CHAPTER 5

Mycolic acid induced anti-mycobacterial mechanisms

5.1 Introduction

5.1.1 A possible role for oxidation of lipoproteins by monocytes / macrophages in TB infection

Low density lipoproteins (LDL) are the principal carriers of cholesterol. They are formed from very low density lipoprotein (VLDL) via intermediate density lipopoproteins IDL. LDL can pass through the junctions between capillary endothelial cells and attach to LDL receptors on the cell membranes that recognize apo B-100 (Brewer, 2000). In Chapter 4 LDL metabolism is explained.

Already in 1984, Steinbrecher, *et al.* proposed that endothelial cells could oxidize LDL, and that macrophages then recognize the modified LDL. In their view reactive oxygen species caused transformation of LDL into atherogenic entities, rather than metal ions. Although it is well known that free ferrous or cupric ions catalyse lipid peroxidation reactions *in vitro,* leading to oxidation of LDL, this can hardly happen *in vivo,* as any free transitional ions become bound and renderred inactive. Chisolm *et al.* (1999) argued 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 can induce *in vitro* oxidation of LDL in medium free of metal ions, unlike smooth muscle and endothelial cells. There are a number of ways in which macrophages may possibly 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 rather act as a potent oxidant of LDL. Cp is over-expressed and secreted by macrophages and a possible role for LDL oxidation by Cp was shown by Chisolm *et al.* (1999).

- **Superoxide (O_2^-):** The role of superoxide in LDL oxidation has been debated for many years. The dependence of LDL oxidation on O_2^- is much less in cell systems that contain free metal ions. Most researchers found that oxidation of LDL by macrophages need a source of extra-cellular cell-derived superoxide - alone it is not enough to mediate changes (Chisolm *et al.*, 1999).

- **The role of 15-lipo-oxygenase (LO):** These non-heme iron-containing enzymes catalyse the direct insertion of molecular oxygen into polyenoic fatty acids, which leads to hydrogen peroxide formation. LO oxidises cellular fatty acids, cholesterol or phospholipid substrates and the hydrogen peroxide products could transfer to LDL, making it prone to 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, leads to oxLDL in a cell free environment. Moreover, LO inhibitors inhibited this cell mediated oxidation process.

- **Myeloperoxidase:** This abundant heme protein is released by activated neutrophils and monocyte/macrophages, especially those found in vascular lesions where foam cells are found. MPO can amplify the oxidizing potential of H_2O_2, the dismutation product of superoxide, by using it as a co-substrate to generate oxidant radical species, reactive halogens, aldehydes, and nitrating agents (Chisholm *et al.*, 1999). MPO catalyses 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 catalyze the conversion of L-tyrosine, into the tyrosyl radical, and initiate LDL lipid peroxidation and dityrosine cross-linking of proteins. Another potential MPO-dependent pathway of monocytes/macrophages that may result in 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.

Aleshina *et al.* (1998) demonstrated that serum MPO was raised almost 10 times in patients with *Mycobacterium tuberculosis* infection due to neutrophil granulocytosis. Brennan *et al.* (2001) showed that MPO knockout mice showed an increase of 50% in atherosclerotic lesions. This shows then a protective role for MPO in atherosclerosis in mice. It therefore appears as if macrophages respond to local lipid disturbances by generating mechanisms of LDL oxidation to enhance uptake of the defective or even toxic LDL. Podrez *et al.* (1999) reported that reactive nitrogen species generated by MPO - H_2O_2 - NO_2^- system of monocytes converts LDL into NO_2 -LDL that is avidly taken up by macrophages, leading to massive cholesterol accumulation and foam cell formation. OxLDL is recognized by macrophage scavenger receptor(s) (SR-AI/II), resulting in an enhanced uptake. Such uptake might account for foam cell formation (Hajjar and Haberland, 1997). As the MA of *M. tuberculosis* also induces foam cell formation (Chapter 4) it is an open question whether this situation is an outcome of the macrophage's battle against TB, or whether it is chaos orchestrated by local TB infection to facilitate progression of infection.

Activated macrophages feature in inflammation. Macrophage derived foam cells are not uncommon, and are also found in diseases other than atherosclerosis, namely:

- **Lepromatous leprosy**: The mainstay of lepromatous leprosy (Th2 activation) is the presence of disorganized infiltrates of foam cells. In tuberculoid leprosy, the Th1 pole of reaction to *Mycobacteria leprae,* no foam cells are found (Volc-Platzer *et al.*, 1990).

- ***Chlamydia pneumonia* infection:** Although infection is associated with atherosclerotic heart and vessel disease, a causal relationship was only established when 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:** Increased presence of foam cells was found in patients that were immunocompromised and had opportunistic infections. The mechanism of foam cell formation here was not clear to the authors (Ishihara *et al.*, 1986).

All of the above included the presence of pathogens, as in the case of tuberculosis, indicating a critical role of LDL oxidation in the pathogenesis of disease or the immune defense to it.

5.1.2 Immune mechanisms of susceptibility and protection against *Mycobacterium tuberculosis*

Acquired resistance against tuberculosis rests on cell-mediated immunity, whereas the humoral responses were found not to be of importance. The most important cell types involved in cellular immunity are mononuclear phagocytes (MP), and T lymphocytes, both playing dual roles. The interaction between an intracellular pathogen and the host immune system occurs as protein from the pathogen are degraded in the phagosome and the resulting peptides are presented via the MHC class II molecules to CD4$^+$ cells.

The organism is exposed to an array of killing mechanisms after internalization by the macrophage. Following lysosome fusion there is a transient rise in pH before acidification. Killing of some organisms may be due to acidification but more likely related to lysosomal enzymes. Cationic proteins that have antibiotic-like properties called defensins are present inside the phagosomes and act before acidification takes place. Both oxygen independent and oxygen dependent mechanisms of bacterial killing exist in the monocytes, but oxygen

dependent mechanisms were found to be of major importance (Lee *et al.,* 1993). Because full eradication of the pathogen is not always accomplished, imbalances of the immune system at a later stage will lead to reemergence of the bacilli, resulting in clinical disease (reactivated TB).

$CD8^+$, cytotoxic lymphocytes, natural killer cells and activated lymphokine killer cells depend mostly on perforin / granzyme systems to kill their targets, while CD4+ utilizes Fas and Fas ligand (part of the TNF family of death receptors) to induce cell death (Shresta *et al.,* 1998).

Canaday *et al.* (2001) showed that after *in vitro* stimulation with *Mycobacterium tuberculosis*, both $CD4^+$ and $CD8^+$ T cells up-regulated mRNA expression for granzyme A and B, granulysin, perforin, and CD95L (Fas ligand). Both T cell subsets seemed to lyse tuberculosis-infected monocytes. Biochemical inhibition of the granule exocytosis pathway in $CD4^+$ and $CD8^+$ T cells decreased cytolytic function by >90% in both T cell subsets. Antibody blockade of the CD95-CD95L interaction decreased cytolytic function for both T cell populations by 25%. However, inhibition of perforin activity, the CD95-CD95L interaction, or both mechanisms did not affect $CD4^+$ and $CD8^+$ T cell mediated restriction of *Mycobacterium tuberculosis* growth. Thus it seems that infected cell lysis and apoptosis are not involved in $CD4^+$ and $CD8^+$ T cell mediated restriction of *Mycobacterium tuberculosis* growth

Huge disparities currently exist in published studies regarding the mechanisms of eradication of *Mycobacterium tuberculosis in vivo.* Some of the disparity in the studies may be contributed to the different animal models that are used, as different species have different effector mechanisms to kill bacilli. From the literature, it seems that nitric oxide synthetase-mediated killing is the main mechanism in murine models, while in humans reactive oxygen intermediates (ROI) are more important (Manca *et al.,* 1999). Tuberculosis is characterized by granuloma formation to contain the infection and prevent further spread of the

bacilli in infected tissues. The ability of cells involved in granuloma formation to produce high levels of ROI differs from individual to individual.

Pathogenic organisms have ways to evade microbicidal responses of the host. Despite the antimicrobial mechanisms of vertebrate phagocytes, *Mycobacterium tuberculosis* can survive within the phagosomes of macrophages, despite the latters' normal antimicrobial function.

Several mechanisms have been proposed whereby mycobacteria could achieve this :

- their residence in altered phagosomes that do not fuse with lysosomes and are only mildly acidified. This involves the TACO protein and cholesterol (Clemens *et al.*, 2000).

- alteration of the phagosome by the persistence of Rab5, which might enable the phagosome to retard maturation (Clemens *et al.*, 2000).

- by binding only to receptors that don't activate innate bactericidal activity (Astarie-Dequeker *et al.*, 1999).

- by mycobacterial production of ammonia (concentrations of up to 20mM), which impairs phagolysosome fusion (Gorden *et al.*, 1980).

- by mycobacterial production of a catalase-peroxidase protein (katG), and alkyl hydroperoxidase reductase protein (AhpC) to accomplish survival in phagosomes even in the presence of H_2O_2 at concentrations as high as 1 mM (Manca *et al.*, 1999).

Gamma interferon (INFγ) is known to increase the oxygen burst in human monocytes leading to the destruction of phagocytosed bacteria (Nathan, 1983). It was speculated that the induction of a Th1 response through continuous secretion of IL12 and concomitant production of INFγ, may release an oxygen burst sufficient to kill all mycobacterial bacilli in granulomas (Manca *et al.*, 1999). Attempts to treat TB by inhaling INFγ were only partly effective, however, and it is now known that the macrophages which endocytose mycobacterial bacilli, are resistant to the bactericidal effect of INFγ (Flynn, 1999).

5.1.3 Phagocytic oxidative pathways to kill mycobacteria

Catalase and peroxidase are both seen as virulence factors for mycobacteria. Adding catalase or peroxidase to the cultures of murine macrophages, can increase survival of *Mycobacteria tuberculosis* (Manca *et al.*, 1999). This is supported by the finding that *M. bovis* BCG vaccination had a fatal outcome in patients with a genetic disability to generate peroxides by causing disseminated disease. Indeed, *in vitro* studies have shown that exogenously added H_2O_2 is very important to limit survival of the bacilli.

Phagocytic killing of foreign organisms is part of the innate immune defense to fight infections. When comparing the relative size of the phagocytosed particle to the phagosome size, it appears that the phagosome is filled almost entirely by the ingested particle. A cleft of less than 500 nm is left surrounding the particle. Various granules are transferred into this cleft, discharging their contents, which kill and degrade the particle. In the chemical arsenal of the phagocytic cell, the hydroxyl and chorine radicals have the highest oxidation potential. The enzyme NADPH oxidase, which is membrane bound and orientated toward the ingested particle, produces superoxide, the precursor of both the hydroxyl and chlorine radicals. Superoxide is converted into radicals in myeloperoxidase dependent or independent ways. Hydroxy radicals are produced independently from myeloperoxidase, while chlorine radicals are products of the myeloperoxidase pathway.

Myeloperoxidase is a tetrameric, highly glycosylated, basic (PI>10) heme protein of about 150 kDa. Myeloperoxidase is abundant in neutrophils and monocytes, and can account for 5% and 2% respectively of the dry weight of these cells. The heme protein is stored in the primary azurophilic granules of a leucocyte, and is secreted in the extracellular, as well as the phagolysosomal compartment following phagocyte activation.
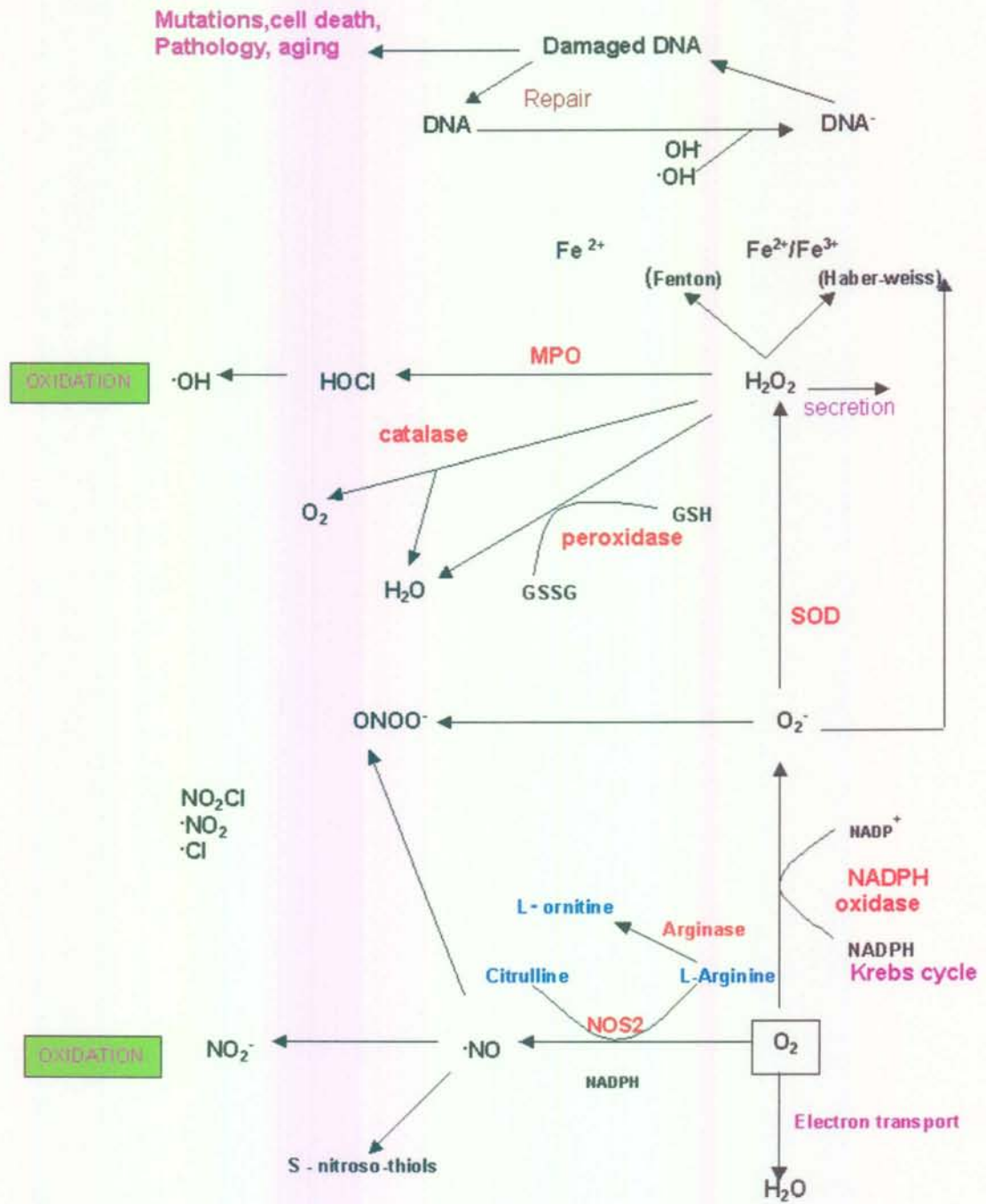
Figure 5.1. Reactive oxygen and nitrogen pathways.

