs

Liposomes containing fluorescently labelled MA were injected i.p. into mice and allowed to be taken up for 2 hours. After removal of the PECs using 10% sucrose, the cells were washed three times using full medium. Fc receptors were blocked for 30 minutes using 5% rat and 5% rabbit sera in RPMI 1640 medium. Surface labelling was done using 2 µl anti-F4/80-PE antibody in 100 µl containing $2x10^5$ cells at 4°C for 30 minutes.

4.3.9 Morphological changes of macrophages loaded with MA

Macrophages were loaded *in vivo* by injecting 100µl of either of the following samples i.p. into mice:

- PBS
- Liposomes
- Liposome-mycolic acids

All samples were diluted or prepared in LPS-free PBS. The PECs subsequently obtained were either used for culture and light microscopy (4.3.11), or fixed and prepared for transmission electron microscopy (4.3.12).

4.3.10 Culturing and preparation for light microscopy

Culturing of macrophages was done in 24 well plates in which 13mm sterile glass slides were put at the bottom. To each well $1x10^5$ cells and 1ml full medium were added. After the appropriate time, the medium was removed and the cells were fixed with 1% paraformaldehyde for 15 minutes. The wells were washed in 2 ml PBS and the glass slides were removed. The above samples were stained using hematoxilin (20 seconds) and counter-stained with eosin (20 seconds).

4.3.11 Preparation for electron microscopy

PECs were fixed using 2.5% glutaraldehyde for 20 minutes followed by osmium fixation for 10 min. The cells were then dehydrated in a graded series of alcohol and embedded in Quetol. Thin sections were cut on a Reichter Jung Ultracut E microtome. Thin sections were examined in a Phillips EM301 transmission electron microscope.

4.3.12 MA dose dependency to activate macrophages

The effect of MA concentration, *in vivo* loading time and *in vitro* culturing time on the development of activated macrophages were evaluated. MA was solubilized in liposomes at concentrations ranging from 0 to 250 µg /ml in PBS. The PECs were loaded for 24 h *in vivo* by injecting 100 µl samples i.p. into mice. After

isolation, the cells were put into culture using RPMI 1640 medium and 10% FCS. The development of activated cells in the adherent fraction of PECs was evaluated over 72 hours using light microscopy. Activated cells were increased in size and showed typical vacuoles.

4.3.13 Time dependency of macrophage activation by MA

MA was solubilized in liposomes at a concentration of 250 μg in PBS. PECs were loaded for 3, 24 and 48 hours *in vivo* by injecting 100 μl samples i.p. into mice. After isolation, PECs were put into culture using RPMI 1640 medium and 10% FCS. The development of activated cells in the adherent fraction of PECs was evaluated over 72 hours using light microscopy.

4.3.14 Comparison between activation of macrophages using MA, or dead *Mycobacterium tuberculosis*

Macrophages were loaded with 250 μg MA in liposomes, or 2.5 $X10^7$ desiccated *Mycobacterium tuberculosis* in 100 μl PBS for 48 hours before removal and culturing of PECs for 24 and 48 hours. Macrophages containing dead bacteria were stained using Giemsa stain to evaluate the formation of multinucleated cells.

4.3.15 Neutral lipid staining of macrophages loaded with MA

Peritoneal mouse macrophages were loaded with either

- Beads
- Beads-MA
- Liposomes
- Liposomes-MA
- Desiccated *Mycobacterium tuberculosis* H37 RA

in vivo for 48 hours. PECs were obtained and put into 24 well culture plates on glass slides. The slides were fixed as before (4.3.10), and stained for 30 min with 0.5% (w/v) Oil Red O in propane-1,2-diol. Stained cells were then washed three

times with 0.5% (v/v) water/propane-1,2-diol and tree times with water alone. Glass slides were then evaluated under a light microscope.

4.3.16 Cholesterol determination in MA-treated PECs

Mice were loaded intraperitoneally for 48 hours with 100 µl of either

- Liposomes
- Liposomes-MA

From the above mice, as well as from a native group of mice, PECs were isolated as in 4.3.2. Cells were washed three times with PBS containing 20µM butylated hydroxytoluene per ml and diluted to $2x10^6$ with the same. Extraction of lipids was done by vortexing the cells using methanol (2ml) and hexane (5ml) at 4°C. Cholesterol heptadecanoate was added to all the samples before extraction as an internal standard.

A part of the hexane layer (1ml) was evaporated and diluted to 0.5 ml in the eluant buffer. Cholesterol and cholesterol esters (linoleate, arachidonate, oleate) were separated on a Vidac C18 reverse phase high performance liquid chromatography (HPLC) column at room temperature and monitored with a 210nm UV-detector. The eluant consisted of acetonitrile-isopropanol 30:70 (v/v).

4.3.17 Macrophage proliferation after MA loading

Macrophages were loaded *in vivo* with 100 µl liposomes and liposomes-MA (25 µg) as before (4.3.7). After 48 hours, PECs were isolated, washed three times and put into culture in 96 well plates ($1x10^5$ cells in 200 µl full medium) and also in 24 well plates ($5x10^5$ cells in 1 ml full medium).

4.3.17.1 Macrophage proliferation measured by thymidine incorporation

PECs samples were divided in two. One half was washed after 3 hours incubation at 37°C in full medium, to remove all non-adhered cells. The other half was used without washing. DNA synthesis was measured by incorporation of [methyl-^{3}H] thymidine (TdR) (2 µCi/ml) over 24 hours. Uptake was stopped by

freezing the plate at –20°C. After thawing, thymidine incorporation was measured using an Inotech cell harvester and a digital autoradiographic counter Inotech AG Cell Harvester system (Switzerland).

4.3.17.2 Macrophage proliferation measured by propidium iodide staining and FACS

All non-adhered cells were washed away after 3 hours of incubation at 37°C using full medium. To each well 200 µl DNA-stain (containing propidium iodide (PI), RNAse and Triton X100) was added. After staining for 2 hours at 4°C in the dark, PI fluorescence was measured using a fluorescence activated cell sorter (FACS Calibur flow cytometer, Becton Dickinson). Cell cycle analysis was performed on a gated, singlet population by using Modfit LT cell cycle analysis software (Verity Software House, Inc). Data acquisition was restricted to 10 000 events for each sample.

4.3.18 Cell surface labelling of foam-like cells

Cell surface markers of macrophages loaded *in vivo* with unlabelled mycolic acids were evaluated using flow cytometry. Mice were injected i.p. with 100 µl beads in PBS or liposomes, each containing 25 µg MA. PECs were collected after 48 hours *in vivo* incubation and used immediately. Controls consisted of beads in PBS or liposomes, both without MA. The cell surface marker F4/80-PE was used to distinguish the macrophages from the lymphocytes by gating the population with fluorescence. This macrophage population was then further characterised with the following cell surface marker antibodies: Anti-MHC class II, -CD1d, -CD11b and -CD36. All above markers were rat anti-mouse antibodies except CD36, which was rabbit anti-mouse IgG. Labelling was according to Table 4.4.

Table 4.4 Summary of antibody markers for surface labelling of foam-like cells.

Antibodies against	Label used*	Amount of antibody per $5x10^5$ cells
MHC II	DL-FITC	1µl
CD1d	DL-PE	1µl
CD11b	DL-FITC	1µl
CD36	NL	1µl

* Antibodies were directly labelled (DL) to fluorescein (FITC) or phyco-erythrin (DL-PE) or not labelled (NL) .

All reactions were carried out on ice. Blocking was done using 5% rat and 5% rabbit sera as applicable for 1 hour before specific antibodies were added. Incubations of primary antibodies were done for 30 min at 4°C. Streptavidin was diluted 1:100 in blocking buffer for 30 min. CD36 was blocked in 5% rat serum alone and anti-rabbit FITC labelled antibody (1:200) was used to detect anti-CD36 binding. Readings were done on a Becton Dickenson flow cytometer and 5000 gated events were counted.

4.3.19 CD1d expression on alveolar macrophages after MA immunization

MA carried on liposomes or serum was used to immunize C57Bl/J6 mice. The immunization protocol consisted of four intravenous liposome+/-MA (100µl/25µg) or serum+/-MA (100µl/25µg) injections, 10 days apart, over 40 days. Alveolar macrophages were obtained as described (4.3.3), and diluted to $1x10^6$ cells/ml. Blocking was done using 5% rat and 5% rabbit sera in wash medium over 1 hour. The anti-CD1d-PE (0.1µg/ml) antibody was diluted to 2µl per 100 µl blocking solution containing $5x10^5$ cells. Incubation was done on ice for 30 min and washed three times between each step. Flow cytometry was done on a Becton Dickenson flow cytometer and 5000 alveolar cells were counted.

4.4 Results

4.4.1 Lipo-polysaccharide (LPS) testing of mycolic acids and carriers

To ensure that mycolic acids and carriers were free from LPS contamination that could adversely influence the results, the LPS detection kit was applied to test the reagents.

