Immunological properties of mycolic acids, the major lipid cell wall component of *Mycobacterium tuberculosis*

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Summary

The immunological effects of mycolic acids (MA) from *Mycobacterium tuberculosis* on mouse peritoneal macrophages were studied. MA was solubilbized using various carriers. Phagosome uptake and maturation (into late stage phagolysosomes) were compared using fluorescent markers and the confocal microscope. During assessment on the effects of MA on mouse macrophages, changes in morphology and activation of the macrophages were found. This indicated that the MA was immune reactive towards macrophages. The phenotype of cell that develops after *in vivo* loading with MA was characterized by using cell surface markers: it was found that MA-loaded macrophages developed into foam cells. Cell survival, proliferation and macrophage cytokine production were examined to characterize the foam-like cells. The effect of MA-induced foam-like cells on living *Mycobacterium tuberculosis* was evaluated and increased bactericidal activity was found. The roles of reactive oxygen and nitrogen intermediates via myeloperoxidase were also examined and a theoretical mechanism for the formation of foam cells proposed. The possible role of myeloperoxidase in activation of macrophages, foam cell formation and killing of *Mycobacterium tuberculosis* is discussed. It is postulated that a possible relationship might exist between tuberculosis and atherosclerosis that is facilitated by mycolic acids.

Opsomming

Die immunologiese effekte van mikoolsuur (MA) geïsoleer vanaf *Mycobacterium tuberculosis* op muis peritoneale makrofaag selle is ondersoek. Mikoolsuur is in oplossing gebring deur van verskeie draers gebuikte maak. Fagositose en maturasie van die fago-lisosoom is met behulp van fluoresente merkers en die konfokale mikroskoop ondersoek. Ondersoek na die effek van mikoolsuur op makrofae, het aan die lig gebring dat makrofae verandering in aktivering en morfologie ondergaan. Dit het die immunologiese aktiwiteit van mikoolsuur op makrofae aangetoon. Die fenotipe van die selle wat deur mikoolsuur geïnduseer is, is met behulp van oppervlakte merkers ondersoek: daar is bevind dat mikoolsuur-gelaaide makrofae in skuimselle ontwikkel. Selproliferasie, -oorlewing en sitokienproduksie is ook ondersoek ten einde die skuimselle te karateriseer. Die effekte van MA-geïnduseerde skuimselle op lewende *Mycobacterium tuberkulose* is getoets en 'n verhoogde bakteriosidiese effek is gevind. Die moontlike rol van miëloperoksidase in die aktivering van makrofae, skuimselvorming en uitwissing van *Mycobacterium tuberkulose* word bespreek. Die rol van reaktiewe suurstof en stikstof radikale op lewendige tuberkulose basille is ook ondersoek en 'n teoretiese meganisme vir die ontstaan van die skuimselle is gepostuleer. Soos reeds bewys is vir ander organismes, is die vraag of tuberkulose ook moontlik 'n rol mag speel in aterosklerose.

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Abbreviations

A:

ACAT : acyl co-enzyme A cholesterol-acyltransferase
ACEH : acid cholesterol ester hydrolase
Ag : antigen
AhpC : alkyl hydroperoxidase reductase protein
AIDS : acquired immunodeficiency syndrome
APC : antigen presenting cell
ARDS : adult respiratory distress syndrome
ATP : adenosine triphosphate
Av-HRP : Avidin-Horseradish peroxidase
AZT : 3'-azido 3'-deoxythymidine

B:

BCG : Bacille Calmette Guerin
BMF : (5 –bromo) methylfluorescein
BSA : bovine serum albumin

C:

CD : cyclodextrin
CE : cholesterol ester
CEH : cholesterol ester hydrolase
CH : carbohydrate
CLPS : *Chlamydia pneumonia* lipopolysaccharide
CMC : critical micellar concentration
Cp : ceruloplasmin
CR : complement receptor
CTL's : cytotoxic T cells

D:

DLiPC : dilinoleoyl phosphatidylcholine
DLPC : dilauroyl phosphatidylcholine
DMF : dimethylformamide
DN : double negative

DNA : deoxyribonucleic acid
DNP-Cap-PE: dinitrophenyl-epsilon-aminocaproyl phosphatidyl
DSPC : distearyl phosphatidylcholine
DTH : delayed-type hypersensitivity

E:

EE : early endosome
ELISA : enzyme linked immunosorbent assay
ER : endoplasmic reticulum
EtOH : ethanol

F:

FACS : fluorescence activated cell sorter
FasL : Fas-ligand
FAT : fatty acid tanslocase
FBS : foetal bovine serum
FC : free cholesterol
FCS : foetal calf serum
FITC : fluorescein-iso-thiocyanate

G:

α GalCer : α galactosylceramide
GM-CSF : granulocyte monocyte colonizing factor
GMM : glucose monomycolate

H:

HIV : human immunodeficiency virus
HOX : hypohalous acid
HPLC : high performance liquid chromatography
HS : hydroxystearate

I:

ILDL : intermediary low density lipoprotein
IL : interleukin
IFNγ : interferon γ
IP : intraperitoneal

ISPF : isonitrosopropiophenone
IV : intravenous

K:

katG : catalase-peroxidase protein

L:

LAM : lipoarabinomannan
LAMP : lysosome associated membrane protein
LDL : low density lipoprotein
LE : late endosome
LipoMA : liposome-mycolic acid
LO : lipo-oxygenase
LPL : lipoprotein lipase
LPS : lipopolysaccharide
LRP : LDL receptor-related protein
Lyso-PC : lysophosphatidylcholine
m/v : mass/volume

M:

MA : mycolic acid
mAU : milli-absorption units
MAC : maximum additive concentration
MBP : mannose-binding protein
M-CSF : macrophage colony stimulating factor
MDR : multi drug resistance
MHC : major histocompatibility complex
MIIC : MHC class II compartment
MP : mononuclear phagocytes
MPO : myeloperoxidase
MR : mannose receptor
MTP : microsomal transfer protein
MW : molecular weight

N:

NADP : nicotinamide adenine dinucleotide
NBCS : newborn calf serum
NCEH : neutral cholesterol ester hydrolase
NK-cells : natural killer cells
NO : nitric oxide
NOS : nitric oxide synthetase
NRAMP : natural-resistance-associated macrophage protein