During phagocytic activation and MPO secretion, the oxidative burst which occurs, is directed by the NADPH oxidase complex to form superoxide and hydrogen peroxide (H_2O_2) (see figure 5.1). The MPO amplifies the oxidizing potential of the hydrogen peroxide using it as a co-substrate to generate a variety of reactive oxidants and diffusible radical species. The active site of MPO is at the base of a deep narrow hydrophobic cleft, restricting access of substrates. Thus, low molecular weight compounds primarily serve as substrates. Substrates that occur naturally are nitrates, tyrosine, ascorbate, urate, catecholamines, oestrogens and serotonin as well as halides and thiocyanate (SCN^-) (Podrez *et al.*, 2000).

The diffusible oxygen products formed are potent signaling molecules for bactericidal cellular processes such as initiation of lipid peroxidation. The regulation of MPO is thought to rely primarily on the rate of the superoxide production, the availability of H_2O_2 and other co-substrates and the abundance of anti-oxidant species such as ascorbate or methionine. However, NO, synthesized by nitric oxide synthetase (NOS), has also been shown to play a role in regulating MPO peroxidase activity. In Figure 5.2, a recently proposed enzyme kinetic model is shown for MPO, displaying multiple intermediate states.
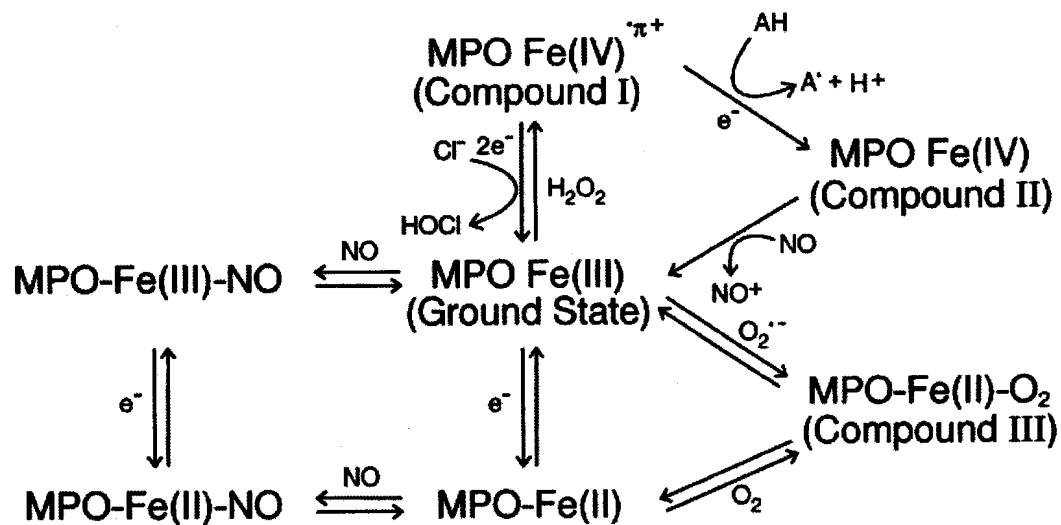

Figure 5.2. Enzyme kinetic model for myeloperoxidase (Taken from Podrez *et al.*, 2000)

These states are influenced by availability of superoxides, hydrogen peroxide and nitric oxide. MPO at ground state exists in the ferric Fe(III) form, which upon

addition of H_2O_2, goes into an active ferryl radical intermediate form. In the presence of halides (such as Cl^-, Br^- and I^-), compound I is readily reduced in a single step, producing MPO-FeIII and the corresponding hypohalous acid (HOX). MPO-Fe(III) can be reduced to inactive ferrous form MPO-Fe(II). MPO-Fe(III) can bind to superoxide and MPO-Fe(II) to oxygen, forming an intermediate ferrous-dioxy compound, MPO-Fe(II)-O_2 (compound III).

The inducible form of NOS and MPO are both found together in the primary granules of leucocytes. During bacterial ingestion, both molecules are secreted into the phagolysosomal and extracellular compartments, leading to nitration of bacterial proteins. Rapid kinetic studies show that at low levels of NO, the starting rate of MPO catalyzed peroxidation of compound is enhanced. At high NO levels, MPO is reversibly inhibited by formation of a nitrosyl complex (MPO-Fe(III)-NO). MPO can serve as a catalytic sink for NO, limiting its bio-availability. Finally NO can bind reversibly to MPO-Fe(II), forming MPO-Fe(II)-NO, which is in equilibrium with MPO-Fe(II) and MPO-Fe(III)-NO. This demonstrates the antagonism between the MPO and NOS mediated pathways of ROI production.

Together with MPO, superoxide is discharged into the phagolysosomal compartment at high concentrations. MPO is a cationic enzyme and will bind via electrostatic binding to the surface of bacteria, which usually bears a negative charge. (See Figure 5.3).
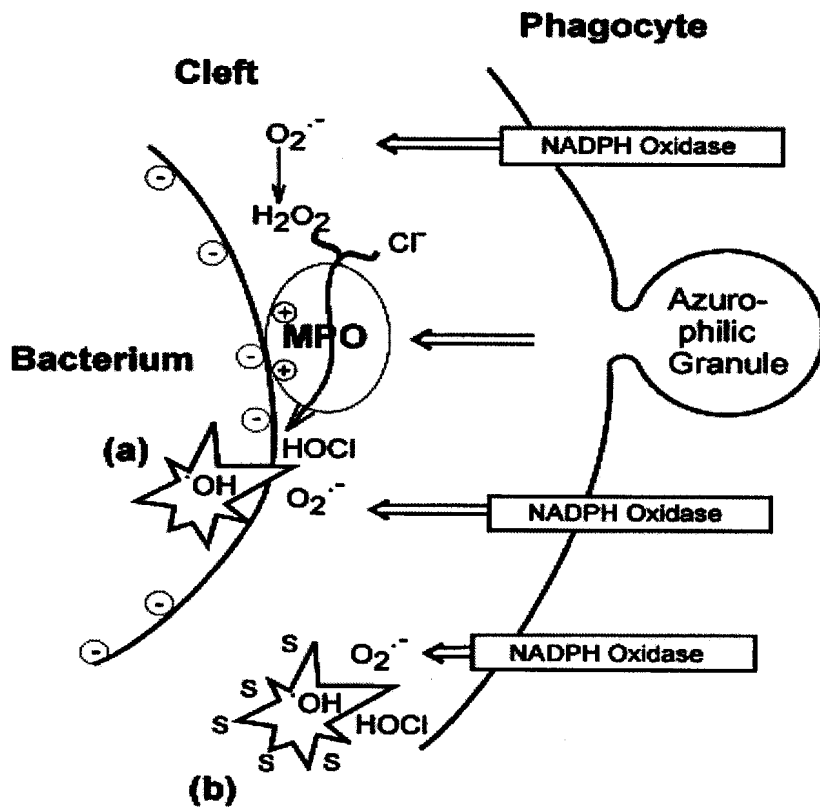
Figure 5.3. The geometry of MPO-mediated bacterial killing within the phagosome. (Taken from Saran et al., 1999).

If attached to the negative surface of a bacterium, MPO would have its active site close to the bacterial cell wall or membrane. It is assumed that O_2^-, being a rather stable radical, diffuses from the site of its generation in the phagocytic membrane to the location at MPO, where newly formed HOCl is set free. The subsequent interaction of O_2^- with HOCl may produce ˙OH radicals in close vicinity to bacterial targets (a). In case (b), where the ˙OH radical is generated further away from the bacterium, it will simply be scavenged by scavenger molecules (Saran et al., 1999). The oxidative pathways induced by the TB infection may affect the immediate environment and cause oxidation of LDL in various forms.

5.1.4 Different oxidative forms of LDL

Figure 5.4 shows the native LDL and different possibilities in oxidative forms of the particle. LDL is extremely oxidation-prone and labile. Native LDL is characterized by it:

- containing one intact, underivatised polypeptide (apoB-100),
- having no lipid peroxides or aldehydes,
- being enriched with polyunsaturated fatty acids (PUFA) and anti-oxidants

The various stages of oxidation of LDL can be compared as follows:
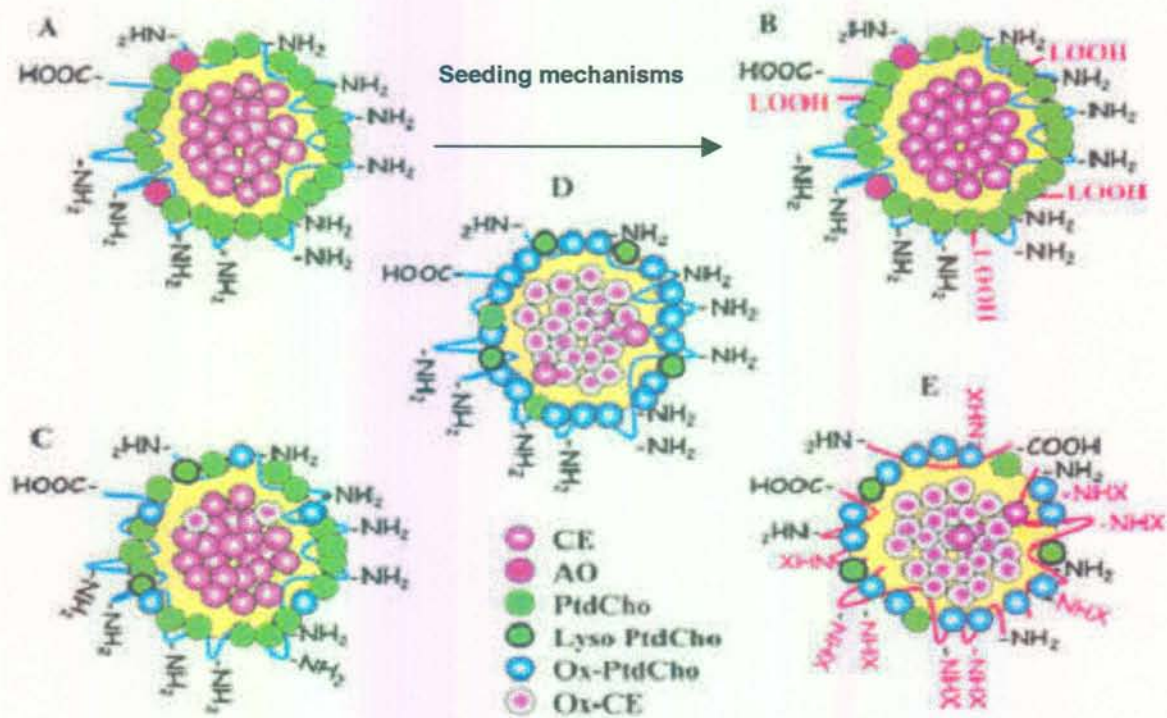

Figure 5.4. Different forms of oxidized LDL: (A) native LDL, (B) seeded LDL, (C) minimally oxidized LDL (D) extensively oxidized LDL (E) oxidatively modified LDL. See detail in text. CE – cholesterol esters, AO - Antioxidants, PtdCho - phosphatidyl choline, Lyso Ptdcho l-lysophosphatidyl choline Ox- PtdCho - oxidized phosphatidyl choline, Ox-CE – oxidized cholesterol esters (Esterbauer *et al.*, 1997).

Seeded LDL: Dietary lipids, and pathological conditions can influence circulating LDL, which may become associated with oxidized lipids, such as lipid hydroperoxides (LOOH) and other degradation products. This LDL could represent the "seeded" LDL, which might have an increased tendency to undergo further oxidation. As the LDL particle itself has not interacted with an oxidant, it:

- still contains one intact, underivatized polypeptide (apoB-100) and
- is enriched in PUFA and antioxidants.

Minimally oxidized LD: MPO, xanthine oxidase and peroxynitrite, under normal and pathological conditions may lead to oxidation of intrinsic LDL. When such oxidation is carried out to a minimal degree, the resulting particle might represent the "minimally ox-LDL" or "mm-LDL" and, as such, might be physically indistinguishable from the native lipoprotein, except for the expected loss of polyunsaturated fatty acids and antioxidants. ApoB-100 is intact and little protein damage or modification is detected. The lipids, particularly phospholipids, are however affected. There is little evidence to support the theory that cells internalize these mildly oxidized lipoproteins by pathways other than the LDL receptor-mediated pathway. This particle contains:

- one intact, underivatized polypeptide (apoB-100),
- decreased amounts of PUFA and antioxidants.

Extensively oxidized LDL: When LDL is oxidized, for example by copper, it undergoes oxidation after an initiation, propagation and termination sequence as suggested by Esterbauer *et al.* (1997). When oxidation plateaus, all the oxidizable fatty acids are consumed and the particle should be enriched in oxidized fatty acids. This particle contains:

- derivatized and crosslinked polypeptides,
- only trace amounts of PUFA and antioxidants,
- extensive oxidation of lipids of LDL,
- massive amounts of lipid peroxides and new lipids such as lyso PtdCho.

Extensively oxidized and modified LDL: Like the true native LDL, the true oxidatively modified LDL is unlikely to occur even in the atherosclerotic artery. Such a particle would be completely devoid of PUFA, mono-unsaturated fatty

acid (MUFA), and antioxidants (even MUFA will undergo co-oxidation under such conditions). Such particles are very likely to be cleared from plasma by the liver, even if they were generated in the plasma compartment. In the fatty streak lesions, it is more likely that macrophages would have cleared moderately oxidized particles long before such extensively oxidized and modified particles are generated. Extensively oxidized and modified LDL contains:

- derivatized and crosslinked polypeptides,
- alterations in detectable amino acid composition due to side chain oxidation,
- loss of detectable lysine due to covalent modifications,
- practically depleted PUFA and antioxidants,
- presence of degraded lipids (core lipid aldehydes and lyso PtdCho).

The body responds to oxidized LDL (oxLDL) in various ways:

- It is recognized by macrophage scavenger receptor(s) (SR-AI/II), resulting in an enhanced uptake. Such uptake might account for foam cell formation (Lipton *et al.*, 1995).
- It is antigenic and results in the generation of autoantibodies. The antigenicity might play a major role in immune clearance as suggested by Calara and coworkers (Calara *et al.*, 1998, Rajavashisth *et al.*, 1990).
- It induces the expression of Interleukin-1 by foam cell macrophages, as well as GM-CSF induction (Lipton *et al.,* 1995).
- It may result in ceroid accumulation in macrophages (Ball *et al.,* 1988). Ceroids are autofluorescent lipid–protein complexes, which accumulate in degenerative tissues including macrophage foam cells.
- It may be chemotactic to monocyte/macrophages (acetyl LDL and other modified LDL forms are chemotactic) (Quinn *et al.,* 1987).

A number of compounds have been identified in oxLDL that has the ability to damage the macrophage lysosomal membrane leading to necrosis and apoptosis (Yuan *et al.,* 2000). Cholesterol oxidation compounds (ChOx) present

in oxLDL can exceed the threshold of toxicity and may account for most of the cytotoxic effect of oxLDL leading to foam cell death.

5.1.5 Macrophage response to MA

Particle uptake is initiated by interaction of specific receptors on the surface of the phagocyte with ligands on the surface of the particle. Entry of *M. tuberculosis* into human mononuclear phagocytes resembles receptor-mediated phagocytosis (Schlesinger *et al.*, 1990). Mycolic acids, as the most abundant lipid antigen of the outer cell wall of mycobacteria, may well play a determining role in the initial contact of the macrophage with the mycobacterium.