No LPS could be detected in any of the following samples tested:

- PBS
- Phospatidyl choline
- Cholesterol
- Mycolic acids

All the samples above were done according to the protocol in the kit and in duplicate. In addition, LPS was added to all the samples to evaluate the possibility of inhibition of the assay by unknown factors in the samples. All samples tested LPS positive after spiking with LPS. Thus, all above samples are LPS free up to the detection limits of the kit and MA do not influence the assay.

4.4.2 Coupling of 5-BMF to mycolic acids

MA was labelled with 5-BMF to allow their detection during uptake in macrophages. Using thin layer chromatography, the coupling of 5-BMF to mycolic acids was evaluated (Fig 4.3). By using the optimal derivatization condition suggested by Mukherjee *et al.* (1995) for the labelling of palmitic acid with 5-BMF, the molar ratio of 1:1 for MA:5-BMF, and 5-BMF:18-crown-6-ether (0.25:1), seemed to work adequately. The excitation wavelength maximum was compatible with the 488 nm emission wavelength of the argon ion laser used in confocal microscopy (LSM 410 invert, Zeiss, Germany).

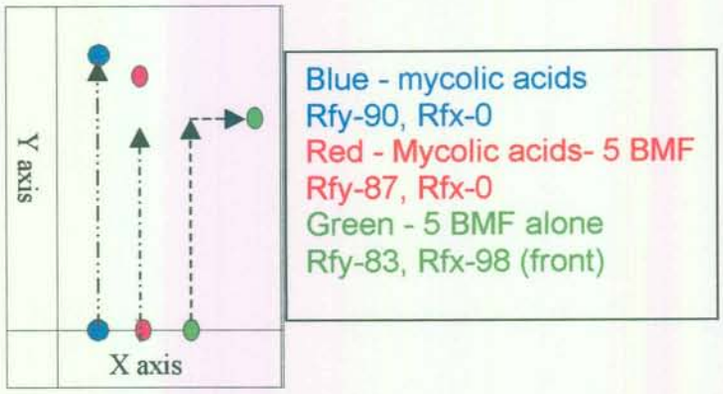


Figure 4.3. Reconstruction of the two-dimensional thin layer chromatographic system to assess the labelling of MA with 5-BMF. The samples tested were mycolic acids, mycolic acids coupled to 5-BMF, and 5-BMF alone. The Rf values for the Y and the X-axis are given. The mobile phase for the Y-axis was chloroform: methanol: water 60:25:4 and the X-axis methanol 100%. Iodine vapor was used to identify the mycolic acids. 5-BMF is bright yellow and could be seen by eye even when coupled to mycolic acids.

Mycolic acids only moved in the Y axis direction when chloroform:methanol:water 60:25:4 was used. No movement was seen in the X axis when methanol alone was the eluant. The 5-BMF alone moved in both the X and the Y axis. In comparison, the fraction that contained the MA coupled to the 5-BMF, only moved in the Yaxis. The results imply that coupling of 5-BMF to MA proceeded quantitatively.

This 5-BMF labelled MA was then used in studies to evaluate uptake and maturation of beads, micelles and liposomes into macrophages.

4.4.3 Uptake of liposomes-MA or beads-MA by PECs: properties of the active cells and the conditions for phagocytosis

Liposomes-MA appeared to be less dense than full medium, as they tended to accumulate on the surface of the medium with time. The *ex vivo* loading of cells with liposomes-MA was therefore difficult to control. This was not a problem when serum-MA or beads-MA were used *in vitro*. In the latter case, the beads settled on the bottom of the flask, in close contact with the cells.

4.4.3.1 MA uptake and LysoTracker co-localization studies in macrophages

The 5-BMF-MA was taken up into macrophages, irrespective of whether the carriers were beads, micelles or liposomes. By using LysoTracker (red and green separately) and MA (green), it was demonstrated that MA on beads, serum or liposomes, co-localized with Lysotracker intracellularly. LysoTracker accumulates in vesicles containing Rab5, a late stage endosomal marker. Thus, the MA could be shown to be taken up inside the cell and to end up in mature endocytic vesicles inside the cell.

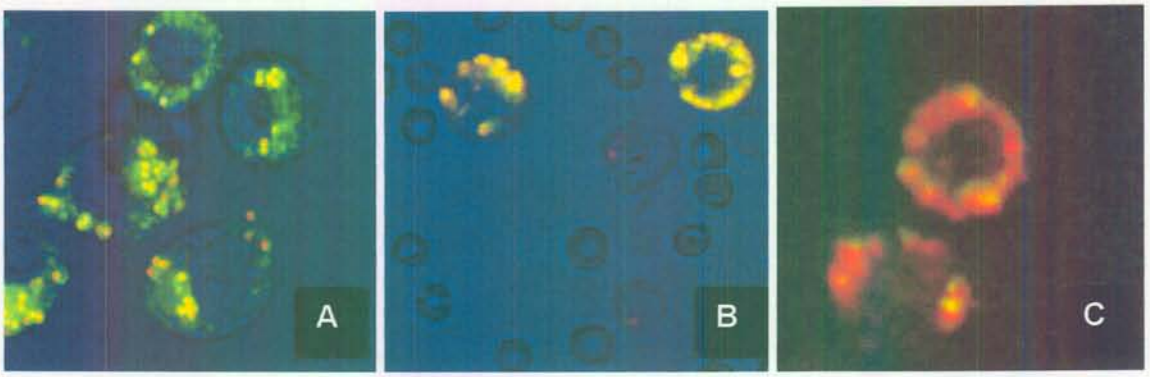


Figure 4.4 Maturation of phagosomes by co-localization with LysoTracker. In (A) LysoTracker green was co-incubated with unlabelled MA coated on 1μm red fluorescent beads. (B) LysoTracker red co-incubated with fluorescent green labelled MA solubilized in serum. (C) LysoTracker red co-incubated with fluorescent green labelled MA solubilized in liposomes. If the red and green co-localize it gives a yellow color.

From Figure 4.4 it is seen that the maturation of the phagocytosed MA particles are not dependent on the carrier that was used. All the MA samples co-localized with the LysoTracker indicating maturation to late stage phagosomes. In Figure 4.4 (A) often more than one bead is present per phagosome demonstrating the beads sticking to each other when covered with MA.

4.4.3.2 Phagocytosis of liposomes by macrophages

To determine whether peritoneal macrophages took up liposomes-MA, and to characterize the active cells, PECs were obtained from mice that were administered fluorescein-labelled liposomes-MA 72 hours before sacrifice. The macrophages were labelled with the F4/80 macrophage marker. These cells

were then subjected to FACS, where the larger activated and granulomatous cells were gated (gate 1) and compared to the smaller non-granulomatous, inactive cell population (gate 2). (see Fig 4.5).