O:

OD : optical density
oxLDL : oxidized low density lipoprotein

P:

PBM : peripheral blood monocyte
PBS : phosphate buffered saline
PC : phophatidylcholine
PCR : polymerase chain reaction
PCv/v : packed cell volume/volume
PEC : peritoneal exudate cell
PEG : polyethylene glycol
PGE_2 : prostaglandin E_2
PI : propidium iodide
PI_3 : phosphatidylinositol 3
PLA_2 : phospholipase A_2
PLGA : poly lactic-coglycolic acid
PPD : purified protein derivative

R:

RT-PCR : reverse transcriptase polymerase chain reaction
RES : reticulo-endothelial system
RNI : reactive nitrogen intermediates
ROI : reactive oxygen intermediate
RT : room temperature

S:

sCD14 : soluble CD14

SDS : sodium dodecyl sulphate

SMase : sphingomyelase

SP-A : surfactant protein A

SP-D : surfactant protein D

SR : scavenger receptor

T:

TAP : transporter associated with antigen presentation

TB : tuberculosis infection

TCR : T cell receptor

TdR : [methyl-^{3}H] thymidine

TG : triglyceride

TGFβ : transforming growth factor β

TGN : trans-golgi network

Th1/2 : T-helper 1 or 2 cell

TI : thymus independent

TNF : tumour necrosis factor

U:

UK : United Kingdom

USA : United States of America

v/v : volume/volume

V:

VLDL : very low density lipoprotein

W:

WHO : World Health Organization

CHAPTER 1

Tuberculosis: the disease and the immune response to it

1.1 Introduction

The most frequent and important causative agent of human tuberculosis is *Mycobacterium tuberculosis*. Occasionally tuberculosis is caused by the closely related organisms *Mycobacterium bovis* (transmitted by unpasteurized milk) and *Mycobacterium africanum* (seen in small proportions in West and Central Africa). Another important disease caused by a pathogen of the genus *Mycobacterium* is leprosy, for which the agent is *M. leprae.* Recently, a number of other species of *Mycobacterium* have become important opportunistic pathogens in immuno-compromised individuals, mainly AIDS-sufferers, where they can cause tuberculous or non-tuberculous symptoms of infection *(*Kaufmann and Andersen, 1998*)*.

1.2 Properties of the etiological agent

M. tuberculosis is an aerobic, rodshaped, non-spore-forming bacillus of about 0.5 x 3 μm, classified as acid-fast due to its ability to retain dyes when treated with acid. The reason for the acid-fastness can be found in the chemical wax that surrounds the mycobacteria and which consists mainly of mycolic acids (McNeil, *et al.*, 1991). In the mycobacterial cell wall, mycolic acids (MA) are linked to underlying arabinogalactan and peptidoglycan. Lipoarabinomannan, another component of the cell wall, is involved in the pathogen-host interaction and facilitates the survival of the bacilli in the host macrophages. Cord factor (trehalose-dimycolate) seems to play a role in the virulence of the bacteria by mediation of cytokines and is responsible for the growth of the organism in rope like arrangements (Saita *et al.,* 2000). During infection and disease, cord factor is also partly responsible for the formation of granulomas.

Pathogenic bacteria belonging to the *Mycobacterium tuberculosis* complex cause tuberculosis. In two-thirds of cases, the lungs are affected, although any other organ in the body can be involved (Antonucci *et al.,* 1995). Mycobacteria grow

very slowly and normally take up to 24 hours to double in number in cell culture media (Antonucci *et al.*, 1995).

1.3 Epidemiology of Tuberculosis

According to the WHO report on the *M. tuberculosis* epidemic and anti-tuberculosis drug resistance in 1997, the projected figures for the next decade are alarming. In the 216 reporting member countries of the WHO, an estimated 7.4 million tuberculosis cases occurred in 1995 with nearly 3 million deaths. The eleven countries in the Southern African sub-region contribute about 275 000 new cases every year.

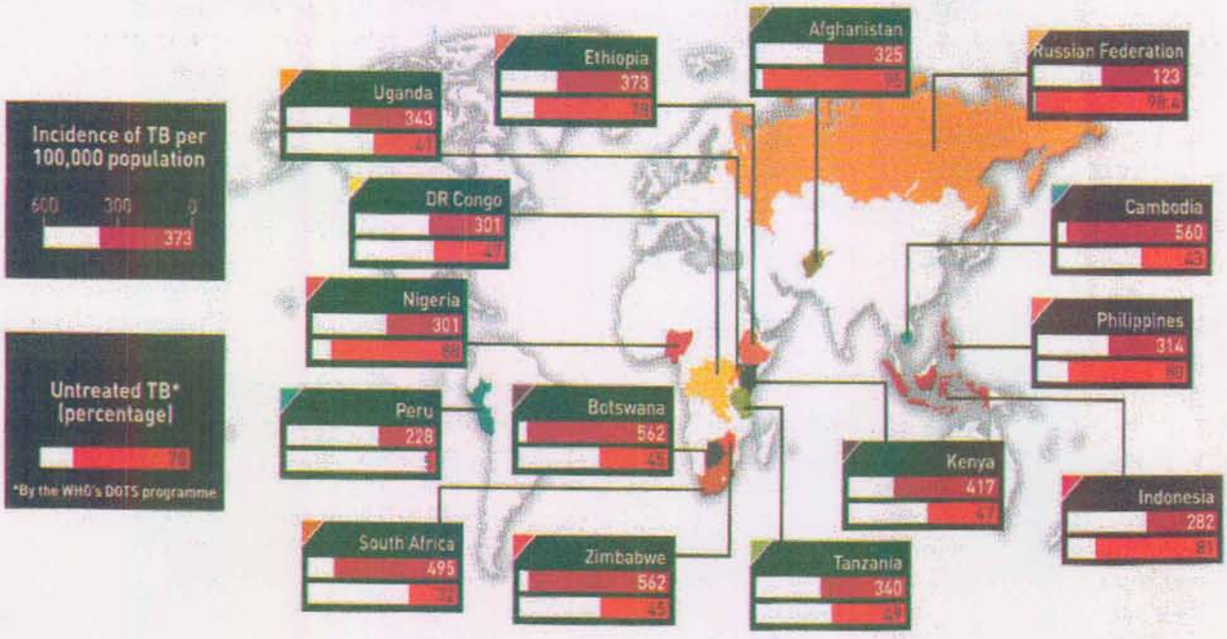


Figure 1.1 Incidence of TB cases per 100 000 of the population for developing countries worldwide and the number of untreated cases as percentage of occurrence. Taken from Coglan and Concar, 2001.