Macrophages have a restricted number of phagocytic receptors available. All these receptors induce rearrangements in the actin cytoskeleton for internalization of the particle. The receptors differ in their mechanisms of uptake, maturation of the vacuole and their ability to induce inflammatory responses. After internalization is complete, shedding of the actin based mechanism preceeds phagosome maturation via a series of vesicle fusion and fission events. Macrophages can bind pathogens directly as a manifestation of innate immunity in host defense. Janeway (1992) has proposed that activation of the innate system is initiated when pathogens bind to non-clonal pattern-recognizing receptors on immune cells. These receptors bind structural motiffs typically displayed on the surface of micro-organisms or mutated host cell surfaces, eg. cancer cells.

Elimination of infectious agents relies on the course of inflammatory reactions and pro-inflammatory mechanisms. It is however mandatory, that the activated inflammatory reactions are contained to allow healing and prevent escalation and extreme tissue damage. Thus, pro-inflammatory and anti-inflammatory reactions must be activated transiently and in a balanced interplay.

Detrimental inflammatory diseases are antecedent to overwhelming secretion of pro-inflammatory cytokines, and dysfunction or failure of anti-inflammatory control mechanisms. Neuro-inflammatory interactions try to counteract the pro-inflammatory effects of IL1 and TNFα, and finally secrete glucocorticosteroids to exert their pleiotropic anti-inflammatory effects (Wilckens and De Rijk, 1997). Macrophages and dendritic cells play a major role in the regulation of both immune–mediated and non-specific inflammation. Interferon γ and LPS were identified as major pro-inflammatory regulators, and IL4 and glucocorticostroids as anti-inflammatory regulators.

More recently it was found that IL4, IL10, IL13 and prostaglandin secretion activate macrophages in an alternative way, leading to increased expression of macrophage mannose receptor, enhanced antigen presentation and increased capacity for endocytosis. Alternatively activated macrophages express a special set of molecules to actively participate in anti-inflammatory processes, tolerance induction and healing. Alternatively activated macrophages participate in the three stages of healing, i.e. down-regulation of inflammation, angiogenesis and elimination of tissue debris (Goerdt et al., 1999, Goerdt and Orfanos, 1999). The molecular repertoire of alternatively and classically activated macrophages is given in Table 5.1.

Table 5.1. Differences in alternatively and classically activated macrophages (Goerdt and Orfanos, 1999).

	ALTERNATIVE ACTIVATION	CLASSICAL ACTIVATION
Cytokines	IL1R antagonist IL10	IL1 IL6 IL12 TNFα
Chemokines	DC-CK1/AMAC-1	MIP-1α
Immune receptors	FcεRII Macrophage mannose receptor Scavenger RI β-glucan R CD163	FcγRI (CD64) FcγRII (CD32) FcγRIII (CD16)
Killer molecules	Arginase	NO, iNOS O$_2$

Classical activation of macrophages proceed by two signals, *i.e.* interferon γ (IFNγ) and membrane bound tissue necrotic factor-α (TNFα), or a small amount of bacterial lipopolysaccharide (Janeway and Bottomly, 1994). Inflammation is regulated antagonistically by IL4 and IFNγ. Alternatively activated macrophage molecules are induced by IL4 and inhibited by IFNγ, while classically activated macrophages are induced by IFNγ, and inhibited by IL4.

Immunosuppressive macrophages and alternatively activated macrophage populations partially overlap. Alveolar macrophages and placental macrophages are the best examples of alternatively activated macrophages. The suppressive efficacy of alveolar macrophages is so strong, that antigen-presenting capability of dendritic cells can become totally diminished. This suppression of alveolar

macrophages protects the lung from unwanted environmentally induced inflammation. IFNγ has been shown to directly inhibit suppressor macrophage activity. Alternatively activated macrophages inhibit mitogen-induced proliferation of peripheral blood lymphocytes and $CD4^+$ T cells. These findings confirm that alternative activation generates immunosuppressive macrophage populations (Goerdt *et al.*, 1999).

Besides lipocortin I and PGE_2, which are well-known suppressive mediators, macrophage derived IL10 and TGFβ have been shown to exert down-modulating and anti-inflammatory reactions. The balancing function of alternatively activated suppressor macrophages in schistosoma egg granuloma formation, changes the Th1 dominant reaction by expression of IL10 to induce clonal anergy. Thus, the alternatively activated macrophages seem to act in alleviating disease activity, or induce tolerance as a protective function.

5.1.6 Aims

MA is known to provide some form of protection against TB and its associated side-effects *in vivo*. In this chapter the most likely mechanisms of anti-mycobacterial action of MA-activated macrophages are investigated, focusing on oxidative mechanisms and the cytokine response.

5.2 Materials

5.2.1 Labelling of dead mycobacteria with FITC

Lyophilized *Mycobacterium tuberculosis* H37Ra (Difco), were diluted to $1x10^7$ cfu/ml in medium containing penicillin/streptomycin (1%, v/v) and RPMI 1640 containing 10% foetal calf serum and penicillin/streptomycin (1%, v/v) medium and supplements were purchased from Pharmacia, Freiburg, Germany. FITC was obtained from Molecular Probes, Leiden, The Netherlands.

5.2.2 Inhibition of mycobacterial growth in MA treated macrophages

Serum, serum-MA, liposomes and liposomes-MA were prepared as discussed in Chapter 3. *Mycobacterium tuberculosis* H37Rv was obtained from the Medical Research Council, Pretoria, South Africa. RPMI 1640 containing 10% foetal calf serum and penicillin/streptomycin (1%) v/v were obtained from Pharmacia, Freiburg, Germany). PECs were harvested from C57Bl/J6 mice as described in Chapter 4.

The BACTEC medium consisted of Middlebrook (7H12) medium, supplemented with polymyxin B, amphotericin B, nalidixic acid, trimethroprim, and azlocillin supplied by Becton and Dickinson.

5.2.3 Cytochemical staining of adherent macrophages

Trizmal™ 6.3 buffer concentrate : Trizma™ maleate 200 mmol/l with chloroform(2%,v/v) added as preservative was obtained from Sigma-Aldrich company, Steinham, Germany.

Myeloperoxidase indicator reagent: p-Phenylenediamine plus catachol was obtained from Sigma-Aldrich company, Steinham, Germany (indicator reagent). Hydrogen peroxide (3%) was made up in phosphate buffered saline and 0.2 ml added to indicator reagent in 10 ml Trizmal™ 6.3 buffer (pH- 6.3) concentrate just before use.

5.2.4 Effect of catalase on cell proliferation of foam-like cells

Catalase was obtained from Sigma-Aldrich company, Steinham, Germany. [Methyl-^{3}H] thymidine (TdR) (2 μCi/ml) was obtained from Amersham. RPMI 1640 culture medium containing 10% foetal calf serum and penicillin/streptomycin (1% v/v) was obtained from Pharmacia, Freiburg, Germany.

5.2.5 Alexa Fluor 660 (CY5) labelling of catalase

Catalase was obtained from Sigma-Aldrich company, Steinham, Germany. Alexa Fluor 660 (CY5) dye was purchased from molecular probes Leiden, The Netherlands.

5.2.6 Cytokine ELISA.

Antibodies against GM-CSF, TNFα, IL6 and IL10, biotinylated anti-GM-CSF, -TNFα, -IL6 and -IL10 cytokine detection antibodies and avidin-horse radish peroxidase (Av-HRP) conjugate, GM-CSF, TNFα, IL6 and IL10 standards as well Corning Easy Wash ELISA plates used were obtained from Pharmingen, San Diego, USA.

Binding Solution: Na_2HPO_4 (1 M), adjusted to pH 9.0 with 0.1 M NaH_2PO_4 was made up as a one litre stock solution.

PBS Solution: NaCl I80.0 g, Na2HPO4 (11.6 g), KH_2PO_4 (2.0 g) and KCl (2.0 g) was made up to 1000ml and the pH was adjusted to 7.0 using 1N HCl. All reagents were obtained from Sigma-Aldrich company, Steinham, Germany.

PBS/Tween®: Tween ® 20 (0.5 ml) was made up to 1000 ml in PBS.

Blocking Buffer: A PBS solution containing 10% foetal bovine serum (FBS), 10% newborn calf serum (NBCS) or 1% BSA (immunoassay grade) was prepared. The blocking buffer was filtered to remove particles before use (Sigma-Aldrich company, Steinham, Germany).

Blocking Buffer/Tween®:) To 1000ml Blocking Buffer 0.5 ml Tween 20 ® was added. (Sigma-Aldrich company, Steinham, Germany.)

Substrate Solution: To 500 ml of 0.1 M anhydrous citric acid in dd H_2O; 150 mg 2,2'-Azino-bis- (3-ethylbenzthiazoline-6-sulfonic acid) was added and the pH adjusted to 4.35 with NaOH. Aliquots of 11 ml per vial were made and stored at - 20°C. Prior to use 100 µl 3% H_2O_2 was added. All reagents were from Sigma-Aldrich company, Steinham, Germany.

3% H_2O_2 Solution: To 90 ml of H_2O 10 ml of 30% H_2O_2 was added.The solution was protected from exposure to light by wrapping the tube with aluminum foil.

Stopping Solution: The stopping solution consisted of 20% SDS/50% DMF:

To 50 ml dd H_2O, 50 ml of dimethylformamide (DMF) and then 20.0 g sodium dodecyl sulfate (SDS) (Sigma-Aldrich company, Steinham, Germany), was added.

5.2.7 Quantification of arginase activity in cultured PECs
Solutions:

- 0.1% Triton X-100 (stored at RT)
- 25 mM Tris-HCl, pH 7.5 (stored at 4°C)
- 10 mM $MnCl_2$ (stored at 4°C)
- 0.5 M L-arginine, pH 9.7 (stored at 4°C)
- Acid mix: H_2SO_4, H_3PO_4 and H_2O in the ratio 1:3:7 (v/v/v) (stored at RT)
- α-isonitrosopropionophenone (ISPF) 9% in ethanol (stored at –20°C)
- Stock solution of urea (30mg/ml) in PBS (stored at –20°C)

All above reagents were obtained from Sigma-Aldrich company, Steinham, Germany.

5.2.8 Quantification of Nitric Oxide (NO)

Greiss solution: 0.5% sulphanilamide, 0.5% N-1 Naphtylethylenediamide hydrochloride in 2.5% H_3PO_4, all obtained from Sigma-Aldrich company, Steinham, Germany.

5.3 Methods

PECs used in all the following assays were from C57BL/J6 mice. The isolation was perfomed as described in Chapter 4.
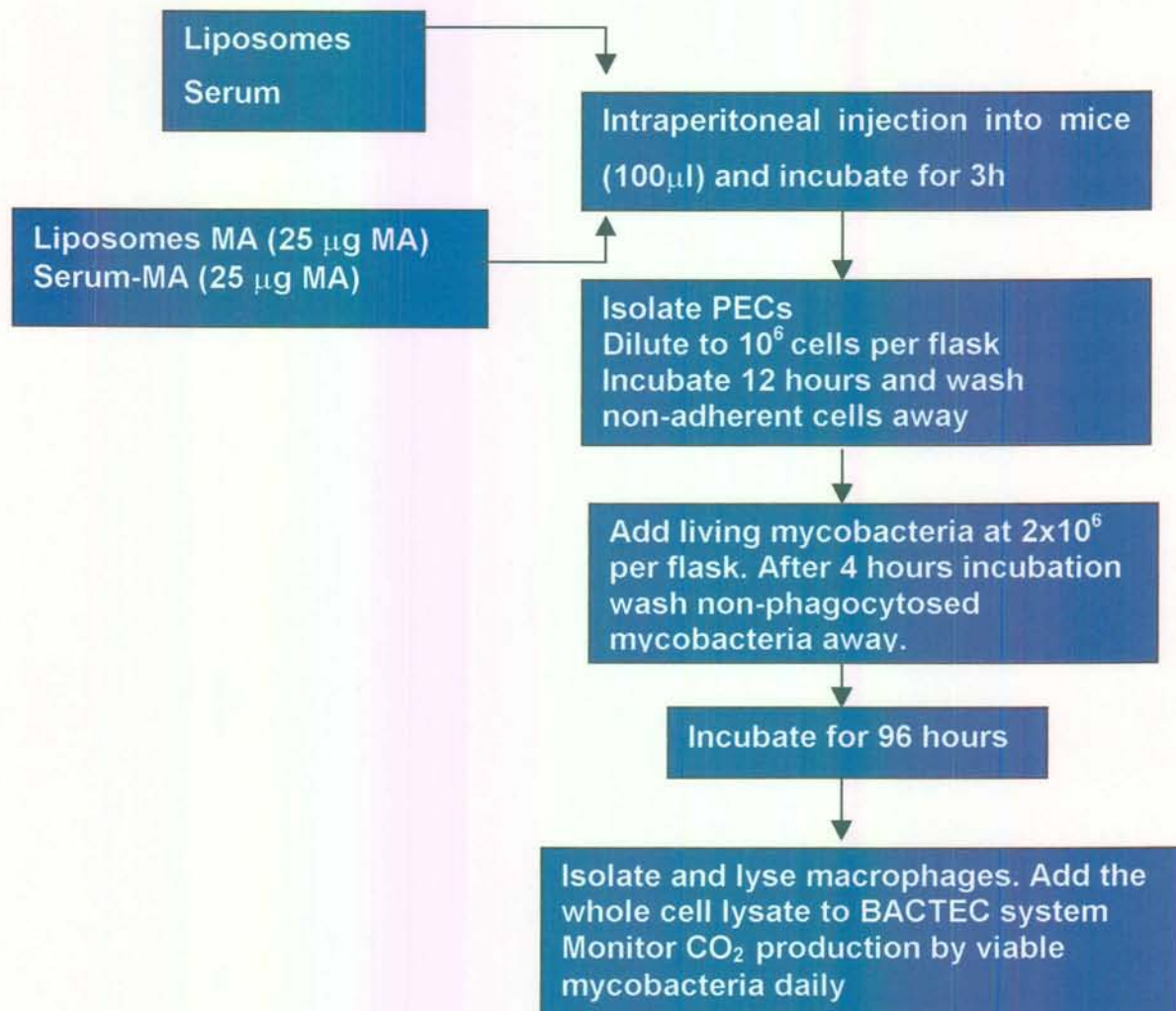
5.3.1 Fluorescein-iso-thiocyanate (FITC) labelling of mycobacteria

Lyophilized mycobacteria were suspended in normal saline. The suspension was spun down to remove aggregates (10 min, 2000 g, 4°C). The supernatant containing single cell bacteria was washed twice again (10 min 2000 g, 4°C). The bacteria were then co-incubated with 0.2 mg/ml FITC in PBS for 2 h at 37°C in the dark, washed twice with PBS, adjusted to a concentration of 1 x 10^7 cfu/ml

and resuspended in RPMI 1640 containing 10% foetal calf serum and 1% v/v penicillin/streptomycin (Durek *et al.*, 1999). Macrophages were loaded *in vivo* with 100μl liposomes and liposomes-MA (25 μg) (see Chapter 4). After 48 hours, PECs were isolated, washed three times and put into culture in 6 well plates (1×10^6 cells in 3ml full medium). Non-adherent cells were removed after 2 hours in culture by washing it 3 times, using full medium at 37°C. Three different concentrations of dead MtbH37Ra bacteria were added to the wells and incubated for 1 hour at 37°C in a humidified atmosphere containing 5% CO_2.