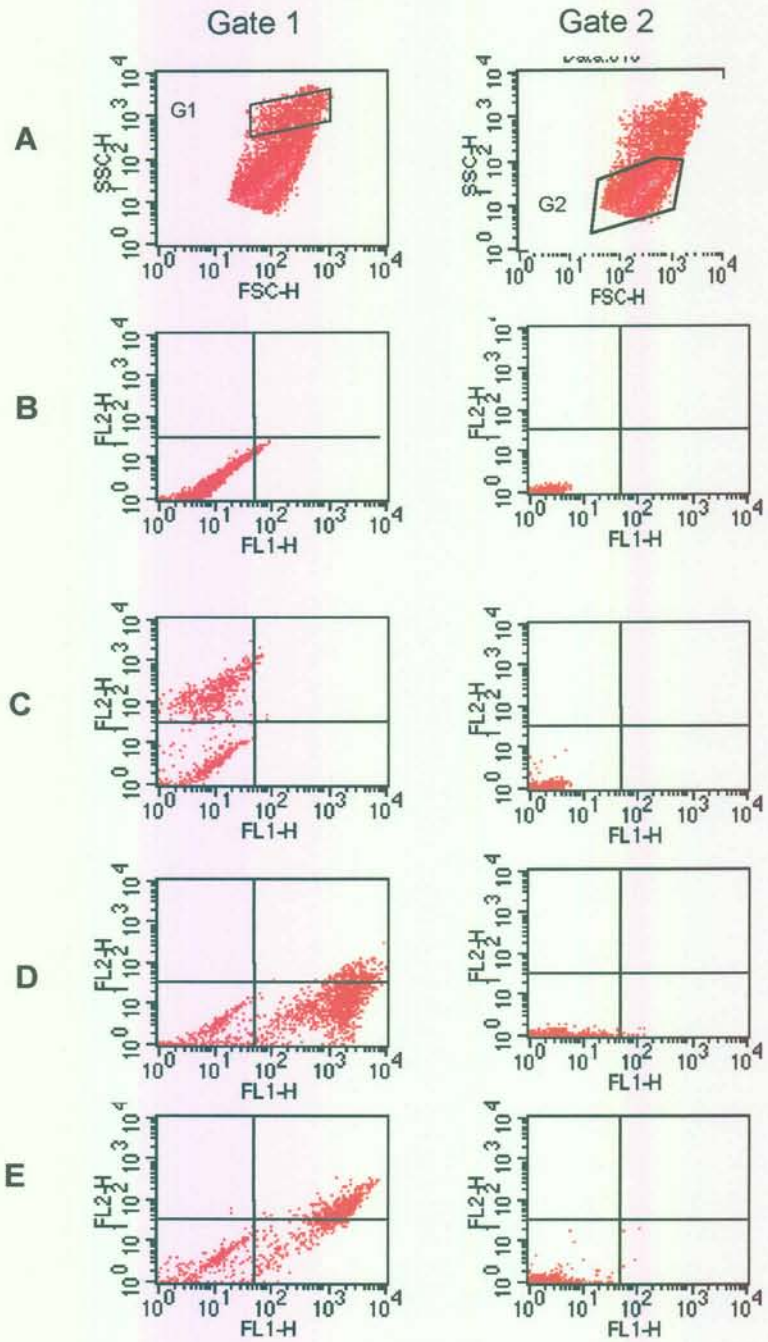


Figure 4.5 FACS data showing uptake of 5-BMF in peritoneal macrophages. (A) Gate selection (B) Unlabelled cells in gate 1and gate 2, (C) Cells in gate 1 and 2 labelled with F4/80 (FL2), (D) Cells in gate 1 and 2 with phagocytosed 5-BMF labelled MA (FL1), (E) Cells in gate 1 and 2 labelled with F4/80 (FL2) and phagocytosed 5-BMF labelled MA (FL1).

Gate 1 selected the cells that were labelled by F4/80-PE, while gate 2 excluded all F4/80 labelled cells. It was seen that gate 2, selecting the smaller cells that didn't label with F4/80, also didn't take up any liposomes-MA. Only the cells that were labelled with the F4/80 (Gate 1), bound and took up the labelled MA, thereby confirming that macrophages efficiently took up liposomes-MA. Cells selected by gate 1 exhibited auto-fluorescence. It is known that macrophages auto-fluoresce. This implies that 5-BMF-mycolic acids are taken up selectively by specific mechanisms attributable to cells that are characterized as professional phagocytic cells.

4.4.3.3 Morphological changes of macrophages following MA uptake

Beads or liposomes with or without MA, demonstrated a spectrum of cell loading from nothing, to huge amounts per cell (Figure 4.6 A and B).

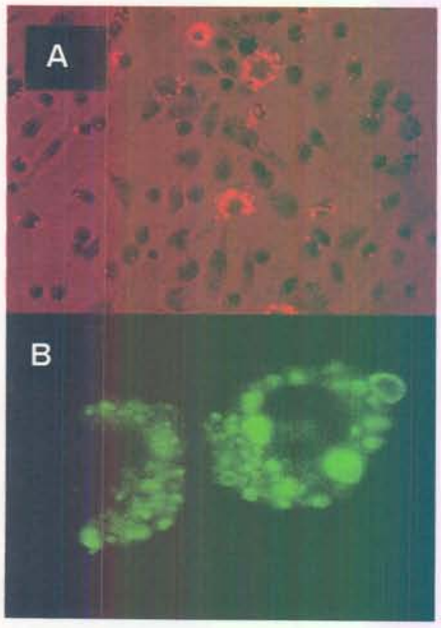


Figure 4.6. Demonstration of (A) bead-MA overloading and (B) liposome-MA.

This may have been due to the variable activation status of the macrophages or existence of subpopulations of macrophage types, differing in phagocytic capability. Whether overloading could influence the normal cell mechanism and antigen presentation capability was a concern. In subsequent experiments, the number of beads and amount of liposomes were reduced without altering the amount of MA introduced per animal. In the case of liposomes, cholesterol was

excluded, such that lecithin alone was used as base for the liposomes, while the amount of MA was doubled. The final lecithin liposome product was diluted 1:2 with PBS to maintain the dose of MA at 25μg MA/100μl. In the case of beads, the same amount of MA was introduced onto half the number of beads.

Cells that received MA in liposomes or on beads, developed into large cells (up to 5 times larger than cells receiving empty liposomes or beads) with vacuoles of various sizes, as shown in Figure 4.7.

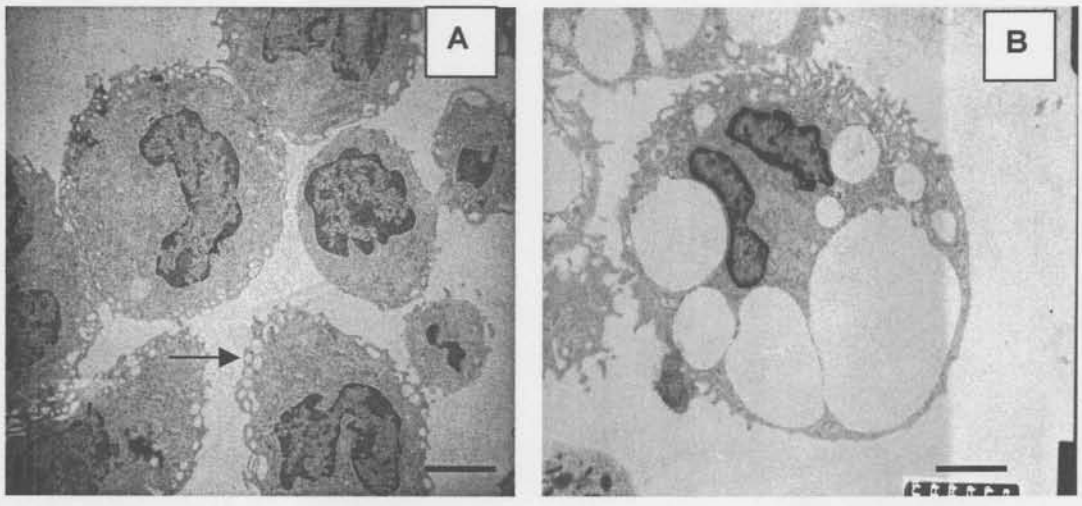


Figure 4.7. Electron microscope images of (A) liposome - and (B) liposome-MA treated mouse peritoneal macrophages. The liposome treated cells contained small vacuoles near the surface of the macrophages (see arrow). In comparison the MA treated cells have large vacuoles.

When the beads-MA were injected, it was noticed that cells that do not contain beads, also developed into activated macrophages (Figure 4.8). As the beads were unbleachable and cells appeared not to secrete the beads after loading, it seems likely that a soluble factor present in the medium signals macrophages to the activation state.

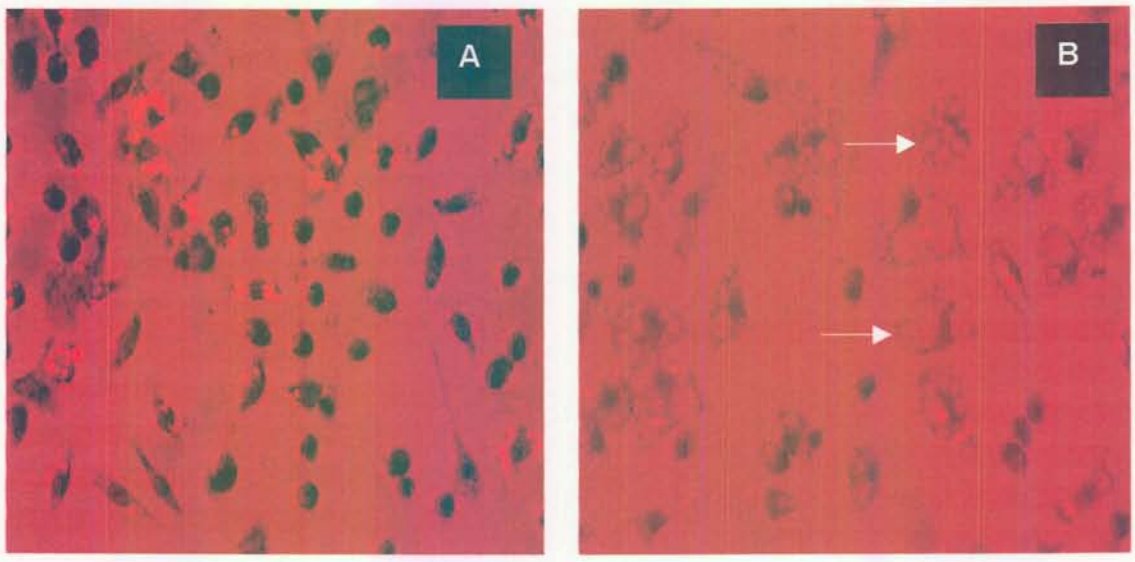


Figure 4.8. Uptake of (A) beads and (B) beads-MA in peritoneal macrophages evaluated by fluorescence microscopy. In (B) the formation of vacuolated cells are seen even when they don't contain any beads (see white arrows).