Compared to other developing countries, South Africa has the fourth highest incidence of TB cases per 100 000 of the population. This represents a TB frequency that is dramatically higher, compared to the USA and Western Europe.

In April 2000, South Africa had an estimated 103 700 new cases, representing 495 cases per 100 000 of the total population (Coglan and Concar, 2001).

HIV infection appears to play an important role in the alarming rate of TB infection in Southern Africa (Verschoor and Onyebujoh, 1999, Corbett and De Cock, 2001). If the rate of increase of TB stays the same in South Africa, the prediction for the year 2006 will be 1117 cases per 100 000 of the population, with 848 per 100 000 cases directly attributable to HIV infection. This means that at least 3.5 million new cases and 90 000 deaths due to tuberculosis are expected in the next decade. Already, hospitalizations for tuberculosis increased 350% from 1991 to 1998 (WHO, Fact sheet no 104, 2000), implying a pending disaster for the next decade.

1.4 Infection

M. tuberculosis is most commonly transmitted from a patient with infectious pulmonary disease to another by droplet nuclei, which are aerosolized by coughing, sneezing or speaking. As many as 3000 infectious nuclei remain suspended in the air for several hours after one cough, from where they may gain access to the terminal air passages by inhalation (Fenton and Vermeulen, 1996). Less than 10% of the inhaled bacilli reach the respiratory bronchioles and the alveoli. The external innate immune systems, *e.g.* the mucus and the epithelium of the upper respiratory tract, filter out and remove most of the bacteria.

Other routes of infection, such as trans-dermal or trans–placental, are uncommon and of no epidemiological significance. Patients with cavitary pulmonary disease or tuberculosis of the respiratory tract (endobronchial or laryngeal), produce sputum with as much as 100 000 bacilli per millilitre.

Up to 75-80% of people exposed to *M. tuberculosis* bacilli do not develop active disease. The risk of acquiring *M. tuberculosis* infection is determined mainly by exogenous factors as explained in a later section.

In contrast to the risk of acquiring TB infection, the risk of developing the disease after being infected depends largely on endogenous factors, including innate susceptibility to the disease and the level of functioning of cell-mediated immunity (Fenton and Vermeulen, 1996). Innate susceptibility is determined by genetic factors which, although not related to immune function, does confer differing degrees of susceptibility in different populations of experimental animals (McMurray , 2001).

1.5 Clinical stages of tuberculosis

Mycobacteria reaching the lung are phagocytosed by alveolar macrophages. Some of the bacilli are destroyed within the phago-lysosomes of the macrophages and the rest remain inside the macrophage vacuoles for an undetermined period of time. Inside these vacuoles the bacilli can replicate and then escape by destroying the macrophage after 2 to 3 weeks, to infect other macrophages (Fenton and Vermeulen, 1996).

Granulomas, formed at the sites of infection by the attraction of monocytes, lymphocytes and neutrophils during the inflammation reaction, contain macrophage-derived giant cells and epitheloid cells. The formation of granulomas is a result of an interaction between macrophages and T lymphocytes and the secretion of cytokines by stimulated cells of both types. The degree of granuloma formation depends upon the number of mycobacteria present in the tissue and on the number of macrophages present at the infection area.

In the cytokine/chemokine cascades that develop around the tuberculosis infection, the relationship between the tissue-destructive delayed-type of

hypersensitivity (DTH) and the T cell-enhanced specific bactericidal killing of mycobacteria by macrophages usually determines the outcome of the disease (Kaplan and Freedman, 1996). How this balance can be manipulated chemically, is currently not fully understood.

1.6 Primary tuberculosis

The majority of primary infections are asymptomatic. The development of a positive tuberculin test between 3-6 weeks after infection is usually the only evidence. A peripheral lung lesion (Ghon focus) accompanied by ipsilateral hilar lymphadenopathy can sometimes be seen on röntgen images (Fenton and Vermeulen, 1996). The pulmonary complex is about 10 mm in diameter and consists of a central zone of caseous necrosis surrounded by palisaded epithelioid histiocytes, Langerhans cells and lymphocytes (Fenton and Vermeulen, 1996). In almost all the cases, a primary lesion will organize. Clinical signs at this stage are unusual except if a lung collapse, caused by enlarged lymph glands, develops. Live tuberculous bacilli may still be present in these scarred foci and may persist for years (Bloom and McKinney, 1999). Complications of primary infection include epituberculosis (pneumonitis and collapse), obstructive emphysema, bronchiectasis, pleural effusion, and associated hypersensitivity phenomena (like Erythema nodosum).

1.7 Secondary tuberculosis

After primary infection, the pathogens may lie dormant and the disease quiescent without any symptoms for decades. Secondary tuberculosis could either start from direct progression of primary disease, inhalation of additional bacilli (exogenous re-infection), reactivation of quiescent primary disease or haematogenous spread of bacilli to the lung. The subsequent lesions nearly always develop in the apices of the lungs because of the high oxygen concentration.

Post-primary tuberculosis progresses despite the existing immunity aquired during the primary exposure, and is considered the most prevalent form of the disease (Fenton and Vermeulen, 1996).

If the healing process is impaired, the lesions do not calcify. They may erode and rupture into adjacent bronchi to form cavities in which *M. tuberculosis* and/or other opportunistic fungi can multiply freely (Nardell, 1993). Leakage of living bacilli into adjacent bronchi will spread the disease in the host and in sputum, discharging it into the air.

Early in the disease, symptoms may be non-specific, such as loss of weight, night sweats, persistent cough, fatigue and loss of appetite (Fenton and Vermeulen, 1996). Systemic features of tuberculosis include anaemia, fever, weight-loss and clubbing of the fingers. In acute pulmonary tuberculosis, lung symptoms become prominent. Upper zone crackles may be heard and haemoptysis may be seen as a result of consolidation. Other complications include pleurisy, pleural effusion, empyema, laringitis, Poncet's arthropathy airflow obstruction, cor pulmonale, aspergilloma, amyloid, carcinoma of the lung, Adult Respiratory Distress Syndrome (ARDS) and eventually death.
Tuberculosis is a multi-system disease and involvement of liver, urogenital system, adrenal glands, spine, joints, larynx and central nervous system has been reported. Haematogenous spread of postprimary tuberculosis starts when infected lesions erode and rupture into vessels leading to infection of any organ of the body (Raviglione *et al.*, 1995).