5.3.2 Inhibition of mycobacterial growth in MA treated macrophages

The flow diagram shows the protocol followed in the survival experiment looking at the effects of foam-like cells on *Mycobacterium tuberculosis*.
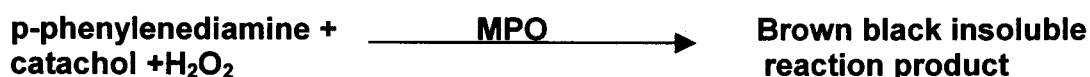
Liposomes
Serum

Liposomes MA (25 μg MA)
Serum-MA (25 μg MA)

Intraperitoneal injection into mice (100μl) and incubate for 3h

Isolate PECs
Dilute to 10^6 cells per flask
Incubate 12 hours and wash non-adherent cells away

Add living mycobacteria at 2×10^6 per flask. After 4 hours incubation wash non-phagocytosed mycobacteria away.

Incubate for 96 hours

Isolate and lyse macrophages. Add the whole cell lysate to BACTEC system Monitor CO_2 production by viable mycobacteria daily

The BACTEC medium (present in an enclosed glass vial) consisted of an enriched Middlebrook broth. It contained a ^{14}C labelled substrate (fatty acid) utilized by the mycobacteria releasing $^{14}CO_2$ into the atmosphere above the medium. The automated BACTEC 460 machine aspirated the $^{14}CO_2$ from the vial through a rubber septum using a needle. Radioactivity was daily quantified and used to plot the growth index. (Middlebrook *et al.*, 1977)

The BACTEC vials were equipped with rubber septums. Care was taken to ensure that leakage of the $^{14}CO_2$ did not take place.

5.3.3 Cytochemical staining of adherent macrophages for MPO

Macrophages were loaded *in vivo* with either 100 µl saline, liposomes or liposomes-MA (25 µg MA) as before. After 48 hours, PECs were isolated, washed three times and put into culture at $5x10^5$ cells in 1ml full medium in 24 well plates, layered with a glass plate at the bottom. Non-adherent cells were removed after 2 hours in culture by washing 3 times using full medium at 37°C. Dead *Mycobacterium tuberculosis* (1×10^7/ml) in full medium was added and incubated for 2h at 37°C in a humidified atmosphere containing 5% CO_2. All non-phagocytosed bacteria were washed away. Macrophages were cultured overnight (16 hours), and then tested for the presence of myeloperoxidase. Cells were washed three times in wash medium at 37°C, and fixed at room temperature for 30 seconds. Slides were then washed using 30 ml water and allowed to dry in the dark. Glass slides were put into myeloperoxidase indicator reagent solution containing Trizmal™ 6.3 buffer concentrate and hydrogen peroxide for 30 minutes at 37°C in the dark, using a water bath.

The reaction consisted of:

p-phenylenediamine +   **MPO**   →   **Brown black insoluble**
catachol +H_2O_2   **reaction product**

Slides were then washed in running water for 30 seconds and examined under a light microscope.

5.3.4 Effect of catalase on cell proliferation of foam-like cells

Macrophages were loaded *in vivo* with either 100 µl liposomes or liposomes-MA (25 µg MA) as before. 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). Non-adherent cells were removed after 2 hours in culture by washing it 3 times using full medium at 37°C. Catalase (10µl of an 800 U/ml suspension in RPMI wash solution) was added to each well. DNA synthesis was measured by incorporation of [methyl-^{3}H] thymidine (TdR) (2 µCi/ml) over 18 hours at 37°C in a humidified atmosphere containing 5% CO_2. Cells were harvested onto glass fiber plates and the incorporated radioactivity was estimated using an Inotech digital autoradiographic counter (Inotech AG Cell Harvester system, Switzerland).

5.3.5 Alexa Fluor 660 (CY5) labelling of catalase

Catalase (10mg) was dissolved in 1 ml 0.1M bicarbonate buffer pH 9. Five milligrams of the Alexa Fluor 660 (CY5) dye was dissolved in 0.5 ml DMF and 100 µl added to the protein solution while vortexing. The protein dye mixture was incubated with continuous stirring for one hour at room temperature, before the coupling reaction was stopped by adding 100 µl hydroxylamine buffer (1.5 M, pH 8.5). Separation of unreacted labelling product from conjugated protein was done using a Sephadex G-25 column, with PBS as eluant. Conjugated protein concentration was diluted to 1mg/ml using PBS and stored at 4°C in the dark until used.

5.3.6 *Ex vivo* loading of macrophages with labelled catalase

Macrophages were loaded *in vivo* with 100 µl liposomes-MA (25 µg) as before. After 24 hours, PECs were isolated, washed three times and put into culture in confocal microscope plates (1x10^5 cells in 200 µl full medium). Labelled catalase (5µl) was added and incubated for 2h at 37°C in a humidified atmosphere containing 5% CO_2. Non-adherent cells and catalase were removed by washing

it 3 times using full medium at 37°C. Cells were then viewed under the confocal microscope (LSM 410 invert, Zeiss, Germany).

5.3.7 Cytokines

GM-CSF, TNFα, IL6 and IL10 determination in supernatant from macrophage cultures

Macrophages were loaded *in vivo* with 100 μl liposomes-MA (25 μg MA) as before. After 48 hours, PECs were isolated, washed three times and put into 24 well culture flasks (1×10^6 cells in 1ml full medium). Non-adherent cells were washed away after 2 hours, LPS or dead MtbH37Ra bacteria added, and the macrophages incubated for 96 hours at 37°C in a humidified atmosphere containing 5% CO_2. Supernatants were removed and frozen at –20°C, and thawed before use in ELISA assay.

5.3.8 ELISA Protocol General Procedure

Capture antibody:

The purified anti-cytokine capture antibody was diluted to 2 μg/ml in binding solution. Diluted antibody (50 μl) was added to the wells of an enhanced protein-binding ELISA plate. The plate was sealed to prevent evaporation and incubated overnight at 4°C.

Blocking:

The plate was brought to RT, the capture antibody solution removed, and non-specific binding blocked by adding 200 μl of blocking buffer per well. The plate was sealed and incubated at RT for 1-2 h. The plate was washed 4 times with PBS/Tween ® .

Standards and Samples:

A standard dilution of GM-CSF, TNFα, IL6 and IL10 ranging from 0 to 1000 ng per ml were made (diluted in blocking buffer/Tween ®). Samples and standards were added at 100 μl per well. The plate was sealed and incubated for 2-4 h at room temperature or overnight at 4°C. It was washed 6 times with PBS/Tween ®.

Detection antibody:

The biotinylated anti-cytokine detection antibody was diluted to 1 µg/ml in blocking buffer/Tween ®. Diluted antibody (100 µl) was added to each well. The plate was sealed and incubated for 1 h at RT. It was washed 6 times with PBS/Tween ®.

Avidin-Horseradish Peroxidase (Av-HRP):

The Av-HRP conjugate was diluted 1:10 000 in blocking buffer/Tween ®. 100 µl per well was added, the plate sealed and incubated at RT for 30 min. It was washed 6 times with PBS/Tween ®.

Substrate:

2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) substrate solution was thawed within 20 min of use. To this 100 µl of 3% H_2O_2 per 11 ml of substrate was added and vortexed, and the mixture immediately dispensed at 100 µl per well. It was incubated at RT (5-80 min) for colour development. The colour reaction was stopped by adding 50 µl of stopping solution. The optical density (OD) for each well was read with a microplate reader set to 405nm.

5.3.9 Quantification of arginase activity in cultured PECs

- **Culture of adherent cells:**

  PEGs were loaded *in vivo* with PBS, liposomes or liposomes-MA (25 µg) and isolated as before. The isolated PECs were then seeded at 1×10^6 / 500µl cells in 48 well plates and incubated to adhere for 4 hours at 37°C. All non-adhered cells were washed away with medium without serum.

- **Preparation of cell-lysate:**

  To each well 500µl PBS (room temperature) was added and the plate centrifuged at 1200 rpm at 4°C for 10 minutes. The supernatant was removed and 50 µl 0.1% Triton was added. The 48 well plate was then shaken for 30 min at room temperature and the cell lysate was either used directly or stored at -20°C in the dark until used.

- **Release and activation of arginase:**

    To a thin walled PCR tube 17μl of a 25 mM Tris-HCl, 6 μl of a 10 mM MnCl₂ and 17μl cell-lysate were added, mixed and incubated for 10 min at 56°C (PCR machine) to activate the arginase enzyme. A quickspin was performed to collect all the fluid at the bottom of the PCR tube.

- **Arginine hydrolysis:**

    To each tube 40 μl of a 0.5 M L-arginine solution was added and mixed. This solution was then incubated for 60 min at 37° before the reaction was stopped by adding 320 μl acid mix.

Remarks:

- A ten times dilution series of urea (in PBS) ranging from 500 – 5 x 10^{-8} mM was made and used as a standard for the quantification of urea.
- To each 80 μl standard urea solution 320 μl acid mix were added and incubated for 1 h at 37°C.

- **Quantification of urea**

    To each of the 400 μl samples or standards solution, 16 μl 9% ISPF solution was added and incubated at 95°C for 30 min. The samples were removed and left in the dark at room temperature for 10 min after which 200 μl of each sample were transferred to a 96-well plate. The colour change was recorded at 540 nm.

    One unit enzymic activity is defined as the amount of enzyme that catalyses the formation of 1 μmol of urea per minute (and hence 60 μmol per 60 min). As a consequence, one unit of enzyme would generate a concentration of urea of 60 μmol/416 μl = 144 μmol/ml = 144mM.The number of units of enzymatic activity in the total enzymic extract could then be obtained via the formula:

$$EA = \frac{Cu \times Vt}{Ve \times P} \times 1000$$

With:

- o   EA = Enzymic activity (in mU)
- o   Cu = Concentration of urea in the 416 μl reaction mix (in mM)
- o   Vt = Total volume of the enzymic extract (i.e. 50 μl)
- o   Ve = Volume of the extract that was tested (i.e. 17 μl)
- o   P   = Concentration of urea produced by 1 U of enzyme (i.e. 144mM/unit)

5.3.9 Quantification of Nitric Oxide (NO)

Cell-free supernatant (100μl) was added to an equal amount of Greis solution. After 10 min incubation at room temperature, the A_{540} was recorded. A standard curve was generated with known concentrations of $NaNO_2$ in culture medium.

5.4 Results

5.4.1 Fluorescein-iso-thiocyanate (FITC) labelling of mycobacteria

The ability of MA to enhance phagocytosis was tested. For this purpose phagocytosis of dead mycobacteria by MA-induced foam-like cells and macrophages treated with liposomes, were compared. Binding of the bacteria was assessed at 4°C, and binding and uptake at 37°C, using the flow cytometer. The histograms in the graph relate to the percentage of 5000 cells that bound to or took up fluorescent bacteria, upon adding three different amounts of dead bacteria to the culture.
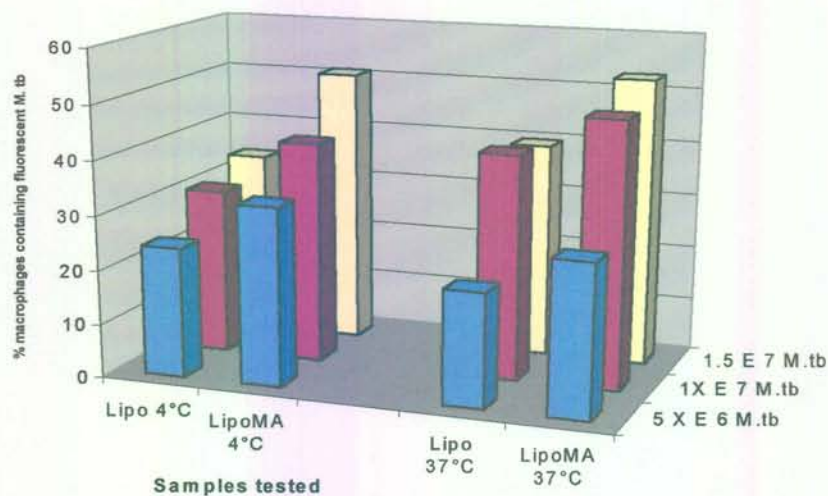

Figure 5.5. Binding (4°C) and uptake (37°C) of FITC labelled MtbH37Ra. No statistical evaluation was done on these samples.

From the data in Figure 5.5, it is seen that about 10 % more cells from the liposomes-MA treated group bound bacteria than those treated with liposomes only (looking at the experiment performed at 4°C). When the cells were also allowed to take up the bacteria at 37°C, then a bacterial concentration-dependent saturation of binding and uptake occurred, but with the MA-treated group remaining at the 10% higher score. It is not known why binding and uptake score lower than binding only at the lowest concentration.

A decay of fluorescence after uptake and chemical attack by the macrophages on the fluorescent bacteria is not excluded. Moreover, this method doesn't compensate for more than one labelled mycobacterium entering the same macrophage. At low concentrations of bacteria, this event will be less frequent.

5.4.2 Inhibition of mycobacterial growth in MA treated macrophages

In order to determine whether MA pretreatment has an effect on the ability of macrophages to kill intracellular mycobacteria, the intracellular survival of *M. tuberculosis* in cultures of macrophages, preloaded *in vivo* with liposomes or liposomes-MA, was measured.

From the data in Figure 5.5, we already know that foam-like cells (cells that received MA), bind and take up at least the same amount of mycobacteria, as macrophages which were treated with liposomes alone. Using BACTEC, the intracellular survival of mycobacteria in foam-like cells was compared to that in macrophages that received only liposomes.

The growth potential of mycobacteria surviving intracellularly after 96 hours of culture, is shown in Fig. 5.6.
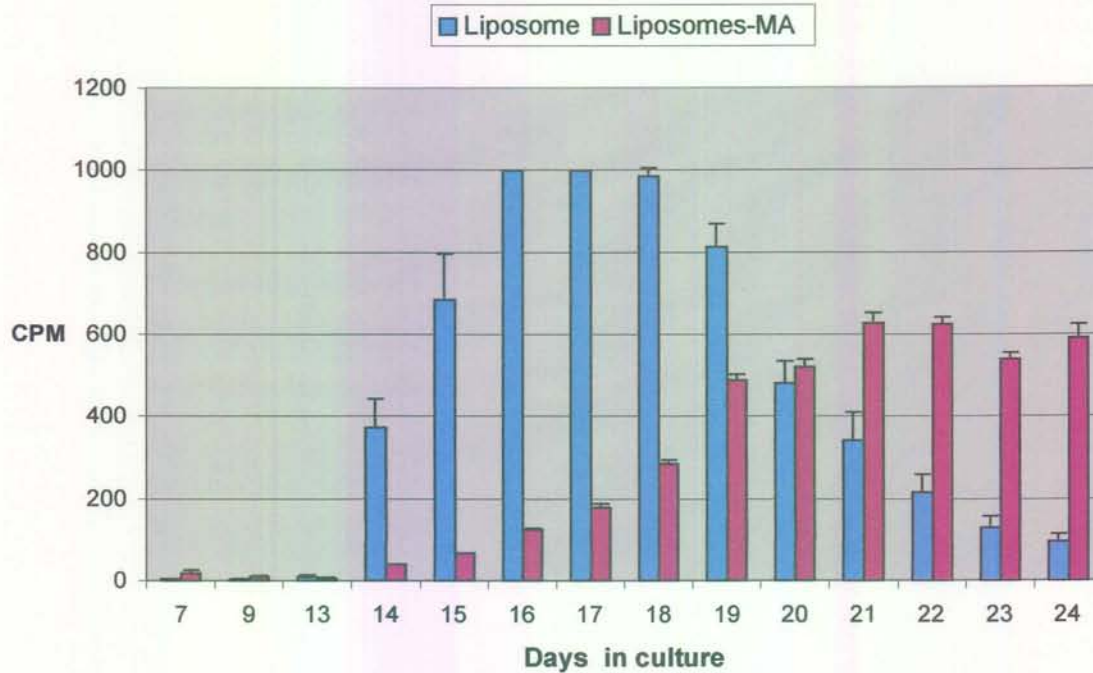
Figure 5.6. Differences in growth potential of *M. tuberculosis* surviving intracellularly in macrophages loaded with liposomes (blue bars) or liposomes-MA (red bars). Each bar represents the mean of 3 samples +/- s.d.