4.4.3.4 Dose dependency of liposomes-MA to activate macrophages

The dose dependency of liposomes-MA to activate adherent cells was determined after 3 hours, 24 hours and 7 days after injection into mice. In Table 4.6 the results are shown of percentage adherent cells found in correlation to the amount of MA (in liposomes) after 24 hours of *in vivo* loading. The time of *in vitro* culture (24, 48 and 72 hours) was assessed to determine the optimal time for evaluation of adherence.

Table 4.5. MA-dose dependency of the percentage activated adherent cells from MA treated mice after 24 hours of *in vivo* loading.

Amount of MA injected	Percentage activated adherent cells over time in cell culture		
	24 hours	**48 hours**	**72 hours**
25 µg	40-50	60-70	60-70
6.2 µg	5-10	30-40	50-60
1.5 µg	0	5-10	10-20
0.35 µg	0	0	0
0 µg	0	0	0

The highest concentration of activated cells were seen in the 25 μg concentration, going down to 0% with 0,35 μg MA when the PECs were evaluated after 72 hours of culturing. The number of activated cells increased over 1.5, 6.2 and 25 μg MA, after 24 hours of *in vivo* loading. After 7 days of *in vivo* loading, no activated cells could be found in the PECs, while after 3 hours of *in vivo* loading, not more than 5% of the cells were activated. Thus, the amount of activated macrophages was dependent on MA-concentration, as well as time of *in vivo* exposure. The optimum time for *in vivo* incubation was found to be 72 hours.

4.4.3.5 Comparison between activation of macrophages using liposomes-MA and dead *Mycobacterium tuberculosis*

A comparison was made between PECs exposed to MA, or dead Mycobacterium to evaluate the differences in the type and amount of activated macrophages after 48 hours of *in vivo* loading, and 24 or 48 hours of *in vitro* incubation. Figure 4.9 shows that PECs exposed to dead bacteria clearly had a different morphology in culture than the PECs treated with beads-MA. Cells exposed to dead bacteria were not as big and did not contain large vacuoles, but had elongated shapes with pseudopodia reaching over long distances. No multinucleated cells were observed in any of the MA or TB treated samples. Beads were chosen as the carrier, as beads are inert and only the effect of the MA is seen.

Table 4.6. Comparison of cells activated with dead Mycobacterium or liposomes-MA in treated PECs after 48 hours of *in vivo* loading and *in vitro* incubation of 24 and 48 hours.

Sample injected	Percentage activated adherent cells after 24h	Percentage activated adherent cells after 48h
PBS	0	0
M tuberculosis	10-20	20-30
Liposomes	0	0
Liposomes-MA	50-60	60-70

When the experiment was repeated with liposomes instead of beads as the carrier for MA, the same morphological changes were observed as were induced with beads-MA (results not shown). Liposome-MA activated cells were present at much higher concentrations (Table 4.7) and were not of the same shape as the cells treated with dead mycobacteria. Not much difference was seen in the development of activated cells after 24 or 48 hours of *in vivo* incubation.

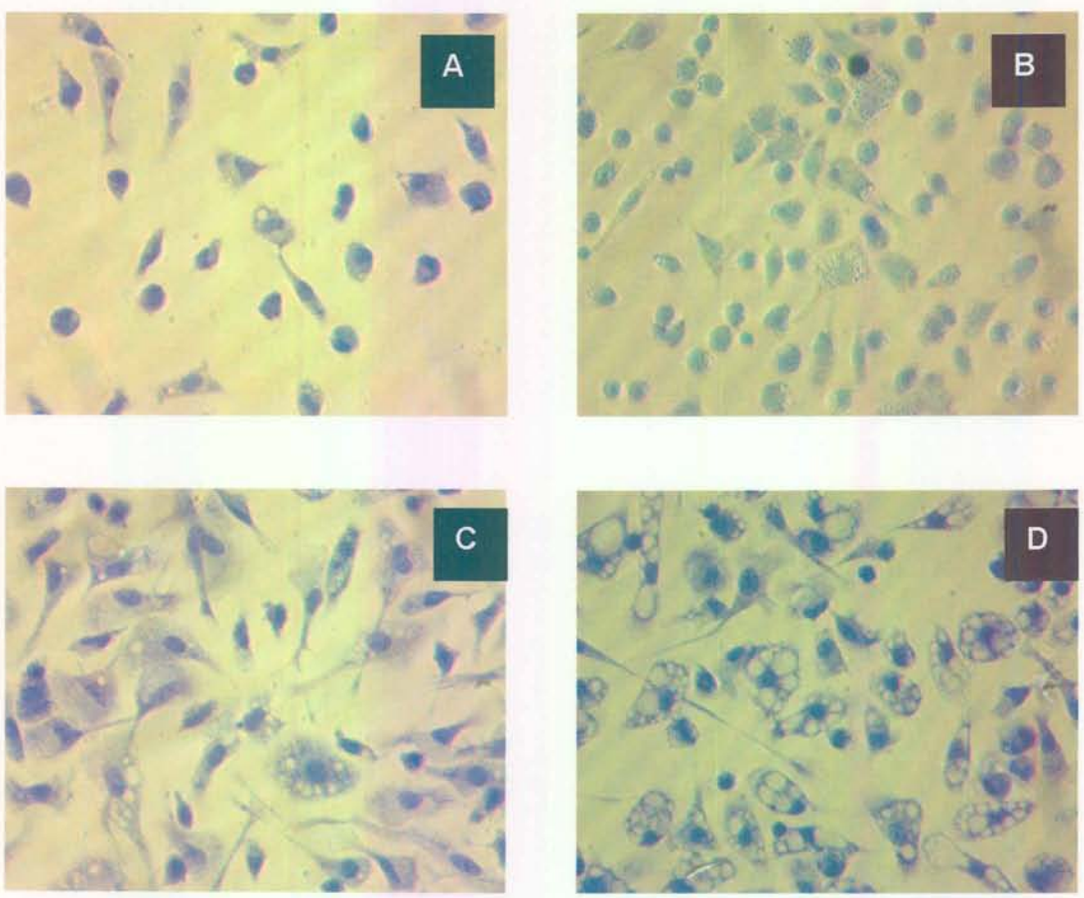


Figure 4.9. Effect of *in vivo* loading of beads, beads-MA and dead *Mycobacterium tuberculosis* on the morphology of mouse peritoneal macrophages. (A) Native macrophages (B) macrophages exposed to beads (C) Macrophages exposed to dead *Mycobacterium tuberculosis* (D) macrophages exposed to beads-MA.

4.4.3.6 Neutral lipid content of macrophages loaded with liposomes-MA

Neutral lipid staining was done on macrophages to evaluate changes in lipid accumulation in MA and *Mycobacterium* treated PECs. The micrographs in

Figure 4.10, show how neutral lipid accumulation inside peritoneal macrophages were induced by 48 hours of *in vivo* exposure to MA carried on either beads or liposomes.