1.8 Miliary tuberculosis

Haematogenous spread of bacilli in patients with poor immunity, resulting in dissemminated disease, is known as miliary tuberculosis.
Miliary tuberculosis can be a complication of either primary or secondary tuberculosis. In high prevalence areas, the majority of cases occur shortly after

primary infection. In comparison, mostly elderly people develop miliary tuberculosis in low prevalence areas, representing reactivation.

The name is derived from the small seed-like appearance of the lesions. The lungs are always involved and any other organ variably so. Two forms exists: acute and chronic "cryptic" miliary tuberculosis.
Complications include meningitis and ARDS. This is an acute medical emergency necessitating prompt treatment to avoid a fatal outcome (Swaminathan, 2000).

1.9 Laboratory detection of tuberculosis

Major disadvantages exist in the traditional laboratory detecting methods. A positive result culture *i.e.* a culture of *Mycobacterium tuberculosis,* may take up to eight weeks for completion but remains a very reliable means of detecting the presence of the bacteria. This in turn leads to further spread of the disease due to the delay in treatment. The normal culture of bacilli can be shortened to 10 -12 days using radiometric determination of bacterial growth in liquid medium, known as the BACTEC technique. This relatively high-tech approach requires a sophisticated infrastructure (Piersimoni *et al.,* 2001)

Tuberculosis is also diagnosed by detection of the whole organism in sputum samples, using the microscope and acid fast stain. The microscope has a sensitivity of detection of at least 5000 – 10 000 bacilli per milliliter of sputum.

Molecular approaches, which were recently introduced, result in more rapid and sensitive detection and drug sensitivity assays, but are expensive and require trained personnel and sophisticated infrastructure. DNA probes are specific, but need 10 000 to 100 000 organisms for a positive signal. At present, the main interest is in the PCR or DNA amplification RT-PCR of parts of the genome of the mycobacteria (Kambashi *et al.,* 2001).

1.10 Tuberculosis Vaccine Research

The only tuberculosis vaccines available for use in humans are attenuated strains of *M. bovis,* i.e. Bacille Calmette Guerin (BCG). This vaccine was introduced in France more than 70 years ago. BCG is still widely used, but controversy surrounds the efficacy of the vaccine (Fine *et al.,* 1994).

New or improved vaccine developments currently investigated are as follows:

- Genetically improved protective antigen BCG or auxotrophic vaccines: improvements relate to genetically engineered attenuation and introduction of cytokines to ensure a Th1 response (Guleria *et al.*, 1996).
- Protein subunit vaccines: this approach involves use of low molecular weight proteins. Typically secreted mycobacterial proteins, are used as vaccines (Wiker *et al.*, 1992).
- DNA subunit vaccines: administration of plasmid DNA expression vectors has been shown to result in protein expression *in vivo.* The translated proteins are immunologically active and elicit humoral as well as cell-mediated immune responses. The approach allows inclusion of cytokine genes to solicit the desired immune response (Donnelly *et al.,* 1994, Lowrie *et al.*, 1994, Lowrie, 1999).

Except for the DNA vaccination method tested in mice, above methods have hitherto met with little success.

1.11 Interaction with HIV infection

Various diseases predispose humans to become vulnerable to the development of tuberculosis. The most important and potent risk factor is HIV infection, which suppresses cellular immunity (Bhatia, 2000).

The risk of developing active disease from latent *M. tuberculosis* is directly related to the degree of immunosuppression in the individual. For non-HIV

infected people, the *lifetime* risk of developing tuberculosis is 10 %. In HIV infected patients it may be 10 to 15 % *per year* (Bhatia, 2000). Other conditions known to increase the risk of developing active tuberculosis among persons infected with the tubercle bacilli include:

1. lymphoma, leukemia and other malignant neoplasm's
2. silicosis
3. haemophilia
4. chronic renal failure and hemodialysis
5. insulin dependent diabetes mellitus
6. immunosuppressive treatment and associative nutritional conditions (Bloom *et al.*, 1994).

The presence of healed, fibrotic tuberculosis lesions constitutes a serous risk of active disease in patients with above conditions.

In patients with HIV infections, endogenous reactivation of pre-existing infection, progression from infection to tuberculosis and transmission of mycobacteria bacilli to non-infected people occurs (Rieder, 1994). In non-African countries, all forms of tuberculosis are now considered an AIDS-defining disease. Tuberculosis is the most common infection in HIV infected patients. Doubling of the current numbers of co-infection is expected in the next decade as a consequence of the impact of the HIV epidemic (Bhatia, 2000).

The pathology and clinical features of tuberculosis are similar in an HIV infected patient prior to acquiring AIDS as in patients without HIV infection. While the $CD4^+$ count declines, the clinical and pathological nature of tuberculosis infection changes and tuberculosis becomes atypical (decreased cavitations, increased extra pulmonary involvement, false PPD test) and disseminated. During this stage, the giant cell and epitheloid granulomas with scanty tubercle bacilli become infrequent.

During the advanced stages of AIDS, the macrophage response is lowered, granulomas become rare and contain abundant mycobacterial bacilli (Bhatia, 2000).

Tuberculosis infected HIV patients are more likely to have extrapulmonary tuberculosis, and greater frequency of other manifestation of HIV disease such as wasting, oral candidiasis and lymphadenopathy (Bhatia, 2000).

1.12 The immune response to tuberculosis

M. tuberculosis is an intracellular pathogen infecting several mammalian cells *in vitro,* but is almost exclusively found in macrophages *in vivo*. Cells can internalize particles and solutes via different strategies including pinocytosis, receptor–mediated endocytosis, patocytosis and phagocytosis. The ingestion of bacilli by macrophages is generally regarded as part of a non-antigen-specific process, broadly known as the innate immune response, which then orchestrates an antigen-specific response, broadly known as the adaptive immune response (Kruth *et al*., 1999).