It is seen that the growth of bacteria surviving in macrophages that received only liposomes, peaked at day 16. For bacteria surviving in macrophages loaded with MA (foam-like cells), growth peaked at 21 days and the amount of CO_2 production never reached the same level as observed for the liposome-loaded macrophages. After day 18, the medium was spent and growth was not sustained anymore. At day 16, an almost 9-fold difference in CO_2 production was observed.

If a different carrier was used (serum), the same tendency was seen (results not shown). In Figure 5.7 the two different experiments are compared for relative growth index.
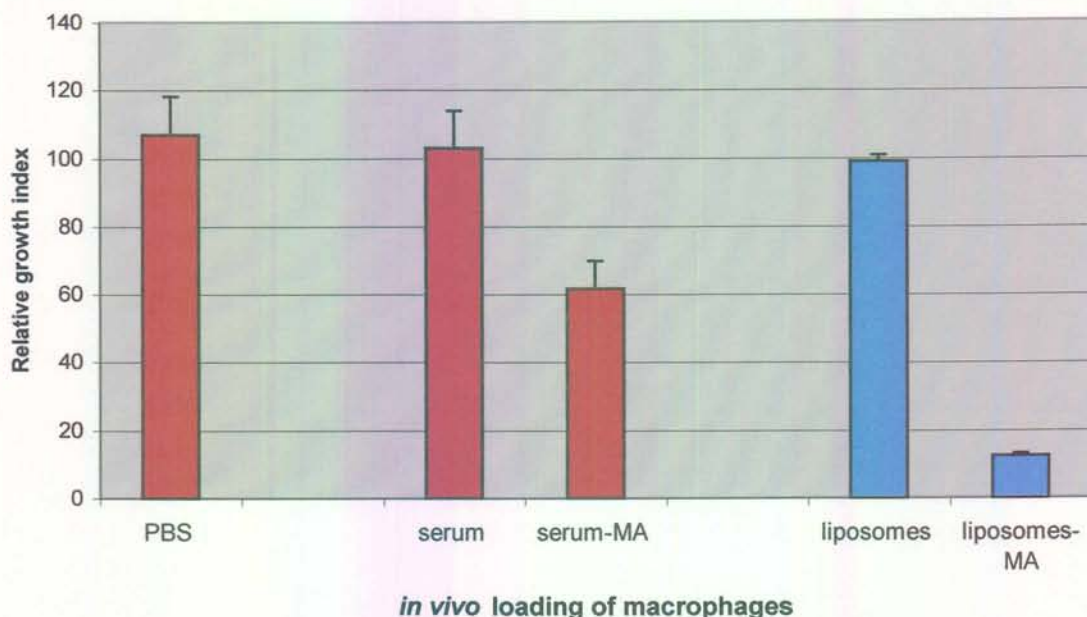
in vivo loading of macrophages

Figure 5.7. Inhibition of growth by *Mycobacterium tuberculosis* surviving intra-
cellularly in macrophages loaded *in vivo* with PBS, serum/serum-MA
(day 10) or liposomes/liposomes-MA (day 16) in 2 separate
experiments (red vs. blue bars). Each bar shows the mean +/-
standard deviation of three to five samples.

From Figure 5.7 it is seen that MA pretreatment reduces the growth potential of
intracellular *M. tuberculosis*, regardless of the carrier used. With serum-MA, a
40% inhibitory effect was seen, whereas with liposomes-MA, an 88% inhibitory
effect was seen.

5.4.3 Cytochemical staining of adherent macrophages

To determine how MA affect the increased binding and uptake of mycobacteria in
macrophages, cells that were treated with MA, liposomes alone, or PBS alone
were exposed to dead mycobacteria in cell culture. The morphology of the cells
was determined after 4 hours, as well as their MPO-activity. Activation of the
MPO pathway of radical formation would imply an enhanced bactericidal activity
induced by MA-pretreatment of the macrophages. The results of microscopy
shown in Fig. 5.8, are statistically summarized in Fig. 5.9.
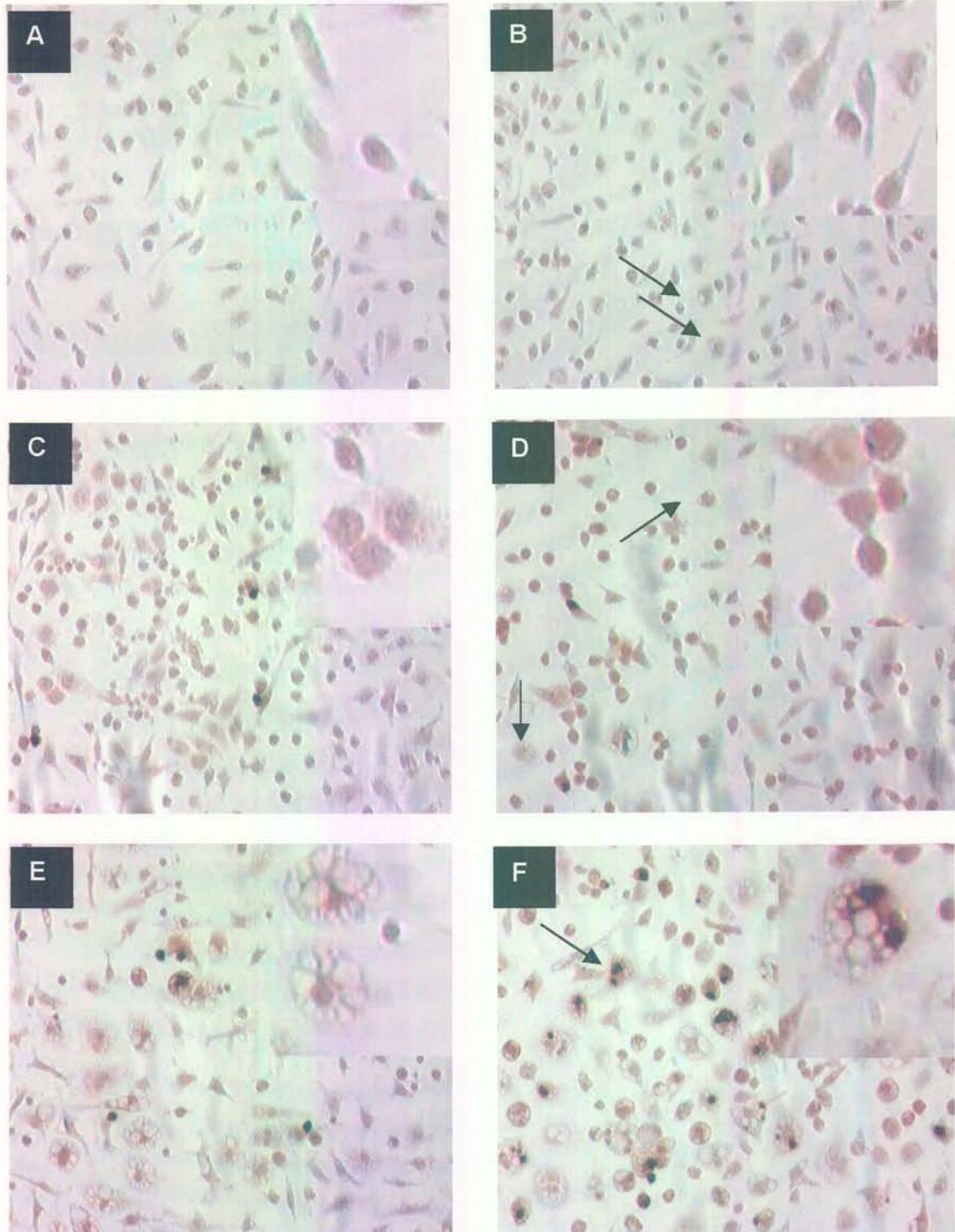
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Figure 5.8. Cytochemical staining for MPO of adherent macrophages. (A) native-(PBS) macrophages (B) dead TB added to native (PBS) macrophages (C) Liposome treated macrophages (D) dead TB added to liposome treated macrophages (E) liposome-MA treated macrophages (F) dead TB added to liposome-MA treated macrophages. Arrows refer to remarks in the text.
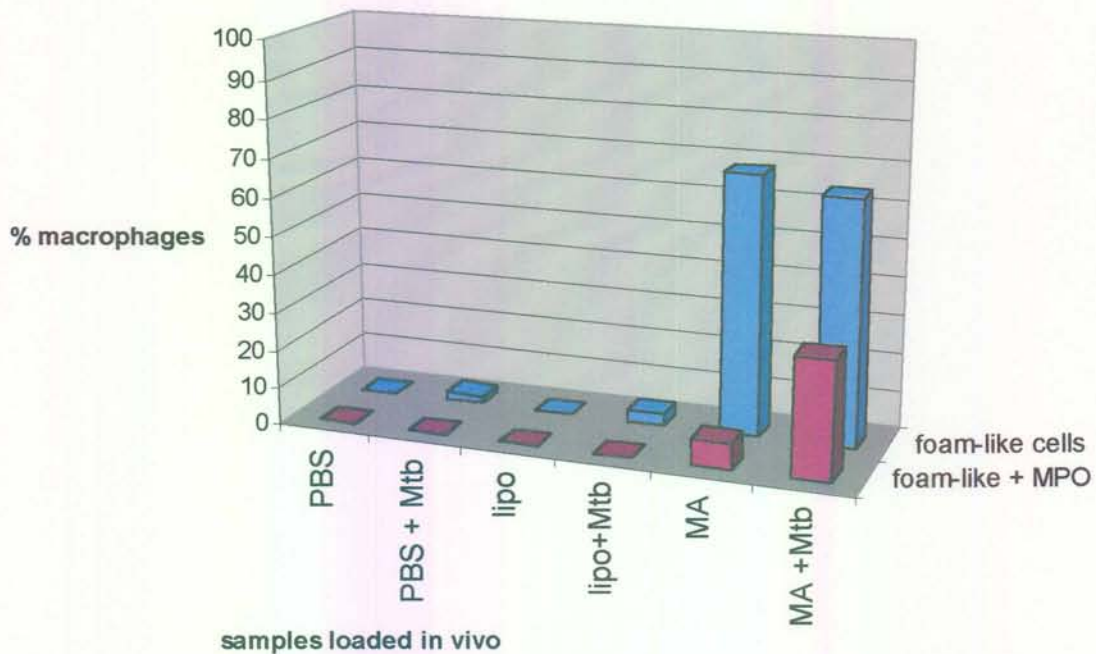
Figure 5.9. Statistics of MPO-staining of macrophages as depicted in Figure 5.8. Three hundred cells for each point were counted under the microscope and plotted.

From Figures 5.8 and 5.9 the following observations can be made:

- (A) Native (PBS treated) macrophages did not contain vacuoles and did not display activation morphology. The macrophages did not stain positive for MPO.

- (B) The addition of dead mycobacteria did not increase MPO staining. Cells (2%) containing small vacuoles were demonstrated (see arrows, Fig.5.8 B).

- (C) If macrophages were loaded with liposomes, they looked similar to native macrophages and did not contain vacuoles. No signs of activation were found.

- (D) By adding *Mycobacteria tuberculosis* to the liposome-loaded macrophages, some cells assumed activation morphology (3%), similar to that seen with native (PBS) treated macrophages exposed to

mycobacteria (B). The presence of MPO was not increased compared to (A) and (B) (see arrows).

- (E) Pre-loading of macrophages with liposome-MA, changed 66% of all the cells into foam-like cells. Huge vacuoles were present in the macrophages. A small amount of cells (7%) stained positive for MPO, all of which were foam cells. If only foam-like cells were counted, this represented 10% that had MPO activity.

- (F) By adding *Mycobacteria tuberculosis* to the foam-like cell cultures, MPO presence was detected in 31% of all cells, as seen by the black precipitate inside the vacuoles (see arrow). If only foam-like cells were taken into consideration, this represented 49% that had MPO activity.

Integrating these results, it seems that MA pre-treatment of cells primed them to a state of activation, but high expression of MPO only took place if another additional stimulus was given, in this case the adding of bacteria. The same effect of increased MPO inside MA induced macrophage foam cells was seen 2 hours after adding concanavalin A to the culture and determining MPO presence as before (results not shown).

No changes were detected in macrophage size or morphology if either liposomes or PBS were loaded into macrophages. Proliferation, or suppression, as measured by thymidine uptake or DNA replication, could not be detected in macrophages that received PBS and liposomes (from Chapter 4). From this it seems that liposomes made from phosphatidylcholine do not activate macrophages after uptake. Looking at the secretion of MPO, no change was detected in the PBS or liposome treated macrophages.

5.4.4 Effect of catalase on cell proliferation of foam-like cells

It is an established fact that macrophage proliferation occurs in tissues of the body, probably mediated by antigen stimulation. Reactive oxygen intermediates play an important role in the process (Kunsch and Medford, 1999).