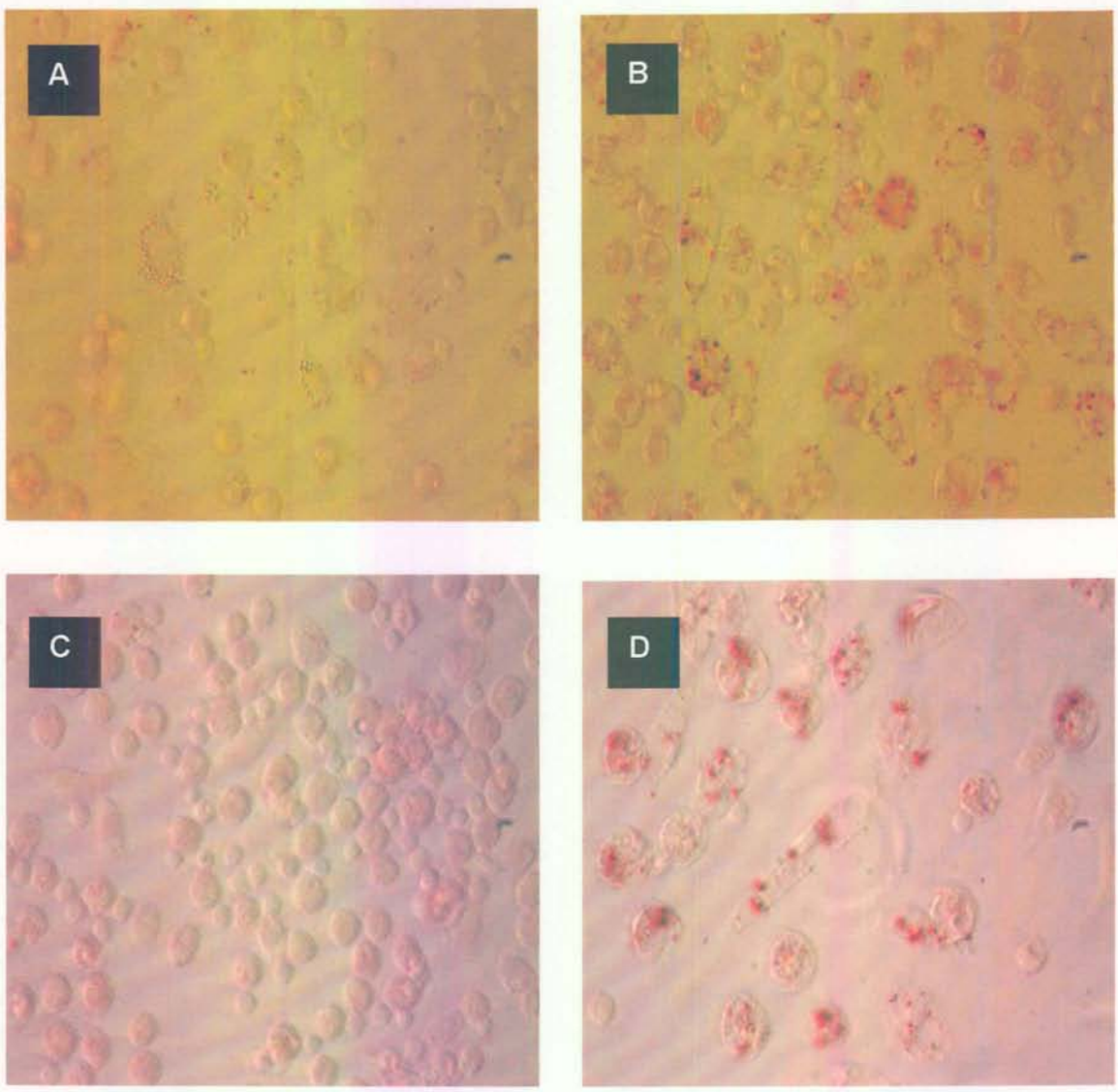


Figure 4.10. Effect of MA on accumulation of neutral lipids inside mouse peritoneal macrophages. (A) beads alone (B) beads with MA (C) liposomes alone (D) liposome containing MA. Note that the red stain is not present in the vacuoles

If dead *Mycobacterium tuberculosis* was loaded in the macrophages, an increase in oil Red O staining was seen, although not in the same amount as with the MA on beads or in liposomes (Fig 4.11).

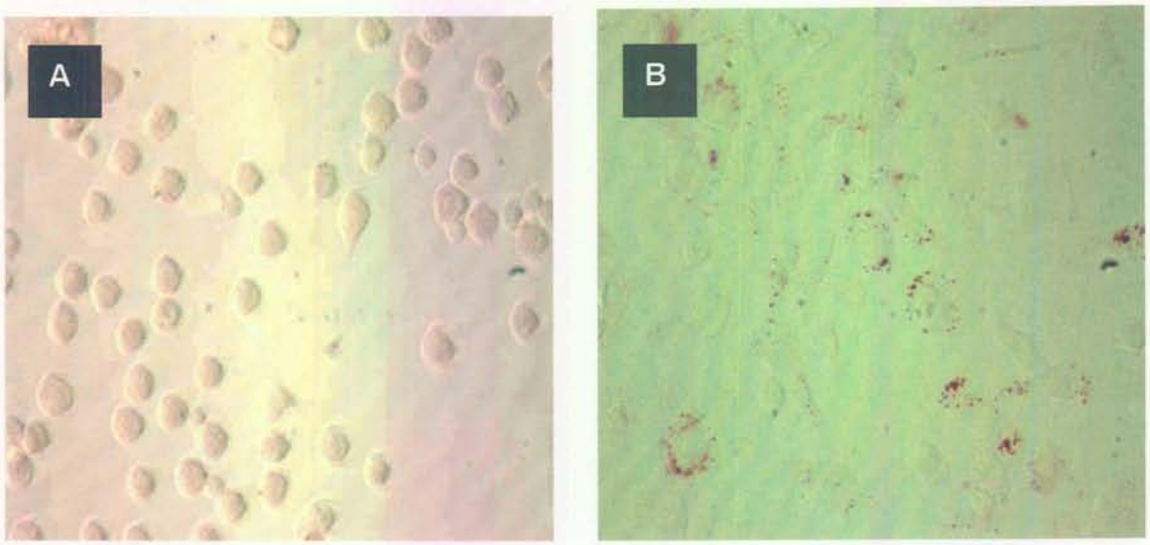


Figure 4.11. Oil Red O staining pattern of mouse peritoneal macrophages exposed to dead mycobacteria (A) Native macrophages and (B) macrophages exposed to dead *Mycobacterium tuberculosis.*

Whereas accumulation of lipid directly from the liposomes is expected to occur in the phagosomes and vacuoles, the figures show that accumulation of neutral lipid occurred in the cytoplasm. This would indicate a propensity of the activated macrophages to accumulate neutral lipids from their environment (as explained in Introduction). This is supported by the evidence shown in Fig. 4.10 (B and D), where MA taken up as MA-coated beads, also show increased cytoplasmic staining for neutral lipid.

4.4.3.7 Cholesterol accumulation in MA-loaded macrophages

MA uptake appears to increase the amount of neutral lipid accumulation in the cytoplasm of activated macrophages. To determine whether the lipids accumulated could be cholesterol, the amount of cholesterol present in the cells was assessed using HPLC. Beads-MA activated cells were avoided in this experiment, as the organic solvents of extraction and chromatography are incompatible with the polystyrene of which the beads are made.

In Figure 4.12 the elution pattern for cholesterol from activated macrophages is shown.

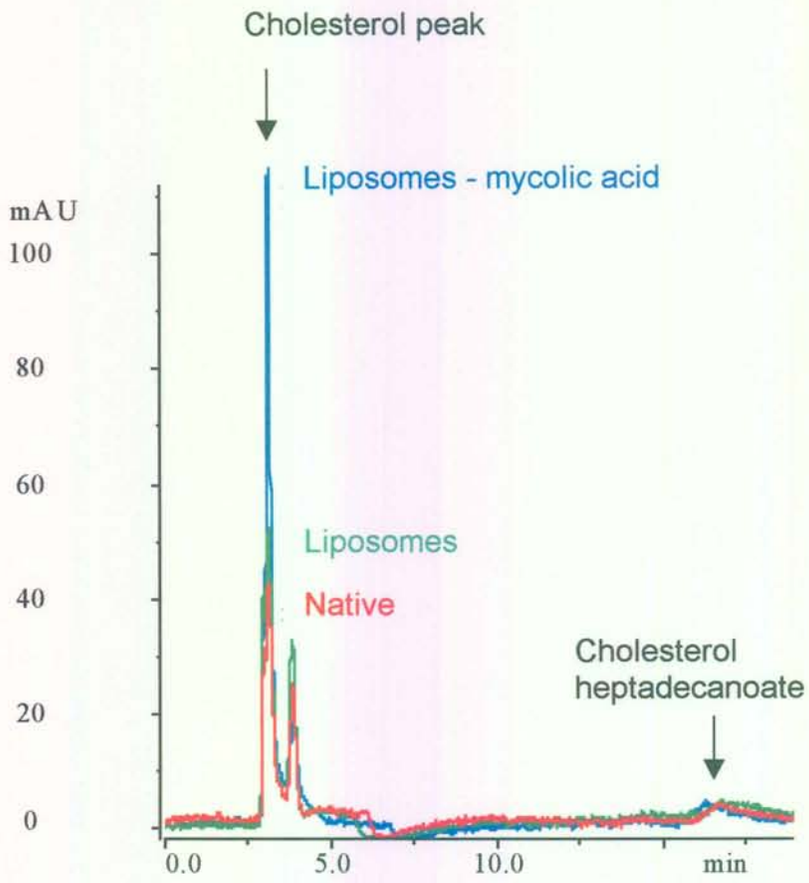


Figure 4.12. Cholesterol elution pattern obtained from neutral lipid extracts from native, liposome and liposome-MA treated macrophages. Cholesterol heptadecanoate was used as internal standard.

From the above pattern, it is clear that cells that were loaded with MA, were richer in cholesterol than cells that had either nothing, or liposomes alone.

4.4.3.8 Macrophage proliferation after loading with liposomes-MA

To determine whether activation of macrophages upon loading with MA concurred with cellular proliferation, thymidine incorporation was measured in PECs cultures derived from native mice or mice exposed for 48 hours to liposomes or liposomes-MA. Incorporation of [methyl-^{3}H] thymidine (TdR) over 24 hours in macrophages with and without MA (25μg) is plotted in Figure 4.13. The test was performed on total PECs, or the adherent cell population derived from them.