1.12.1 Innate immunity

Innate immunity comprises four types of defense barriers, namely anatomical, physiological, phagocytic and inflammatory. Tuberculosis elicits unique responses in the latter three:

Anatomic barriers consist of the skin and mucous membranes. Mechanical, pH and mucus entrapment prevents uptake and entry of the pathogen in the first line of defense (Kuby, 1997).

Physiological barriers make use of temperature, pH, oxygen tension as well as soluble factors to combat spreading of pathogens. Soluble factors such as lysozyme, interferons and complement work together to bring about damage to pathogens, either by destroying them or facilitating their clearance (Kuby, 1997).

Interferon gamma (IFNγ) seems to be the crucial effector molecule in mice and humans against a variety of intracellular pathogen infections, including *Mycobacterium tuberculosis* (Flynn *et al.,* 1993).

By inducing reactive nitrogen intermediates (RNI), IFNγ mediates its protective effect, killing intracellular mycobacteria. In humans, the role of RNI is not as clear as in mice. RNI appears to link the innate and adaptive immunity in tuberculosis. Nitric oxide (NO), generated by infected macrophages, determines the life span and function of specific lymphocytes at the infection site (Sciorati *et al.*, 1999).

Phagocytic barriers comprise the ingestion of extracellular macromolecules via endocytosis and particulate matter via phagocytosis. Endocytosis occurs via either pinocytosis or receptor mediated endocytosis (Kuby, 1997). Macromolecules dissolved in extracellular tissue fluid are internalised by inward folding (invagination) and sealing of small regions of plasma membrane to form endocytic vesicles. These are approximately 0.1μm in diameter. The endocytic vesicles fuse with each other and are delivered to endosomes. Endosomes are intracellular acidic compartments and are involved in sorting. Endosomes fuse with primary lysosomes to form secondary lysosomes. Primary lysosomes contain degradative enzymes that break down the ingested materials into small products.

With phagocytosis the material is 10 to 20 times larger and the plasma membrane expands around the particle to form phagosomes. Once the material is ingested, phagosomes fuse with lysosomes and digestion is via the same route as endocytosis (Kuby, 1997). Phagocytosis of either *Mycobacterium tuberculosis,* or large latex beads induced interleukin 12 (IL12) secretion by the macrophage, whereas tumour necrosis factor (TNF) was produced only in response to *M. tuberculosis* and not to latex beads (Ladel *et al.*, 1997).

Patocytosis is a process whereby aggregated low density lipoproteins, microcrystalline cholesterol and small (<0.5 μm) polystyrene microspheres enter a labyrinth of membrane-bound compartments that remain

connected to the surface. It seems to be triggered by the hydrophobic nature of the particle entering. Mycolic acids and other bio-lipids from the cell wall of mycobacteria could enter the macrophage in this way (Kruth, *et al.*, 1999).

Inflammatory barriers are created by tissue damage caused by a wound or invasion by pathogenic micro-organisms. The cardinal signs of inflammation, *rubor* (redness), *tumor* (swelling), *calor* (heat), *dolor* (pain) and *functio laese* (loss of function), are reflected in three events during inflammation:

1. Vasodilatation
2. Increase in capillary permeability
3. Influx of phagocytes into damaged tissue

Among the chemical mediators of inflammation released into the area, acute phase proteins, histamine, kinins and chemokines abound. The hypothesis proposed for *M. tuberculosis* infection is that erosion of a bacterium into the interstitium of the lungs causes inflammation. Increased capillary permeability promotes influx of macrophages, but also allows haematogenous spread of bacteria. Important to this hypothesis is that bacilli must escape the local environment very early during infection, to explain the kinetics of generation of T cells (Orme and Cooper, 1999). Granulomas start forming from the interstitial pneumonitis within 15-20 days. Interferon gamma secretion in the tissue activates macrophages to stop the further progress of infection. Dendritic cells in lung tissue migrate to lymphoid tissue after activation. Without infection the activation of dendritic cells doesn't occur. Alveolar macrophages control this by IL10, transforming growth factor β (TGFβ) and prostaglandin secretion. If infection is established in the interstitium, macrophages ingest bacilli and release IL12. This drives the maturation of dendritic cells to a Th1 phenotype (Orme and Cooper, 1999).

In mice the Th1 response is followed by a Th2 response, which may be essential to limit the inflammation and minimize tissue damage. The Th2 may also contribute to the immunosuppression that is frequently seen in advanced tuberculosis. In HIV infected tuberculosis patients, this is more apparent, as the immunosuppression affects the Th1 response. Once again the macrophages contribute by producing suppressor cytokines such as IL10 and TGFβ. Th2 cells activate B cells and initiate antibody production in TB infection (Grange and Stanford, 1994, Fine *et al.*, 1994). No protective role for these various antibodies have been found. Th2 cells activate B cells and initiate antibody production - this is also known to occur in TB infection (Grange and Stanford, 1994, Fine *et al.*, 1994).

1.12.2 Acquired Immunity

Acquired or specific immunity reflects a functional system capable of recognizing specific antigens by antibodies and the antigen receptors on T cells, leading to the elimination of the antigens. The acquired immunity is adaptive and displays four attributes:

1) Antigen specificity
2) Practically unlimited diversity
3) Immunological memory
4) Discrimination between self and non-self.

Immunity to tuberculosis consists of a variety of complex interactions between different cell populations and secreted cytokines to combat infection through inflammation.

T cells, rather than antibodies cause protection against tuberculosis. This was already observed in the 1940's. Later, a number of authors confirmed that the presence of antibodies to different antigens of *Mycobacterium* in infected individuals did not correlate to susceptibility or resistance against disease. (Andersen, 1997).

1.12.2.1 T cells involved in *Mycobacterium tuberculosis* infections.

T cells contributing to protection in tuberculosis include the $CD4^+$, $CD8^+$ single positive and $CD4^-CD8^-$ double-negative types, expressing either the $\alpha\beta$ or $\gamma\delta$ antigen receptors. Among these subsets, an array of different mechanisms of antigen recognition and specific effector functions are available to combat disease.