The contribution of H_2O_2 to the MA-induced proliferation was estimated by adding catalase to the cell culture and subsequently determining the replication rate. Macrophages loaded with liposomes and liposome-MA *in vivo*, were put in culture in the presence or absence of catalase, and evaluated for proliferation by using [methyl-^{3}H] thymidine. As the foam-like cells were already formed, catalase didn't influence their morphology (results not shown).
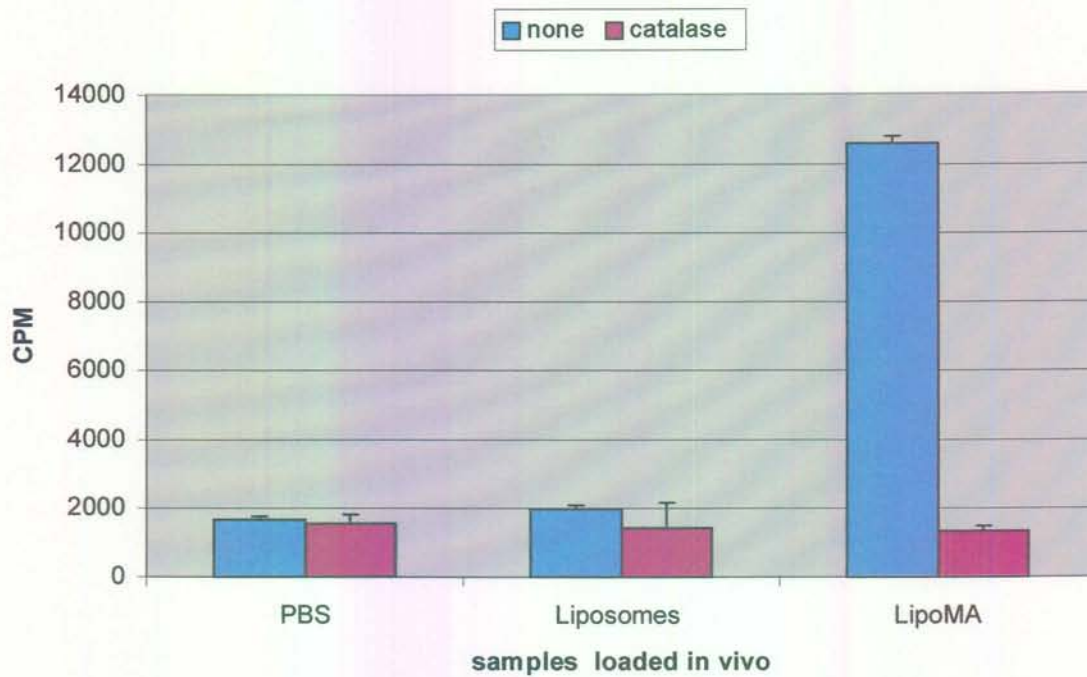

Figure 5.10. Effect of catalase on the uptake of [methyl-^{3}H] thymidine in macrophages loaded with PBS, liposomes or liposomes-MA. Each bar represents the mean (+/- s.d.) of 5 values.

No difference in [methyl-^{3}H] thymidine uptake was seen in cultures that received PBS or liposomes (Fig 5.10). Also, no change in uptake of [methyl-^{3}H] thymidine was seen if catalase was added to these cultures. As seen before in Chapter 4, the *in vivo* loading of macrophages with MA increases the uptake of [methyl-^{3}H] thymidine 6 to 10 fold (experiment was repeated five times). When catalase was added to the culture, [methyl-^{3}H] thymidine uptake was diminished to values similar to that seen in macrophages treated with liposomes alone (Fig 5.10). No

[methyl-^{3}H] thymidine incorporation was seen in PBS and liposome-loaded macrophages if catalase was added.

From this one can conclude that MA-induced proliferation of foam cells proceeded though H_2O_2 and derived ROI.

5.4.5 *Ex vivo* loading of macrophages with CY5-labelled catalase

To establish the fate of the catalase in cell cultures of MA-loaded macrophages, a study using fluorescence-labelled catalase was performed out.

Macrophages were loaded *in vivo* with liposomes or liposomes-MA and cultured *ex vivo* in the presence of CY5-labelled catalase. Fig. 5.11 shows the superimposed images of fluorescence and light microscopy.
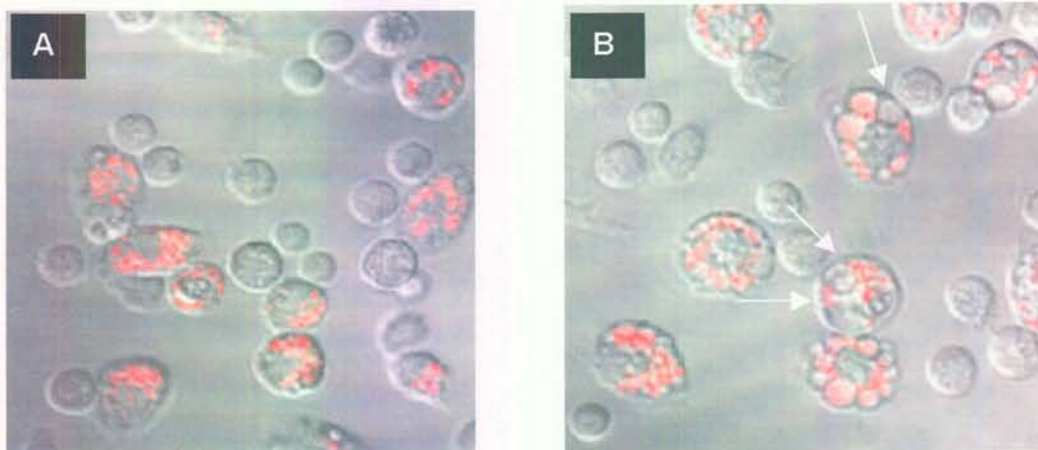

Figure 5.11. Fate of Cy5-labelled catalase in A) liposomes, B) liposomes-MA loaded macrophages. The white arrows indicate vacuoles without any labelled catalase.

From Figure 5.11 it is seen that foam-like cells accumulate catalase in the vacuoles, although not in all of the vacuoles (see white arrows). Combining results from Figures 5.10 and 5.11, it seems that catalase was actively taken up into the vacuoles of foam-like cells and had an influence on uptake of thymidine, which represents active proliferation of the macrophages. By lowering the availability of hydrogen peroxide, proliferation of foam-like cells also disappear.

These results imply that the effect of catalase addition on macrophage proliferation may not be limited to the removal of extracellular H_2O_2. It is not excluded that catalase may retain its activity after uptake in the vacuoles. In

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addition, this experiment shows that the vacuoles of MA-induced foam cells remain physiologically involved in protein uptake.

5.4.6. Cytokine profiles from culture supernatants of macrophages

5.4.6.1. GM-CSF and TNFα

As GM-CSF expression is associated with macrophage proliferation and is found at sites of inflammation, foam-like cells were examined for the presence of this growth factor. TNFα is a pro-inflammatory cytokine that is often expressed in combination with GM-CSF, e.g. in the lungs of TB-infected mice. GM-CSF and TNFα-levels in culture supernatants were determined by ELISA.
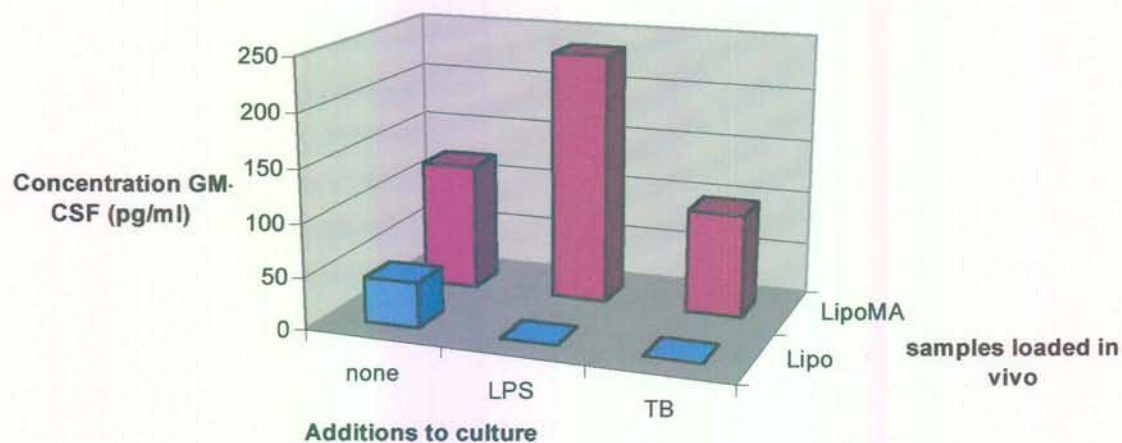

Figure 5.12. GM-CSF values from culture medium of macrophages preloaded *in vivo* with liposomes and liposomes-MA. Cultures were also carried out in the presence of dead MtbH37Ra bacteria or LPS. Results represents single values that were reproducible in at least 2 experiments.

Figure 5.12 shows an increase of GM-CSF in the culture supernatant of macrophages preloaded with liposomes-MA, as compared to macrophages pre-loaded with liposomes. Addition of LPS, but not dead mycobacteria, enhanced

the production of GM-CSF by liposomes-MA loaded macrophages. These additions did not induce the production of GM-CSF in cultures of macrophages preloaded with liposomes.

The ELISA results for TNFα are shown in Fig. 5.13.
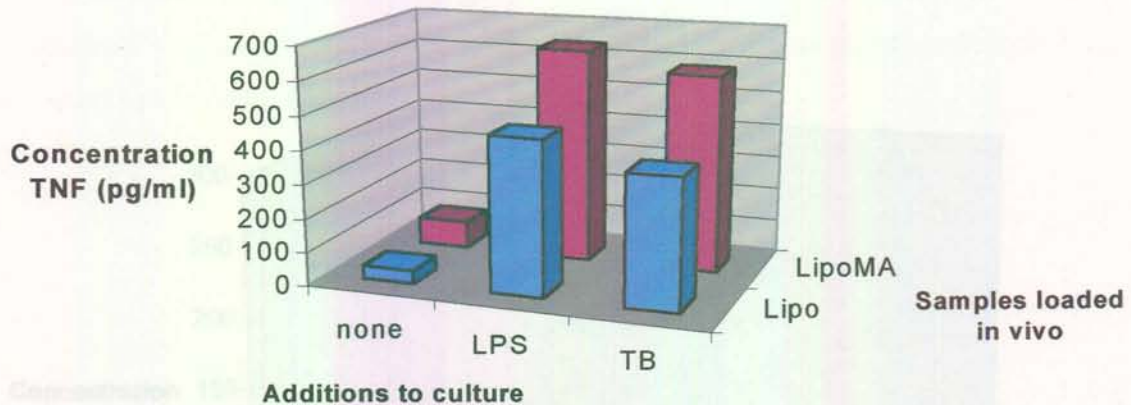

Figure 5.13. TNFα concentration detected in supernatants from macrophage cultures loaded *in vivo* with liposomes or liposomes-MA. Cultures were also carried out in the presence of dead MtbH37Ra or LPS. Results represent single values that were reproducible in at least 2 experiments.

The TNFα values only increased slightly with loading of MA. Dead MtbH37Ra bacteria added to the culture, increased TNFα concentration almost as high as LPS addition. This increased TNFα effect was seen in practically equal proportions in liposome and liposome-MA loaded macrophages. These results show that MA pretreatment of macrophages induced a GM-CSF response without a significant up-regulation of TNFα. Whereas the GM-CSF response to LPS appears to be primed by prior exposure of the macrophages to MA, this two-step response is not observed for TNFα. With the latter, a strong response to both LPS and killed TB bacteria was observed, irrespective of preliminary priming with MA. In none of the MA loaded macrophage cultures could increased levels of INFγ be detected (results not shown).

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These results demonstrate a unique immunostimulatory property of MA. Based on this, other cytokine profiles were also titrated to obtain a better assessment of the type of immunoregulation induced by MA.

5.4.6.2 IL6 and IL10

Whereas IL6 is a pro-inflammatory cytokine, IL10 is generally known as an anti-inflammatory cytokine.
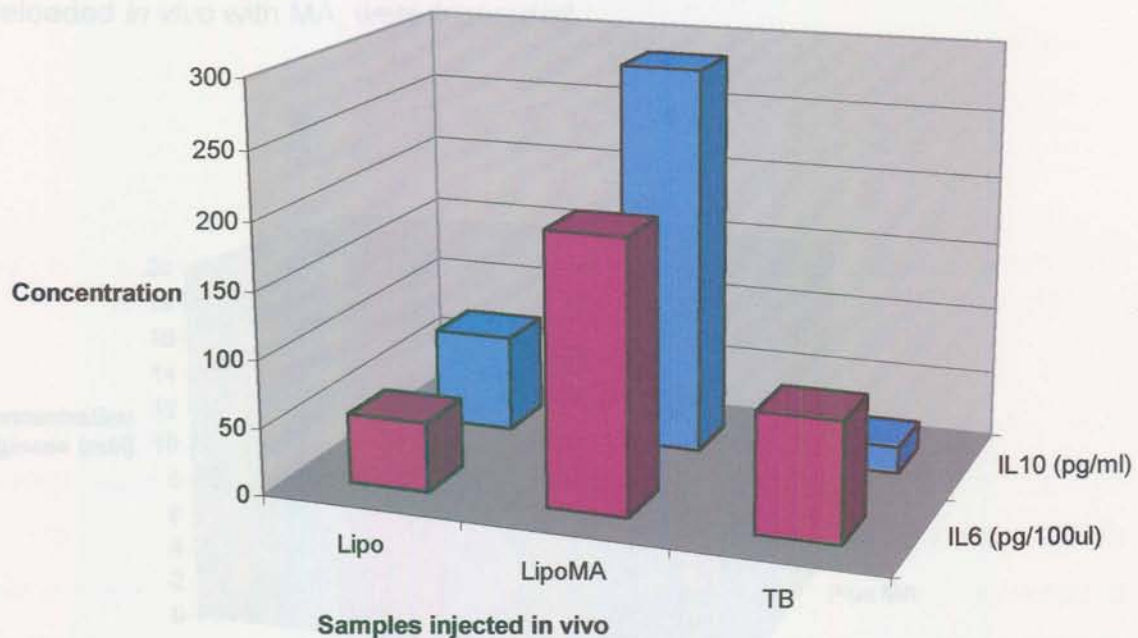

Figure 5.14. IL6 and IL10 concentrations from macrophage cultures loaded *in vivo* with liposomes, liposomes-MA or dead MtbH37Ra bacteria. IL10 is given in pg/ml and IL6 in pg/100 μl. Results represent single values that were reproducible in at least 3 experiments.

From Figure 5.14, it is seen that in MA-pretreated macrophage cultures, both IL6 and IL10 are increased. The amount of IL6 and IL10 detectable in culture medium of macrophages loaded *in vivo* with dead MtbH37Ra bacteria, is about as low as for liposome loaded cells. These results imply that MA induces both pro-inflammatory (GM-CSF, IL6) and anti-inflammatory (IL10) cytokines.

5.4.7 Arginase concentration detected in macrophages from culture

Classically activated macrophages produce NO from arginine as a killer molecule via nitric oxide synthetase as a Th1 response to infection. Alternatively activated macrophages use arginine to produce ornithine. When arginase is the arginine convertase, nitric oxide synthetase is not activated to produce NO. To determine whether MA-induced activation of macrophages has characteristics of alternative or classical activation, NO and arginase levels in cultures of macrophages preloaded *in vivo* with MA, were measured.
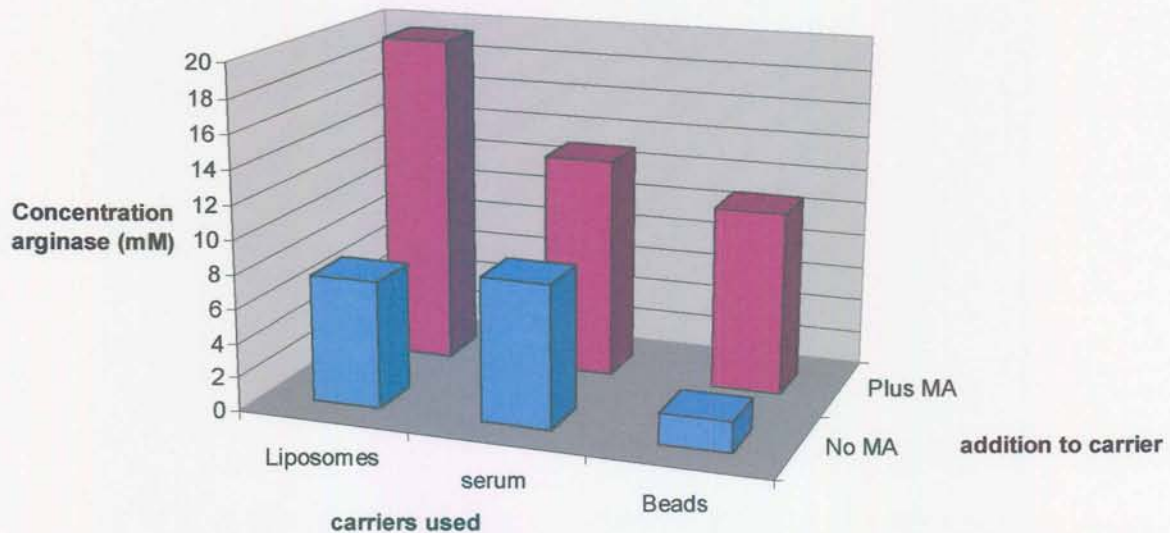

Figure 5.15. Arginase determination from adherent macrophages after MA loading *in vivo,* using different carriers. Results represent the mean of 4 values that were reproducible in at least 2 experiments.