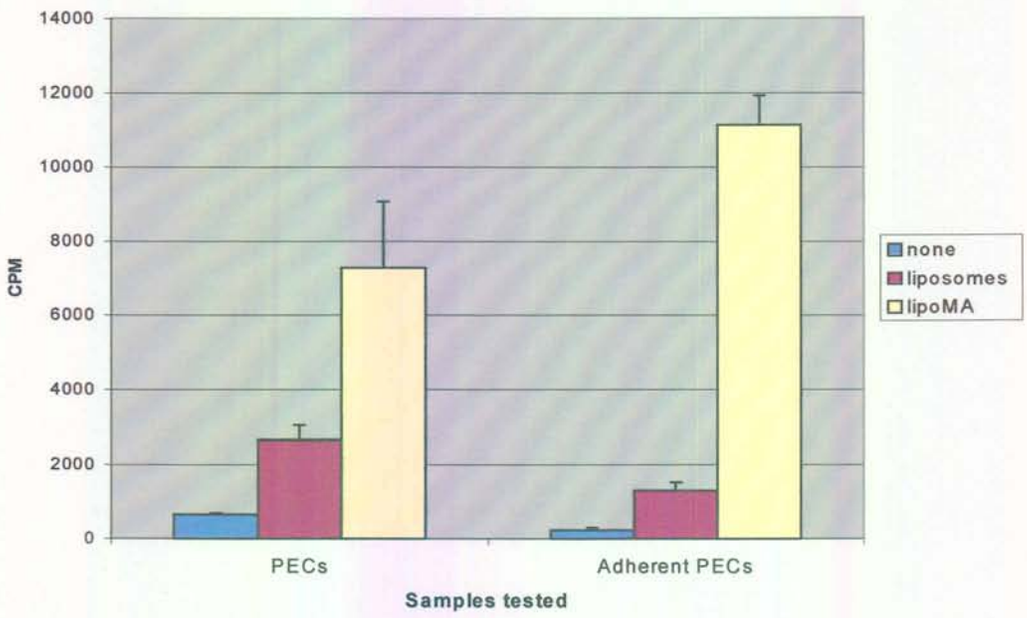


Figure 4.13. Cellular proliferation of PECs after 48 hours of *in vivo* exposure to liposomes or liposomes-MA

It is clear that cells which had received MA, incorporated almost 10 times more thymidine into the cell than native or liposome-treated cells. To ascertain that this was due to cell proliferation rather than DNA repair, the S phase of the growth cycle for the different experimental groups was investigated. Fig. 4.14 shows that MA uptake reduced the number of cells in G0/G1 phase and increased the number of cells in S phase and G2 phase, indicative of proliferation of cells. Shorter DNA fragments are also observed, probably due to enhanced apoptosis.

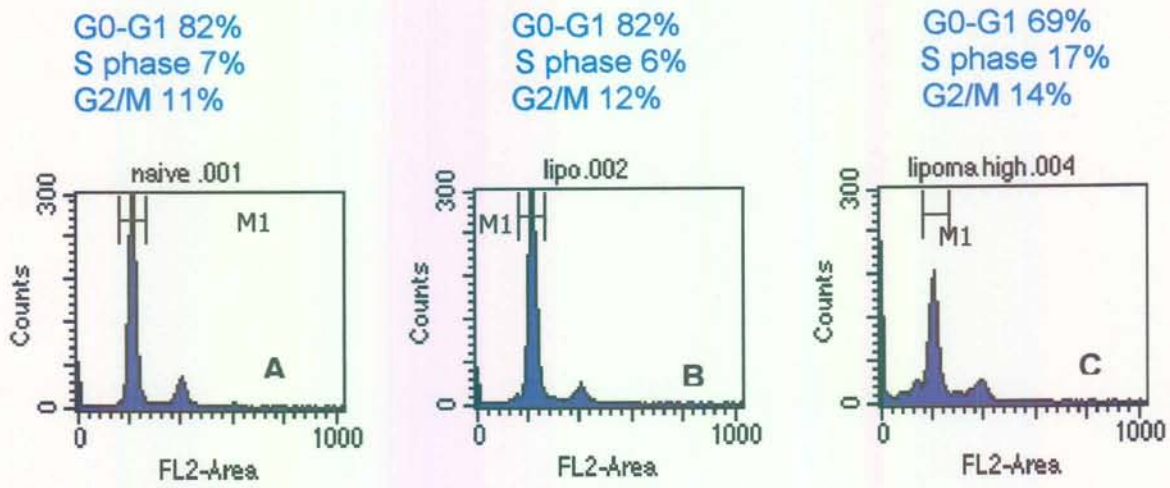


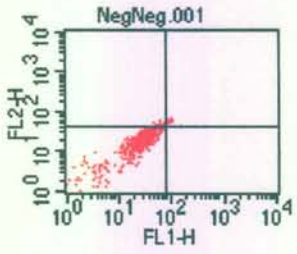
Figure 4.14. FACS analysis of cellular proliferation of PECs upon MA induction. The cells were harvested, stained with PI and analyzed for DNA profile. (A): Native PECs; (B): PECs loaded with liposomes; (C): PECs loaded with 25μg MA-containing liposomes.

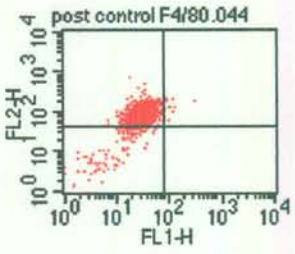
Compared to untreated or liposome treated macrophages that lasted only 9 days in culture, MA induced foam cells survived for up to 30 days in normal macrophage medium conditions.

4.4.3.9 Characterization of MA-activated macrophages according to cell surface marker expression.

Cells loaded with liposomes or liposomes-MA and immunostained for cell surface molecules gave the results that are presented in Figg. 4.15–4.17. Negative control consisted of a gated PECs cell population sorted according to large size and low granularity. This gated population was then labelled with F4/80 (FL2). Only F4/80 labelled cells were used for surface detection of I-A^d, CD11b and CD1d. Most of these cells consisted of macrophages. Flow cytometric analysis showed that I-A^d increased after liposomes-MA uptake in F4/80 positive cells (Figure 4.15). The CD11b was down-regulated on F4/80 positive cells (macrophages) that phagocytosed MA but not on liposomes phagocytosed cells (Fig 4.16). No change was found in CD1 expression on the cell surface of macrophages that phagocytosed liposomes of liposomes-MA (Fig 4.17).

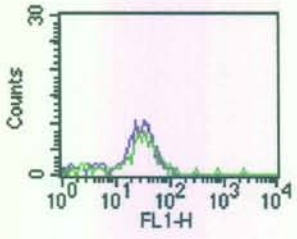
PEC population gated for use - no labelling

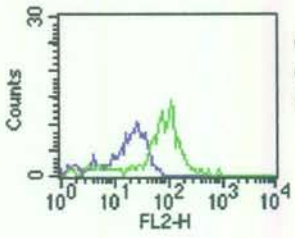




F4/80 - positive cells were used for I-A^d surface labelling

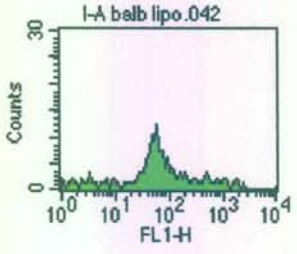
Negative control - overlay of unlabelled (purple) macrophages and isotype control (green)



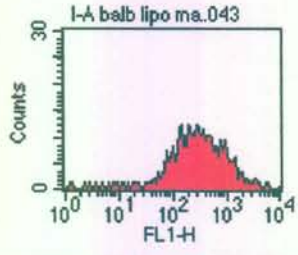


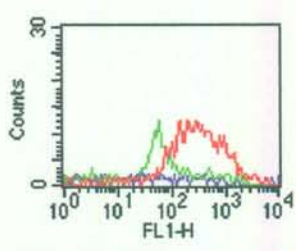
Overlay of unlabelled cells (purple) and F4/80 labelled macrophages (green)

I-A^d surface labelling of liposome treated macrophages (green)



I-A^d surface labeling of liposome-MA treated macrophages (red)

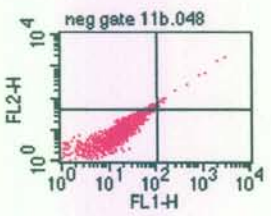


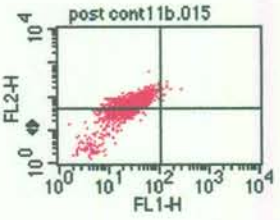


Overlay of I-A^d labelled macrophages (red) – liposome-MA treatment compared to liposome treatment alone (green)

Figure 4.15 Flow cytometry data comparing effects of liposomes-MA and liposomes alone on I-A^d cell surface expression on mouse peritoneal macrophages. F4/80 was used to gate the macrophage population. Anti-I-A^d antibodies were directly labelled.