$CD4^+$ T cells

The importance of T cells in the defense against tuberculosis was demonstrated by aggravation of disease in $CD4^+$ depleted mice (Orme, 1987). Moreover, transfer of $CD4^+$ T cells from immunised animals, protected non-immunised TB infected mice. The dramatic decrease of resistance to tuberculosis in AIDS patients, shows that $CD4^+$ T cells also have an important protective effect in humans (Barnes and Modlin 1996). Protection against *Mycobacterium tuberculosis* infection by T cells seems to be antigen-specific and mediated by secreted cytokines. The cytokines activate macrophages, which kill the bacteria. Among these cytokines, interferon γ appears to be the crucial one. The cytolytic activity of $CD4^+$ T cells against mycobacterially infected monocytes has been described, but how this manifests *in vivo* is not known (Kaufmann and Andersen, 1998). $CD4^+$ cytotoxic T cells (CTLs) isolated from broncheo-alveolar lavage, indicated that they play a role in local immune responses against *M. tuberculosis*. The MHC class II molecule restricted lysis of target cells. It was noted that alveolar macrophages are more resistant to lysis than blood monocytes. It was further observed that CTL clones, specific for *M. tuberculosis*, lyse a number of targets, using the granule exocytosis as well as the Fas - Fas ligand (FasL) pathway (Oddo *et al.*, 1998, Manfredi *et al.*, 1998).

$CD8^+$ T cells

β2-Microglobulin forms part of the structure of MHC class 1 proteins, the ligand for $CD8^+$. The importance of $CD8^+$ T cells is demonstrated in β2-microglobulin deficient mice. These animals lack mature $CD8^+$ T cells and are highly

susceptible to mycobacterial infection. The susceptibility could also derive from defects in the CD1 presenting system, which also contains β2-microglobulin (Porcelli and Modlin, 1999, Moody *et al.,* 1999). Vaccination of mice with a DNA vaccine encoding the mycobacterial heat shock protein 65, revealed that protection was predominantly mediated by $CD8^+$ T cells (Tascon *et al.*, 1996). These cells were detected in high frequency, secreted IFNγ, and were highly cytolytic. In *M. tuberculosis* infected humans, Tan *et al.* (1997) detected CTLs against tuberculosis in the lungs of patients. Using ELISPOT, Lalvani and Hill (1998) demonstrated IFNγ producing $CD8^+$ T cells in the peripheral blood of TB infected patients.

Until recently, it was shown that all $CD8^+$ cell-lines recognize antigens from *M. tuberculosis* in the context of classic MHC class I molecules. It is now realized that non-classical class I (MHC class Ib and CD1) presenting molecules are also recognized. A subset of $CD8^+$ cells, which recognize CD1 presented non-peptide tuberculosis molecules, were able to recognize and lyse TB infected macrophages irrespective of whether they were derived from healthy donors, TB or HIV/TB co-infected persons (Stenger and Modlin, 1998).

IFNγ, produced by $CD8^+$ T cells, contributes to protection by complementing macrophages and $CD4^+$ T cells: Tascon *et al.* (1996), demonstrated that *M. tuberculosis* infection could be controlled by transferring $CD8^+$ cells to MHC class II deficient mice. However, the protection was lost if the donor cells were taken from IFNγ deficient mice. This suggests that IFNγ, rather than cytotoxicity, provides the mechanism of protection.

Double-negative αβ CD1 restricted T cells

Double negative ($CD4^-CD8^-$) T cells with the αβ antigen receptor, recognize non-protein antigens presented on the CD1 molecules (Beckman *et al.,* 1994). These CD1 restricted double negative (DN) T cells, contribute to the response of the

host against mycobacterial infection. As with CTLs, DN T cells lyse macrophages infected with *M. tuberculosis,* and produce IFNγ (Tascon *et al.,* 1998). It was also demonstrated that DN CD1-restricted T cells recognize antigen on the surface of *M. tuberculosis* infected cells (Sieling *et al.*, 1995). Fas-FasL interaction mediated the cytotoxicity of DN CD1-restricted T cells, but no effect was seen on the viability of the bacteria. This is in contrast to $CD8^+$ T cells, which lysed the infected macrophages via a Fas independent, granule-dependent mechanism, killing both the host cell and the bacteria. Thus, the $CD8^+$ and DN phenotypically distinct subsets of human CTLs use different mechanisms to react to the infected cells with distinctly different outcomes.

T cells with the γδ antigen receptor

T cells with the γδ antigen receptor (γδ-T cells), are important in the resistance against tuberculosis: they accumulate in human lesions caused by *M. tuberculosis* infection and respond *in vitro* to non-protein mycobacterial antigens by proliferation (Stenger and Modlin, 1999). Moreover, their ability to produce IFNγ in response to *M. tuberculosis* infected macrophages supports this hypothesis (Tsukaguchi *et al.*, 1995). *In vivo,* protection mediated by γδ-T cells seems to be dependent on the size of the mycobacterial innoculum. γδ-TCR deficient mice can control *M. tuberculosis* infection if the amount of bacteria is not too high. The infection was found to be more pyogenic than granulomatous, hinting at a role of γδ-T cells to regulate cellular traffick at the site of infection, rather than directly combatting the infection or infected host cells. γδ-T cells migrate into the mycobacterial lesions to contain infection. FasL is upregulated on activated γδ-T cells induced by mycobacterial antigens during chronic disease. If the infection turns chronic, the *M. tuberculosis* reactive γδ-T cells are cleared via apoptosis through Fas ligandation. This in turn contains the tissue damage, due to limitation of inflammation, but allows a chronic tuberculosis infection (Li *et al.*, 1998).

1.13 Cytokines in tuberculosis

Cytokines are soluble proteins produced by a range of cells. They work at nano- to picomolar concentrations on cell membrane receptors to regulate and modulate signal transduction, cell proliferation, activation and differentiation. They induce proliferation and effector function of a variety of cells that are involved in the elicitation of both the innate and adaptive immune responses.

Interleukin 1

IL1 is produced upon stimulation of human monocytes with lipoarabinomannan (LAM) from the cell walls of *M. tuberculosis* (Zhang and Rom, 1993, Wallis *et al.*, 1990). IL1 may contribute to the fever that is characteristic of TB infection, as it is an endogenous pyrogen (Dinarello, 1984). It may also enhance the inflammatory response by inducing the macrophages to produce IL6 and tumour necrosis factor α (TNFα). IL1 produced by macrophages, drives IL2 receptor expression by T cells. If peripheral blood monocytes secrete too much IL1, the upregulation of IL2 receptors can have a suppressive effect on T cell proliferation through consumption of IL2 (Barnes and Modlin, 1996).