Figure 5.15 shows the arginase difference found in macrophages when different carriers were used in the *in vivo* loading. MA-loaded macrophages (foam cells) showed an increase in arginase concentration as compared to the controls in Figure 5.15. Equal numbers of cells were used in the arginase assay, but

different arginase levels were found in the samples. The liposome and serum carriers induced almost equal concentrations of arginase, compared to beads that induced very little arginase. The observed increase of arginase activity was not always reproducible. At least in lower liposome containing cholesterol-MA mixture, no arginase activity increase was found. This was observed two times.

The arginase determination protocol makes two assumptions. The assay is based on the number of macrophages present after loading liposomes and liposomes-MA for 48 hours *in vivo*. The PECs are counted and equal amounts of cells are put into culture. After 2 hours, all non-adherent cells are removed by washing. Fresh medium is put onto half of the samples for NO determination, and the other half is used for arginase assay. By comparing the liposome and liposome-MA sample from the peritoneal cavity of the mouse, it was assumed that no differences in the size of the macrophage population took place over two days. Moreover, as foam-like cells are larger, the influence of protein content was not taken in consideration for correction. The question is: should protein content be taken into account, considering that foam-like cells, which consist almost entirely of vacuoles filled with protein (as seen in Figure 5.8), may give a false value to the assay?

In a separate experiment, NO and arginase production by macrophages preloaded *in vivo* with liposomes or liposomes-MA, were compared. The results are shown in Fig. 5.16.
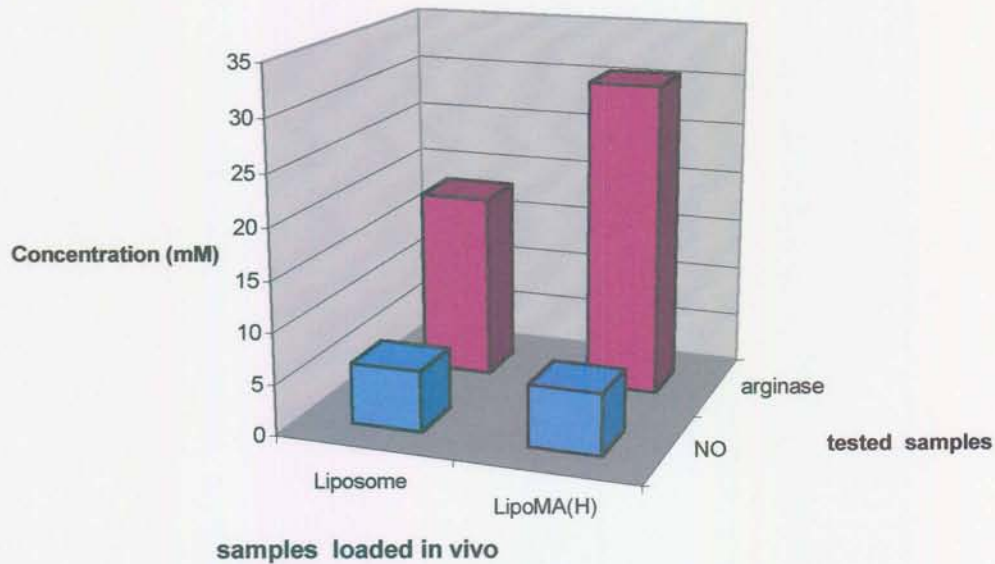
Figure 5.16. NO and arginase production by macrophages that were loaded *in vivo* with liposomes or liposomes-MA. Results represent single values that were reproducible in at least 2 experiments.

Macrophages preloaded with liposomes-MA produced more arginase than macrophages preloaded with liposomes. However, MA pretreatment did not induce an increase in NO production. In a time study performed (results not shown here) it was confirmed that during ties of 2, 24 and 48 hours no change in the amount of NO was detected and the values did not go higher than 5 mM at any time. In this respect, MA-treated macrophages show characteristics of alternative activation, rather than classical activation.

5.5 Discussion

In previous work done at the University of Pretoria on the protective role of MA pre-treatment against *Mycobacterium tuberculosis* infection after administration in mice, some form of protection was found (Pretorius, 1999). This *in vivo* protection corroborated the results presented here, where *in vitro* inhibition of *Mycobacterium tuberculosis* growth in macrophages (PECs) was observed upon MA administration. Whereas the *in vivo* experiments used serum as MA carrier,

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here both serum and liposomes served as carriers for MA to inhibit the mycobacterial growth in macrophages.

One would expect that growth inhibition of *Mycobacterium tuberculosis* occurs by pro-inflammatory mechanisms utilizing ROI, as this is one of the known mechanism for destruction of mycobacteria by the immune system. Borelli *et al.* (1999) showed that MPO from neutrophils, in the presence of hydrogen peroxide, exerts a consistent killing activity against *Mycobacterium tuberculosis*. It was further shown that the killing effect of the MPO was not specific to the strain of *Mycobacterium tuberculosis* that was used, and the activity was found to be time and dose dependent, requiring the presence of chloride ions in the assay medium. Myeloperoxidase, superoxide as well as other ROI's produced by monocytes, macrophages and neutrophils take part in killing of organisms in the phagosomes. However, even today the precise mechanism of killing is not clear (Saran *et al.*, 1999)

Mycobacterium tuberculosis has been shown to be highly resistant to hydrogen peroxide (Laochumroonvorapong *et al.*, 1996, Manca *et al.*, 1999). This hydrogen peroxide resistance seems to be mediated by the catalase-peroxidase protein (KatG) encoded by the gene *katG*. Strains of *Mycobacterium tuberculosis* without KatG expression or catalase activity were sensitive to killing with hydrogen peroxide. However, once catalase activity is present, even at minimal amounts increased survival of *Mycobacterium tuberculosis* was found (Manca *et al.*, 1999).

In the work presented here, it was shown that MA induced the MPO pathway in foam cells, by the positive staining of MA-induced foam cells for MPO. This was enhanced by the subsequent addition of dead *Mycobacterium tuberculosis.* The implication is that the MPO activation signal is amplified by a second signal that is triggered by dead mycobacteria.

Burdon *et al.* (1995) showed that superoxide and hydrogen peroxide inside cells have a growth stimulatory effect. Both these products were found during the respiratory burst in the pathway initiated by the activity of plasma membrane NADPH-oxidase of activated macrophages and neutrophils. Catalase, which destroys the H_2O_2 intermediate in this pathway, was found to abrogate foam cell proliferation. This was in support of an MA-induced ROI pathway towards foam cell proliferation.

Foam cells are also brought about by oxLDL uptake in macrophages. Rajavashisth *et al.* (1990) as well as Biwa *et al.* (1998) demonstrated that the presence of oxLDL could increase the presence of IL1and GM-CSF. IL1 is known to induce the ROI pathway. Reactive oxygen intermediates (ROI) can also be influenced by TNF signaling. TNF is usually produced by macrophages during an inflammatory response, and exhibits both protective and pathologic effects in *Mycobacterium tuberculosis* infection. Human alveolar macrophages and mononuclear cells produce TNFα in large quantities in response to *M. tuberculosis* (Valone *et al.*, 1988).

A simplified pro-inflammatory pathway of MA-induced foam cell formation concomitant with bactericidal activity does not hold. By measuring the inflammatory cytokines GM-CSF and TNFα of MA-induced foam cell cultures, it was found that GM-CSF was induced, but TNFα was only marginally up-regulated. Compared to LPS or dead mycobacteria, liposome-MA only induced small amounts of TNFα.

The effect of MA may also be indirect. It could induce macrophages to form extracellular LDL, which are then taken up by the macrophages/foam cells. This leads to large increases in GM-CSF expression (Biwa *et al.*, 1998). This action is highly specific for oxLDL, as AcLDL or lysophosphatidylcholine (lyso-PC) containing LDL did not induce GM-CSF (Biwa *et al.*, 1998). In a murine

macrophage culture loaded with oxLDL, GM-CSF peaked at 4 hours and then decreased over 24 hours to reach basal levels.

By comparing IL6 and IL10, the expected up-regulation of the pro-inflammatory IL6 and down-regulation of the anti-inflammatory IL10 was observed with PECs pre-loaded with dead *Mycobacterium tuberculosis*, but this profile was reversed in the MA-induced foam cell cultures. The apparently contradictory outcome of our experiments concerning the presence of increased IL10, low IL6 as well as increased TNFα, needs an explanation. Based on the above, it was thought likely that MA induced the alternative pathway of activation of macrophages.

From the literature IL6 is increased up to 10 000 times in cultures of *Mycobacterium tuberculosis* infected macrophages, compared to uninfected controls (VanHeyningen *et al.,* 1997). These authors found that mycobacteria had to be metabolically active to induce this large IL6 induction. This data suggests that the organism must synthesize or process an effector molecule responsible for IL6 production. At this stage, it is not clear what role IL6 plays in *Mycobacterium tuberculosis* infection.

From the literature, *Mycobacterium tuberculosis* induces IL10, which inhibits the Th1 response to the infection, probably by inhibiting the synthesis of INFγ by NK cells and macrophages (Kaufmann, 1995, Gong *et al.,* 1996). Production of IL10 may be part of an alternatively activated macrophage system, in which the Th1 role is downplayed to protect the lungs from disseminated necrotic damage (Goerdt *et al.,* 1999). In this process, macrophages preferentially express the receptors of innate immunity, which have a broad specificity for foreign antigens. IL10 suppresses the immune response by down-regulation of the MHC molecules and inhibition of the production of monokines.

Usually alternatively activated macrophages do not secrete high amounts of IL6. Interleukin 6 decreases lipoprotein lipase (LPL) and monomeric LPL levels in

plasma, which leads to increases in macrophage uptake of lipids (Yudkin *et al.*, 2000). IL10 was originally described as a Th2 cytokine, but is also secreted by alternatively activated macrophages and human Th1 cells (Abbas *et al.*, 1996).

To find more supporting evidence of alternative activation as a mechanism for MA-effects on macrophages, arginase activity was determined in MA-derived foam cells. Arginase was increased in some experiments and NO production was not increased. The variability of the results could be influenced by different carriers, and different constitutions of liposomes, such that not all MA loaded macrophages expressed increased amounts of arginase. These results therefore do not exclude alternative activation as a mechanism of MA-effects on macrophages, but do not unequivocally support it either.

Increased arginase would fit the profile of alternatively activated foam cells inducing macrophages towards becoming bactericidal in nature to combat mycobacteria. Alternatively activated macrophages also do not produce NO, but rather have an increased expression of arginase, which counteracts the NO synthetase production of NO. Moreover, the cytokine profile for alternative activated macrophage shows an IL10 activation compared to classical activation producing IL6, IL12 and TNFα (Goerdt *et al.*, 1999).

In summary, the effect of MA, at least in peritoneal macrophages, to protect against *Mycobacterium tuberculosis,* is to convert the cells into foam cells that have some characteristics of alternative activation. This is supported by pro-inflammatory cytokine secretion of GM-CSF and IL6, but kept under control by a strong up-regulation of IL10. Whether this mechanistic profile would also apply to alveolar macrophages, (most common place of *Mycobacterium tuberculosis* infection) remains unanswered in this chapter. However, a mechanistic model has been developed for the PECs model that can be directly tested in alveolar macrophages.

CHAPTER 6

Summary

Mycobacterium tuberculosis is an intracellular bacterial pathogen that preferentially resides inside resting macrophages. Once activated, the macrophage becomes the most important effector cell against infection. The bioactive, complex outer wall of *Mycobacterium tuberculosis* elicits various effects from the immune system. Components of the mycobacterial cell wall are obvious candidates for study, since this is the interface between the immune system of the host and the mycobacteria. The cell wall determines which mycobacterial components can reach the host and which host components are accrued by the bacteria, thereby influencing the host immune response and relevant potential drug treatments (Daffe and Draper, 1998).

Isolated cell walls of mycobacteria retain the shape of the mycobacteria, indicating that the cell wall determines the size of the bacteria. The shape-forming properties of the cell wall are attributable to peptidoglycan. In mycobacteria this closely resembles the peptidoglycan found in other bacteria. A phospodiester bond attaches a branched-chain polysaccharide, the arabinoglycan. At the distal ends these arabinoglycans are esterified to mycolic acids. Mycolic acids are 1-alkyl-2-hydroxy fatty acids with a typical carbon length of 90 carbons containing cyclopropyl-, methoxy-, keto- and methyl groups. The alkyl branch is commonly 24 carbons in length. This size and structure of mycolic acids are unique to mycobacteria. Peptidoglycan-arabinoglycan mycolate forms the so-called cell wall skeleton, which is also associated with various lipids, glycolipids and proteins (Daffe and Draper, 1998).

Constituents of all parts of the envelope have biological activity, which may be relevant in the combined immune response to the organism. By isolating single components and looking at individual effects on the immune system, we may perhaps understand the complexity of the challenge that mycobacteria pose to the host immune system.

The immune response to lipid molecules is very different to that elicited by protein antigens. Proteins are processed and presented on MHC-molecules to allow participation of B and T lymphocytes from adaptive immune system. Lipids are not presented on MHC and thus preferentially engage the innate immune system, with the macrophage at the center of activity. The cells of innate immunity generally have no specific antigen binding receptors, which excludes the development of specific immune memory, and thus vaccines, to lipid antigens. It is only in the last decade that appreciation grew of the important role that bacterial lipids play in directing adaptive immunity by their activation of innate immunity (Ulrichs and Porcelli, 2000).

Tuberculosis and AIDS are deadly partners in the human disease. The potential that mycobacterial cell wall lipids may have in controlling the progress of tuberculosis in HIV infected patients, was recently proposed by Verschoor and Onyebujoh (1999). The authors emphasize the potential advantage of stimulating protective cellular and humoral immunity against tuberculosis with mycobacterial cell wall lipids without burdening the $CD4^+$ T cell population (the target of HIV-infection). In addition, the expected short-lived immune memory of antibodies against mycobacterial lipids were regarded as beneficial for serodiagnosis of TB infection. If the antibodies to the lipid antigens could be shown to appear and wane in correlation with the mycobacterial infection and independent of the BCG-vaccinated state of the patient, then these antibodies would constitute the ideal surrogate marker for serodiagnosis of tuberculosis.