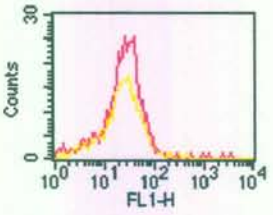
PEC population gated for use-no labelling

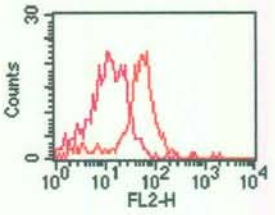




F4/80-positive cells were used for CD11b surface labelling

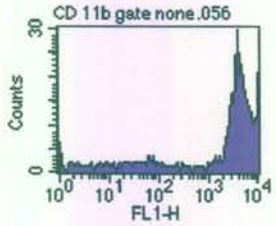
Negative control - overlay of unlabelled macrophages (pink) and isotype control (yellow)

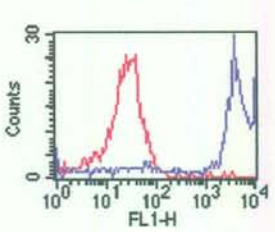




Overlay of unlabelled cells (pink) and F4/80 labelled macrophages (red)

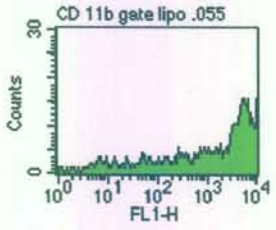
CD11b labelled macrophages– no treatment given

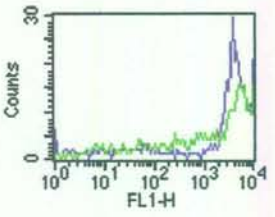




Overlay of isotype control (pink) and CD11b labelled macrophages (purple) – no treatment

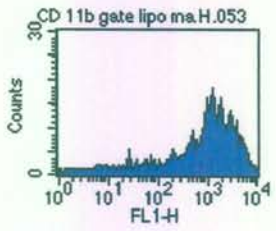
CD11b labelled macrophages – liposome treatment (green)

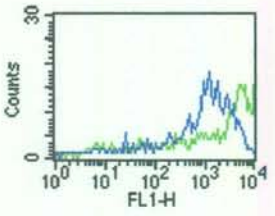




CD11b labelled macrophages. Overlay of liposome treatment (green) to no treatment (purple)

CD11b labelled macrophages– liposome-MA treatment (blue)

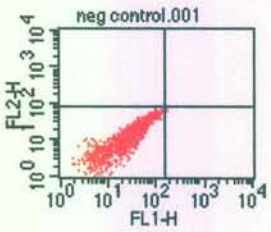


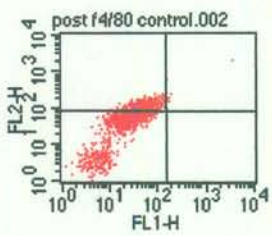


CD11b labelled macrophages – Overlay of liposome-MA treated macrophages (blue) and liposome treated macrophages (green).

Figure 4.16. Flow cytometry data showing effects of liposomes-MA on CD11b cell surface expression using mouse peritoneal macrophages. F4/80 (FL2) was used to gate the macrophage population. Anti-CD11b antibodies were directly labelled with FITC (FL1).

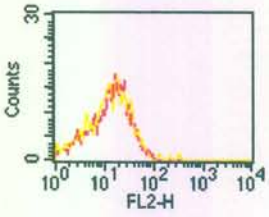
PEC population gated for use - no labelling

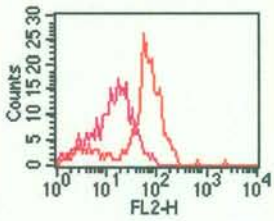




F4/80 - positive cells were used for CD1 surface labelling

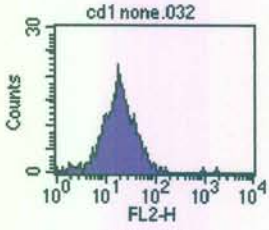
Negative control - overlay of unlabelled macrophages (pink) and isotype control (yellow)

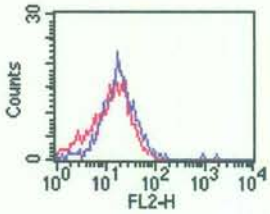




Overlay of unlabelled cells (pink) and F4/80 labelled macrophages (red)

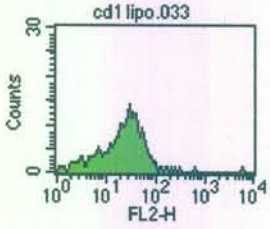
CD1d labelled macrophages (purple) – no treatment

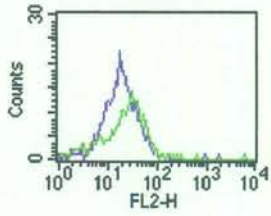




Overlay of isotype control (pink) and CD1d labelled macrophages (purple) – no treatment

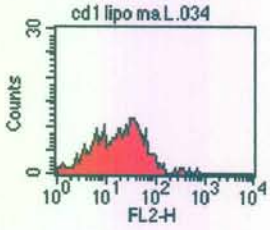
CD1d labelled macrophages (green) – liposome treatment

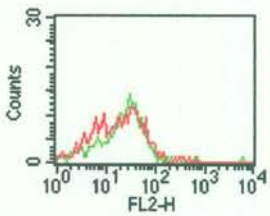




Overlay of CD1d labelled macrophages (purple) – liposome treatment (green) compared to no treatment

CD1d labelled liposome-MA treated macropages (red)





Overlay of CD1d labelled macrophages – liposome-MA treatment (red) compared to liposome treatment (green)

Figure 4.17 Flow cytometry data comparing CD1d surface expression on macrophages loaded with liposomes-MA and liposomes using mouse peritoneal macrophages. F4/80 (FL2) was used to gate the macrophage population. Anti-CD1 was directly labelled with PE (FL2).

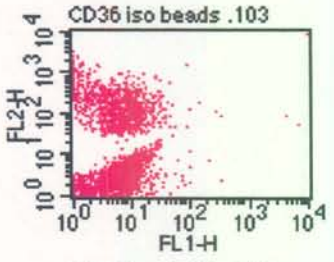
Table 4.7 Changes observed in the surface markers in mouse peritoneal exudate cells upon exposure to MA. (N) shows no change in expression, (+) shows increased expression and (-) shows decreased expression.

Surface markers on foam cells formed by liposome-MA	Change compared to macrophages loaded with liposomes
MHCII	+
CD1d	N
CD11b	-

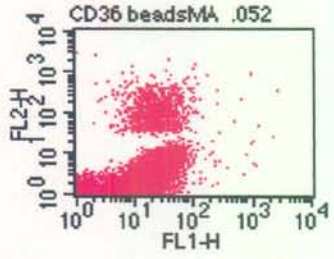
From the above, it is seen that MHCII expression increased, CD11b expression decreased, and the rest stayed the same.

4.4.4 CD36 surface labelling of macrophages loaded with beads-MA

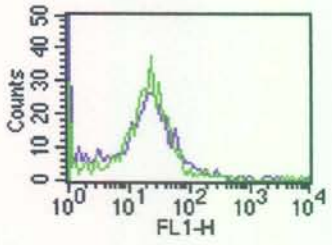
Changes in CD36 expression on bead-MA loaded macrophages was evaluated by flow cytometry. In Figure 4.18 it is shown that no increase in CD36 surface labelling was detected.



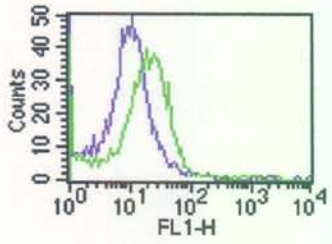
PECs loaded with fluorescent (FL2) beads and incubated with FITC labelled isotype control (FL1)



PECs loaded with beads-MA (FL2) and FITC labelled anti-CD36 (FL1)



Isotype control of macrophages without beads (purple) overlaid with macrophages without beads and labelled with FITC labeled anti-CD36 (green)



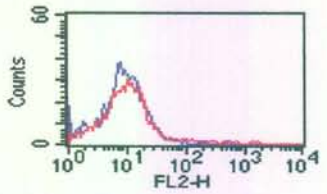
Macrophages loaded with beads isotype control (purple) overlaid with macrophages treated with beads-MA and labelled with FITC labelled anti-CD36 (green)

Figure 4.18. Flow cytometry data showing effects of beads-MA on CD36 surface expression on mouse alveolar macrophages. Beads - containing macrophages were used to gate the macrophage population. Anti-CD36 was reported by a secondary anti-rabbit IgG antibody labelled with FITC.

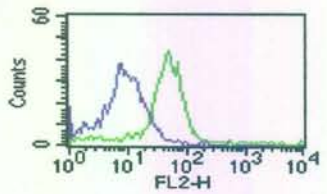
From Figure 4.18 it is evident that CD36 is induced in the macrophages after upake of MA-beads, in comparison to when beads only were taken up.