Interleukin 2

IL2 determines whether a T cell will proliferate and become a functional effector cell. The interaction of the IL2 receptor and its agonist enhances clonal selection and proliferation of CD4 memory T cells to change into IFNγ secreting cells. T cell proliferation can be induced through upregulation of IL2 receptors and IL12 production (Platanias and Vogelzang, 1990).

Interleukin 6

IL6 is a potent T cell proliferation and B cell maturation factor, leading to increased production of immunoglobulins by activated B cells (Hirano *et al.*, 1990). It may therefore mediate the hyperglobulinemia that is characteristic of tuberculosis. IL6 gene-disrupted mice cannot control acute intravenous infection (Ladel *et al.*, 1997). IL6 is increased 10 000 times in cultures of infected macrophages, compared to uninfected controls (VanHeyningen *et al.*, 1997). In

humans, the IL6 from lung lavages of patients with active pulmonary tuberculosis was also found to be high, indicating that IL6 may play a key role in *M. tuberculosis* infection. Lipoarabinomannan (LAM) as well as muramyl dipeptide, both components of mycobacterial cell walls, have been shown to stimulate IL6 production (Zhang *et al.*, 1994a). Moreover, mycobacteria had to be metabolically active to induce this 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 the mycobacterial infection. It seems that low levels of IL6 are necessary for differentiation and growth of infected macrophages, whereas high concentrations of IL6 suppress the macrophages' ability to stimulate T cells (VanHeyningen *et al.*, 1997)

Interleukin 12

Secretion of IL12 by the infected macrophages and the production of IFNγ by natural killer (NK) and other cells, lead to Th1 cell development. Th1 is usually associated with effective immunity leading to containment of tuberculosis. Anti-IL12 antibodies inhibit the proliferation of T cells against *M. tuberculosis* (Zhang *et al.*, 1994b).

Both IL2 and IL12 have profound influence on the adaptive immunity. IL12 induces the development of Th1 cells, which increase immunity to intracellular pathogens (Flynn *et al.*, 1995a). The production of IFNγ, and proliferation of T- and NK cells are also enhanced by IL12 (D'Andrea *et al.*, 1992, Kobayashi, 1989). IL12 binds to high-affinity β1/β2 interleukin receptor complexes on the T- and natural killer cells. Mutations of these receptors lead to severe mycobacterial infections. Both IFNγ and IL12 receptor deficiencies predispose to mycobacterial infections and impair the formations of mature granulomas (Altare *et al.*, 1998). IL12 mRNA and -protein are high in response to *M. tuberculosis* (Zhang *et al.*, 1994b). Thus, IL12 participates in a resistant immune response against *M. tuberculosis.* IL12 has the capacity to induce proliferation of cytolytic T cells only upon co-stimulation of the T cell receptor with antigen or anti-CD3 (Bertagnolli *et al.*, 1992). IL12 might be a candidate to control the cytolytic part of the initial

immune response against microbial pathogens by T cell proliferation, but only in the presence of antigen. The cytolytic activity of $CD8^+$ T cells and NK cells are also enhanced by IL12 (Flynn *et al.*, 1995b, Gately *et al.*, 1994). IL10 and TGFβ in contrast, inhibit IL12 synthesis and suppress Th1 responses (D'Andrea *et al.*, 1992).

Interleukin 10

IL10 has suppressive functions by down-regulation of the MHC molecules and inhibition of the production of monokines. Antibodies against IL10 in TB patients enhanced the production of IFNγ (Gong *et al.*, 1996). This suggests that IL10 may inhibit the immune response against *M. tuberculosis* in humans. Mycobacteria induce IL10, which inhibits the Th1 response to mycobacteria, probably by inhibiting the synthesis of IFNγ by NK cells and macrophages. If the production of IL10 by macrophages and IL4 by $CD4^+$ NK cells takes place at an early stage of the infection when innate immunity plays a critical role, the differentiation of T cells will be biased towards Th2 cell development. This may worsen the prognosis of the disease (Kaufmann, 1995).

IL10 was originally described as a Th2 cytokine, but is also secreted by alternatively activated macrophages and human Th1 cells (Abbas *et al.*, 1996). Production of IL10 may be part of an alternatively activated macrophage system controlling the Th1 effect to protect the lungs from disseminated necrotic damage.

Tumour necrosis factor α (TNFα)

TNFα exhibits both protective and pathologic effects in *M. tuberculosis* infection. Physiological concentrations seem to be important to anti-mycobacterial immune defenses. It has been speculated that physiological concentrations of TNF at the site of infection contribute to granuloma formation, control of the disease and mycobacterial elimination. Orme and Cooper (1999) and Rhoades *et al.* (1995), looking into the relationship between these mechanisms, proposed that protection is cytokine- and DTH driven. Protective immunity is an attempt by the

host to prevent further spread of the infection. This is accomplished by the innate response acting via IFNγ and the adaptive system characterized by clonal expansion of specific T cells. These T cells secrete IL12 and then IFNγ. In granuloma formation, influx of macrophages is regulated via chemokines, mostly produced by leukocytes and local tissue cells. Large amounts of macrophages and monocytes move into the infection site long after protective immunity has been activated. Because DTH and protective immunity occur at the same time, it was always seen as one reaction. According to Orme and Cooper (1999), the key element in both protection and DTH reactions is TNFα. Classically activated macrophages are a good source of this cytokine. TNFα drives the DTH reaction by chemokine secretion, recruiting monocytes into the region. However, TNF is a double edged sword, on one side necessary for granuloma formation and protection, but on the other side leading to continued influx of monocytes and tissue damage (Orme and Cooper, 1999).

If increased TNFα is found in the blood, systemic manifestations like fever and cachexia is found (Barnes and Modlin, 1996, Tracey and Cerami, 1994). Disruption of the gene coding for the TNFα receptor in infected mice, leads to decreased survival (Flynn *et al.*, 1995). Patients with tuberculous pleuritis, who mount a resistant immune response to infection, have concentrated TNFα at the site of infection (Barnes *et al.*, 1990). Human alveolar macrophages and mononuclear cells produce TNFα in large quantities in response to *M. tuberculosis* (Valone *et al.*, 1988).