Of all the lipids present in the mycobacterial envelope, the lipoarabinomannans (LAM) are the best studied. They dissolve well and are even found in the urine of tuberculosis-infected patients (Tessema *et al.*, 2001). Mycolic acids, structurally unique and characteristic of the various mycobacterial species and their most abundant lipid cell wall component, became the focus of this study. Mycolic acids are found in all mycobacteria and some related taxa as nocardia. Different strains

of a given species generally exhibit the same mycolate profile. The long carbon chain substances (>90) mycolic acids are only found in the pathogenic mycobacteria suggesting that it may play a role in virulence (Daffe and Draper, 1998).

In previous work, mice immunized with MA and then challenged with living mycobacteria showed a degree of resistance to infection (Verschoor et al., 1998, International patent no PTC/GB 98/00681). Verschoor and Bey, (1995), (International patent no PTC/GB 95/00856) gave the first indication that mycolic acids are antigenic, i.e. that they induced a specific antibody response. Beckman et al. (1994) discovered that mycolic acids from Mycobacterium tuberculosis stimulate the proliferation of human double negative (CD$_4$CD$_8$) T cells. This indicated that MA could possibly be applied in diagnostics and therapy of tuberculosis.

In contrast to LAM, MA does not dissolve easily. It could however be successfully purified from crude mycobacterial extracts by using counter current distribution, according to a recently published protocol (Goodrum et al., 2001). In this article, some biological properties of the mycolic acids were determined. MA was solubilized in serum by adding a chloroform solution of MA and then removing the chloroform by nitrogen gas. This approach, while technically successful, was not ideal for the study of the innate immune answer to MA, because of the effect that serum itself had on immune responsiveness. Moreover, the extent of the damage that chloroform incurs on serum is unknown.

Finding an alternative carrier for mycolic acids for use in biological experiments proved to be difficult. As the mycolic acids exhibit properties of waxes, solubilizers for hydrophobic elements were investigated. Nine different compounds were tested for their ability to solubilize MA, remain inert to the body and immune system, be non-toxic to cell cultures and avoid haemolysis of erythrocytes.

Surprisingly enough, Solutol HS15, which is commonly used as a drug solubilizer (Fromming *et al.*, 1990), was found to be toxic to macrophage cultures. When Solutol HS15 was used *in vivo,* the toxicity was diminished, but still not ideal for the purpose of this study.

MA could best be delivered to macrophages when adsorbed onto polystyrene beads/microspheres or included in liposomes made from phosphatidylcholine. The advantage beads have over the liposomes, is their total inertness. Unfortunately, their disadvantage is that beads left inside the macrophage cannot be metabolized, leading to macrophages bloated with beads. Liposomes are fully metabolized by the macrophages after uptake. By using the mouse, a tuberculosis resistant animal model, the nature of the protective immune response and mechanisms of containment can be investigated because the mouse develops strong immunity to mycobacterial infections. Susceptible species like guinea pigs can be used for the study of progressive pulmonary disease and the pathology that ensues. *Mycobacterium tuberculosis* infection in C57BL/J6 mice produces a delayed but sustained response in the lung, correlating with granuloma onset and characterized by high induction of IL6, IFNγ, IL1β, IL10, and TNFα (Actor *et al.*, 1999). The histopathology and cytokine response to *Mycobacterium tuberculosis* infection in mice varies among the organs. Increased survival during acute infection may therefore depend on the ability to contain mycobacteria within granulomas in the lung (Actor *et al.*, 1999). The mouse at this stage remains the model of choice to study; not only the cell-mediated response to tuberculosis (Orme, 1996), but also of the vast amount of immunological reagents available for defining murine biological molecules that is lacking in most other animal models.

One disadvantage of the mouse-model is its limited variety of CD1 molecules. Beckman *et al.* (1994) have shown that mycolic acids are presented on CD1b molecules to the immune system, which group together with CD1a molecules as

class I CD1 molecules. Mice lack class I CD1 molecules, and only contain class 2 CD1d molecules. The role of class 2 CD1 molecules is not known, but Szalay *et al.* (1999) demonstrated their participation in *Mycobacterium tuberculosis* infection by modulating CD1 and showing that most type 1 cytokines were affected.

The TB bacillus solicits various strategies to protect itself against the multiple onslaught of the system, which in return, is continuously trying to find a way to rid the body of colonizing pathogens. Mycobacteria have the ability to survive in the host macrophages by blocking maturation of mycobacterial phagosomes into phagolysosomes (Deretic *et al.*, 1997). It has been shown that *M. tuberculosis* resides inside non-acidified vacuoles (Crowle *et al.*, 1991), possibly by hindrance of phagosome maturation at the level of early endosomes. The proton translocating ATPase is usually present in the majority of phagocytosed endosomal vesicles, but is absent in mycobacteria infected phagosomes. The precise trafficking events underlying this exclusion are unknown. This study showed that particles that are surrounded by MA and phagocytosed by macrophages do mature to late stage endosomes. This indicates that the mechanisms of delayed maturation are not dictated by MA. The MA containing phagosomes mature to the late stage endosomes, which contain molecules that can present MA to the rest of the immune system.

By using beads to carry the MA into the macrophages, it was observed that macrophages that do not contain beads also changed into foam-like cells. This implies that there might be another factor other than MA, or induced by MA, that is causing the macrophages to turn into foam cells. GM-CSF may be a candidate. From the literature, it seems that GM-CSF is increased in foam cell cultures and is essential for foam cell development (Biwa *et al.*, 2000). The maximal induction of GM-CSF was noted at 4 hours, followed by a time-dependent decrease to a basal level within 24 hours. MA-induced foam cells may come about by uptake of oxLDL. OxLDL-induced macrophage growth was 75%

inhibited by replacement of the culture medium at 24 hours by fresh medium containing the same concentration of oxLDL. Thus, cytokine(s) other than GM-CSF is participating in the later phase of oxLDL-induced macrophage growth. Biwa *et al.* (1998) observed that GM-CSF plays a priming role in foam cell development, but by itself could not sustain foam cell development and proliferation.

The term oxLDL describes a specific oxidized LDL. As such, it does not describe a well-characterized molecular species. Native LDL is heterogenous and one can hardly expect that the oxLDL will be homogenous. If the complexity of the LDL particle and the huge number of oxidation sensitive components in it is considered, the range of different oxidation products is high (Steinberg, 1996).

OxLDL may come about through extra cellular oxidation by the MA-activated macrophages. Subsequent uptake of oxLDL activates phospholipase D that in return releases phosphatidic acid. Phosphatidic acid is a biological active molecule implicated in regulation of various cellular functions, like stimulation of phospholipase A_2. Phospholipase A2 induces arachidonic acid release and eicosanoid production, leading to (foam) cell proliferation (Gomez-Munoz *et al.*, 1999).

The formation of foam cells raises questions like:

- how does MA change the macrophages into foam cells?
- what happens to the macrophage once turned into a foam cell?
- does this also happen during TB infection and if so what are the implications or purpose of this during tuberculosis?

The first question on the mechanism of foam cell formation is not easy. As foam cells are formed by oxidation of LDL, the obvious question is how does MA oxidize the LDL. Looking at all the possibilities and then at results that were found, the highest probability would come from MPO-induced oxLDL. It seems that MPO is increased in MA-treated macrophages (chapter 5). Jerlich *et al.* (1999) described that MPO, present in atherosclerotic lesions, oxidizes LDL.

Podrez *et al.* (2000) described how MPO-generated reactive nitrogen species convert LDL to oxLDL *in vitro*. Besides the role of GM-CSF and possibly other cytokine(s), and/or the direct activation by MPO through formation and uptake of oxLDL, no other mechanism can currently be put forward to explain the formation of foam cells upon MA exposure.

On the second question as to what happens to the macrophages once turned into foam cells, one needs to consider the surface molecules that are expressed. In 1994, Beckman *et al.* demonstrated that CD1b carries MA. CD1 molecules are highly restricted to the molecules that they carry. Expression of CD1 is not observed in normal artery walls but appears to be restricted to the foam cells of atherosclerotic lesions in humans. Given the abundance of T cells, CD1$^+$ macrophages, and lipid accumulation in atherosclerotic plaques, Melian proposed a role for lipid antigen presentation by CD1 proteins in the generation of the inflammatory component of atherosclerotic lesions (Melian *et al.*, 2000). CD1d expression on macrophages in mice did not change in response to MA treatment. As CD1d from mice is not known to carry MA, this was not necessarily anticipated.

MHCII expression was increased on MA-induced mouse foam cells, compared to control macrophages. Human foam cells also express increased amounts of MHCII compared to macrophages, indicating an active role in linking the innate immunity to adaptive immunity.

The last question as to whether foam cell formation occurs during TB infection and what the possible impact of MA-induced foam cells on disease may be opens up interesting possibilities.

Mycolic acids can experimentally be dissolved or absorbed in serum (chapter 3). Therefore, in the infected mammalian, mycolic acids that are shed from killed mycobacteria may become solubilized in the blood. Moreover, it was recently

cited by Beatty *et al.* (2000) that mycobacterial lipids were found in the cytoplasms of non-infected macrophages. Whether the MA will be in the same chemical form as was used in the *in vitro* studies performed here, is not known, but it is possible due to their chemical resistance and lack of reactive functional groups. Foam cells that may be induced by the MA could display a high preference for cholesterol uptake, therefore providing the resident tuberculosis bacilli with a rich carbon source. The complete genomic sequence of *M. tuberculosis* suggests that it contains sterol biosynthetic enzymes as well as two putative cholesterol-degrading enzymes (Cole, 1998, Bellamine *et al.*, 1999). It is also known that non-pathogenic forms of *M. tuberculosis* can grow on cholesterol as sole carbon source. Furthermore, keeping in mind that the new opinion on TB infections is that the organism is generally dormant inside its host, foam cell formation can be a mechanism for the mycobacterium to stay alive inside the macrophage, nurtured with enough cholesterol as carbon source.

Why have foam cells not been described more often in experimental TB and hardly ever in human tuberculosis (Yuasa and Kanazawa, 1995)? It is important to note that foam cells have been described in lepromatous leprosy, caused by infection with *Mycobacterium leprae* (Kaplan *et al.*, 1983). The possibility of overloading the macrophage with the amount of MA exists, and subsequently what we are looking at is an end pole of what can happen in abundant MA loading inside the macrophage.

Lepromatous leprosy represents the Th2-pole of leprosy. The Th1 pole, known as tuberculoid leprosy, represents the immune resistant phase of the disease and is not characterized by foam cells. It may well be that the overloading of macrophages with MA in the experimental design of this study drives the macrophage into a Th2 type pole, here represented by the appearance of alternatively activated type macrophage/foam cell. These cells may be transitory from the resistant to the dormant stage of tuberculosis. This is displayed as an

initial increased bactericidal effect followed by a cholesterol accumulating nurturing cell for dormant bacteria.

Closer to the "physiological" situation, is the description in this study of induction of activated macrophages with a small amount of vacuoles and accumulation of neutral lipids by exposure to dead mycobacteria. This may apply to dead mycobacteria, but also to dormant active mycobacteria.

The question arises whether the induction of foam cells is unique to MA. At least two studies have shown infectious agents other than mycobacteria evoking molecular and cellular changes associated with foam cell appearance and activity. In one such study it was shown that LPS from *Chlamydia pneumonia* infection, appears to induce foam cells, leading to atherosclerosis (Kalayoglu and Byrne, 1998). In another example, infection with cytomegalovirus was cited as an increased risk factor for the development of atherosclerosis, without knowing what the inducing molecule could be (Epstein *et al.*, 1999). As atherosclerosis is associated with risk factors such as smoking, hypertension, hypercholesterolemia and diabetes, it is clear that MA might just be one of various foam cell-inducing factors.

Mycolic acids never induced multinucleated giant cells typical for tuberculosis in the lung. MA may suppress the ability to induce these cells. Macrophages infected with mycobacteria *in vitro* secrete an array of pro-inflammatory cytokines leading to granuloma formation containing multinucleated giant cells. TNFα is also known to be involved in granuloma formation (Kindler *et al.*, 1989, Flynn *et al.*, 1995). This seems to be directly related to the response to the mycobacterial lipoarabinomannan (LAM) (Chatterjee *et al.*, 1992). It seems that TNFα and INFγ are involved in the formation of multinucleated giant cells commonly seen in *Mycobacterium tuberculosis* infection (Chensue *et al.*, 1996, Heinemann *et al.*, 1997).

In MA-induced foam cells the amount of TNFα secreted is low in comparison to that induced by LPS. In none of the MA-loaded macrophage cultures could increased levels of INFγ be detected. MA-induced foam cells express IL6, suggesting a role for this cytokine. IL6 has both pro- and anti-inflammatory activation properties. IL6 down-regulates the production of TNFα but has almost no effect on IL10. IL6 can also down-regulate the production of GM-CSF and INFγ. Although IL6 stimulates the acute-phase protein response, it is placed among the anti-inflammatory cytokines when its net effect in disease is considered. VanHeyningen *et al*, (1997) found that mycobacteria-infected macrophages could increase induction of IL6 up to 10 000 times. In MA-treated foam cells, IL6 was highly induced, compared to treatment with mycobacteria or liposomes alone. This may explain why MA-infected macrophage cultures appears to be immune unresponsive.

IL10 is the most important anti-inflammatory cytokine and deactivates the synthesis of macrophage pro-inflammatory cytokines. It is a potent inhibitor of macrophage derived IL1, IL6, IL8, IL12 and TNFα. It also inhibits B7 accessory molecules and CD14. In MA-induced foam cells, IL10 was increased and perhaps synergised with the IL6 to create an anti-inflammatory environment. Together with the observed lack of nitric oxide production associated with increased arginase activity, an anti-inflammatory response seems to follow on MA treatment, at least in peritoneal macrophages. Perhaps if macrophages from other areas of the body or organs were used, another type of reaction would have been seen. The low NO could also indicate production of ONOO⁻ that is necessary to produce oxLDL for foam cell formation.

Mycobacterium tuberculosis is a pathogen that uses numerous mechanisms to evade elimination by the host immune response, many of which are still poorly understood. It is possible that the Achilles heel of the pathogen may be found in the innate response following on the early processing of the infectious insult. MA may play an important role at this stage, by not producing a massive increase in TNFα, but IL6 as well as IL10. Granuloma formation may thus be suppressed,

thereby impairing the mycobacterium's immune escape option. Having said this, the role and mechanism of the foam cells formed by MA are far from resolved and worthy of further research that should include comparison with other diseases where foam cells are known to play some role.

The discovery that mycolic acids induced foam cells in mice, links tuberculosis to atherosclerosis. The demonstration of an array of immunoregulatory properties of mycolic acids in murine *in vivo* and *in vitro* experimental models, keep trend with the cutting edge thinking of cardiovascular disease and contributed to a deeper understanding of both tuberculosis and atherosclerosis. Future research may focus on the oxidative disturbance of lipid homeostasis at sites of inflammation in both diseases, according to the mycolic acid model developed in this study.