4.4.5 Effects of intravenous MA administration on alveolar macrophages.

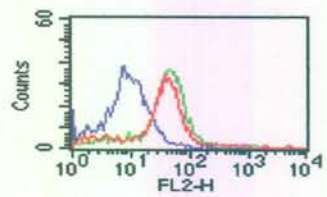
CD1d expression on PECs was found to remain unchanged after beads-MA uptake. In order to assess CD1d expression on alveolar macrophages after intravenous administration of MA on beads, serum or liposomes lung lavages were done on treated animals and cells prepared for FACS analysis, probing CD1. Figure 4.19 shows the results obtained.



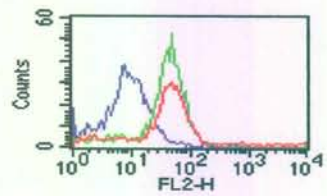
Unlabelled untreated alveolar macrophages (purple) overlaid with isotype control (red)



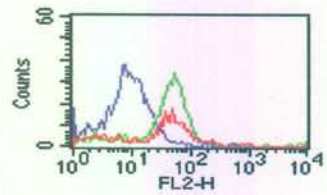
Unlabelled untreated alveolar macrophages (purple) overlaid with CD1d labelled untreated macrophages (green)



Unlabelled untreated alveolar macrophages (purple) overlaid with CD1d labelled untreated macrophages (green) and CD1d labelled beads-MA treated macrophages (red).



Unlabelled untreated alveolar macrophages (purple) overlaid with CD1d labelled untreated macrophages (green) and CD1d labelled liposome-MA treated macrophages (red)



Unlabelled untreated alveolar macrophages (purple) overlaid with CD1d labelled untreated macrophages (green) and CD1d labelled serum-MA treated macrophages (red)

Figure 4.19. Flow cytometry data showing effects of beads-MA, liposomes-MA and serum-MA and controls injected IV into mice on surface expression on mouse alveolar macrophages. F4/80 (FL2) was used to gate the macrophage population. Anti-CD1was directly labelled with PE (FL2).

From the above it is seen that no increase in surface labelling of CD1d molecules was achieved. Nothing can be said about the turnover of the CD1d molecules in alveolar macrophages.

4.5 Discussion

To test the hypothesis that MA administration induces foam cells, the first challenge was to solubilize the MA and to demonstrate uptake into macrophages. Mycolic acids have only the carboxylic group as general characteristic functional group. Approximately 8% of all biological molecules possess a carboxyl group. This complicates the analysis and detection of low-levels of MA during uptake experiments. Radio-immunological detection was considered, but it is extremely expensive, laborious and time consuming to produce radio-active labelled MA. Instead derivatization of the carboxyl group with a suitable chromophore was the option that was used to monitor the MA distribution after uptake. Fluorescence detection has the potential to detect picomolar quantities of the labelled product.

A relatively new derivatisation agent, 5-Bromomethyl fluorescein (5-BMF), was used as flurophore. It is thermo-stable, thereby allowing the dissolution of labelled MA in aqueous liposomes, which involves high temperature. Using the labelled MA, uptake by the cells and progression through to the mature lysosomes could be demonstrated, irrespective of whether the MA was loaded on beads, serum or liposomes. Uptake of MA was demonstrated to occur only with cells expressing F4/80 cell marker, i.e. macrophages, neutrophils and dendritic cells. As seen in the FACS profile, only the larger and more granular cells labelled with F4/80.

When dead *Mycobacterium tuberculosis* strain H37 were loaded into macrophages in the same manner as the liposomes, only a small amount of cells developed into a morphologically activated form that appeared to be differently activated than macrophages receiving liposomes–MA. The *Mycobacterium*-loaded macrophages appeared elongated and had small vacuoles. The morphology of the MA-activated macrophages on the other hand, resembled

foam cells associated with atherosclerosis (De Winther and Hofker, 2000). As foam cells have certain properties distinguishing them from other cells, these properties were investigated.

When the activated cells were tested for neutral lipids by using Oil Red O stain, the cells that received MA showed much more staining of neutral lipids in the cytoplasm, than cells that received liposomes or beads alone. Moreover, accumulation of the neutral lipids was only found in the cytoplasm, and not in the enlarged vacuoles. Cells activated by dead mycobacteria had comparatively small amounts of accumulated neutral lipids and did not have the characteristic appearance and neutral lipid staining pattern of the MA-induced foam cells.

According to Yancey and Jerome (1998) the fate of oxLDL differs from that of acylated LDL in foam cells and differs among various species. Acylated LDL is produced by artificial oxidation of LDL with copper sulphate and accumulates in the enlarged vacuoles found in macrophages with foam cell formation. Different processes can form oxLDL from which neutral lipids accumulate in the cytoplasm of foam cells, as was seen in this chapter. Here morphological evidence argues for the formation of foam cells upon MA activation, followed by cellular proliferation. By extracting the induced foam cells with non-polar organic chemicals, and separating them on HPLC, it was observed that much more cholesterol accumulated in cells from liposome-MA treated animals than in cells that were exposed to liposomes only.

MA-induced foam cell formation was suggested by the changed macrophage morphology, the increased Oil Red O staining and cholesterol isolated from MA loaded macrophages. Other properties of foam cells, such as macrophage survival and DNA synthesis were assessed.

Hamilton *et al.,* (1999) described that oxLDL-induced macrophage-derived cells have extended survival and DNA synthesis. Reid *et al.* (1993) reported that 100μg/ml oxLDL or more induced apoptotic changes in the macrophages. Thus, the concentration of activation agent (eg. oxLDL) appears to be related to survival and apoptotic changes induced in the foam cells. In our study, we found that macrophages loaded with MA, survived 30 days in culture, compared to

untreated or liposome treated macrophages, that only lasted for 9 days. An increased uptake of radioactive thymidine of almost ten times was recorded from cells that received MA, compared to untreated or liposome treated macrophages, correlating with an increased S phase of the growth cycle. This indicated active cell proliferation. The FACS profile of MA-activated cells showed peaks of lower fluorescence, indicative of apoptosis. The number of divisions that the MA treated macrophages and/or lymphocytes undergo, remains unknown. According to the FACS results, it is presumably a balance between survival, proliferation and cell death by apoptosis. The above findings suggest that macrophages are stimulated by MA via a pathway similar to induction of an oxLDL pathway.

Expression of CD1a, b and c were found on monocyte-derived foam cells in cultures treated with oxidized or acylated LDL in the presence of GM-CSF and IL4 (Melian *et al.*, 1999 and Table 4.3). Foam cells also express a number of other cell surface markers as summarized in Table 4.3. As the above-mentioned surface markers are defined for human macrophages and foam cells, it cannot be directly applied to mouse cells. Certain properties of foam cells are, however, the same, independent of the species. The combined data shown here demonstrated that in cells that labelled with F4/80 and treated with MA, MHCII expression increased, the CD11b was down-regulated and CD1d expression stayed unchanged. It has been described previously that CD11b is unchanged in oxLDL-induced human foam cells (Melian *et al.*, 1999). Here, MA affected a decrease in CD11b expression. MA given intravenously in four doses equally distributed over 40 days, yielded alveolar macrophages testing unchanged CD1d expression whereas CD1b molecules are strongly induced in atherosclerotic foam cells. In macrophages induced with beads-MA, CD36 seemed not to be increased on the surface. As described earlier (4.1.6.3), CD36 expression correlates with foam cell fomation in humans (Huh *et al.*, 1996). Atherosclerotic plaques are chronic inflammatory lesions, consisting of dysfunctional, macrophage derived foam cells, T cells and smooth muscle cells. Clinical and experimental evidence suggest that the cellular immune response is playing a

role in atherosclerosis. The inhibition of atherosclerosis in LDL receptor-deficient, as well as in C57BL/J6 hyperlipidemic mice seen upon treatment with monoclonal antibodies against CD4 and CD40, supports this concept (Emeson *et al.*, 1996). Atherosclerotic lesions are characterized by abundant expression of major histocompatibility complex (MHC) I and II molecules present on macrophages and smooth muscle cells. Several investigators have suggested a possible link to infections with pathogenic micro-organisms (Chlamydia, Cytomegalovirus and Mycoplasma) that drives the immune response to a state of atherosclerosis. The unique chlamydial lipopolysaccharide might be the factor inducing foam cell formation in macrophages (Kalayoglu and Byrne, 1998). A convincing association was found between *Chlamydia pneumonia* infection and atherosclerotic heart disease (De Winther and Hofker, 2000). The results shown here suggest that mycolic acids, unique to *M. tuberculosis*, could have a similar effect on foam cell induction.

Taken together, the data presented in this chapter suggest that at least a subpopulation of macrophages are converted to foam cells upon *in vivo* exposure to MA. In the next chapter the mechanisms and effects of foam cell formation, cytokine production and the influences on the innate immunology of tuberculosis are discussed.