Transforming growth factor β (TGFβ)

TGFβ is produced constitutively by monocytes from TB patients and inhibits IL2-dependent T cell proliferation and IL2 receptor expression (Ortaldo *et al.*, 1991). TGFβ down-regulates MHC class II expression on macrophages and inhibits cytokine production by macrophages (Oswald *et al.*, 1992, Toossi *et al.*, 1995). Macrophages are only activated by IFNγ if TGFβ is neutralized, indicating that TGFβ inhibits anti-mycobacterial immune defenses. In macrophages, as in

lymphocytes, TGFβ's level of control is not regulated by mRNA expression, but in secretion and activation of latent forms. Its function may be to contain the extent of inflammation following infection, in order to limit damage to non-infected surrounding tissues (Toossi *et al.,* 1997, Toossi and Ellner, 1998).

Interferon γ (IFNγ)

Interferon gamma is a pleiotropic cytokine, playing a central, but complex role in resistance of the host to pathogens. IFNγ exerts its activities on macrophages and lymphocytes, resulting in classically activated macrophages and differentiated T cells to deliver a Th1 type of immune response. Except for regulation of several aspects of the immune system, IFNγ also:

1. Stimulates bactericidal activity of phagocytes,
2. Stimulates antigen presenting through MHC class I and II molecules,
3. Facilitates leukocyte-endothelium interactions,
4. Influences cell proliferation and apoptosis,
5. Control the expression of a variety of genes whose function is unknown at this time (Boehm *et al*., 1997).

Such a variety of effects by a single cytokine is coordinated by complex patterns of cell specific gene regulation. It is known that over 200 genes are regulated by IFNγ (Boehm *et al*., 1997).

For a long time it was thought that only T cells and NK cells express and secrete IFNγ. As a general rule, both these cell types require cooperation from accessory cells being in some form of activated state. For T cells to produce optimal amounts of IFNγ, the following signals are needed:

1. a ligand for the antigen receptor (specific or aspecific)
2. a balanced cytokine combination (TNFα, IL12, IFNγ)
3. contact of accesory cells through adhesion molecules.

Macrophages are intimately related to the IFN system, so much so that an "IFN-macrophage alliance" has been postulated (Mogensen and Virelizier, 1987). All attention is currently given to the effects of IFNγ on macrophages, assuming that

other cells are the source of the IFNγ. The biological effects of IFNγ are summarized in Table 1.1.

Table 1.1. Biological effects of IFNγ on macrophages. (Gessani and Belardelli, 1998)

Function	Effect
Classical macrophage activation	↑
Alternatively activated macrophages	↓
Cytokines and chemokines production	↑ ↓
MHC class II antigen expression	↑
FcγR1 expression	↑
Nitric oxide production	↑
Differentiation	↑
Antiviral activity	↑

Until a few years ago, it was not believed that macrophages could produce IFNγ. Recently, Fultz *et al.* (1993) and Fultz and Vogel (1998), as well as Gessani and Belardelli (1998) provided proof that the IFNγ gene is constitutively expressed in unstimulated, resting peritoneal macrophages. In addition, if these macrophages were stimulated by LPS treatment, increased levels of IFNγ mRNA, as well as cell associated immunoreactive IFNγ was found. Moreover, it was demonstrated that IFNγ can up-regulate its own gene expression, by accumulation of both its mRNA in the cytoplasma and protein in the culture medium.

Convincing proof is now available that macrophages can indeed produce IFNγ under certain physiological as well as pathological conditions. The functional role of this is not yet understood, but it seems that the regulatory mechanisms activating transcription of this gene differ in macrophages in comparison to lymphocytes. Only a single signal will activate transcription of IFNγ in macrophages as opposed to at least two signals in lymphocytes. This emphasizes the importance of macrophages as early players in the immune

response, giving a direct response to infection. It has been well demonstrated that shortly after infection, IL12 is secreted in huge amounts, giving the major signal for local release of IFNγ. This in turn may act as the auto-stimulatory signal for further cytokine production. In peritoneal macrophages, IFNγ is spontaneously secreted, implicating that under physiological conditions certain macrophages are in a classical activated state (Gessani and Belardelli, 1998).

The finding that macrophages can respond to IL12 by producing IFNγ, adds a new component to the role of macrophages in the relationship between innate and acquired immunity.

It appears that the activated macrophages and the Th1 $CD4^+$ T cells play the major role in TB immunology, leading to an acute phase inflammatory response. Monocytes attracted by many chemotactic factors, ingest the bacilli released by the lysed macrophages. The tissue-damaging stage is a result of a delayed hypersensitivity (DTH) reaction, destroying nonactivated TB infected macrophages.

1.13.1 The role of Th1 and Th2 cytokine responses in *M. tuberculosis* infections

Mycobacterium tuberculosis is a facultative intracellular organism. Eradication of infection is only possible through cellular immunity.

In the Th1 responses, macrophages loaded with *M. tuberculosis* secrete IL12 which in turn activates lymphocytes to secrete IL2 and IFNγ, both being Th1 cytokines. From various sources, it seems that IFNγ alone is not enough for eradication of disease (Flynn, 1999).

In Th2 responses, elevated levels of IL4, IL5, IL6 and IL13 are found together with IgE secretion and activation of eosinophils and mast cells. IL4, possibly derived from basophils, mast cells or NK1.1 T cells, induce the Th2 response.

IL10 is found in both Th1 and Th2 responses and in alternatively activated macrophages. In murine mycobacterial infection, the Th1 response found in the first three weeks after infection, changed to a mixed response after day 50. Abbas *et al.* (1996) proposed that the Th2 mostly present in chronic infections, down-regulate the tissue damage found in pure Th1 responses.

The type, dose and route of antigen, can also influence the polarization toward a Th1 or Th2 response (Table 1.2). Mycobacterial antigen such as purified protein derivative (PPD) induces a strong Th1 response, whereas *Toxocara* excretory antigen induces a Th2 response. Immunization with 10^7 *Mycobacteria vaccae,* induces a Th1 response, compared to 10^9 bacilli which leads to a mixed Th1/Th2 response (Hernandez-Pando and Rook, 1994). Low or high dose soluble protein with uptake via pinocytosis induces a Th2 response (Constant and Bottomly, 1997). As will be discussed later, phagocytosis via the different receptors tends to polarize the immune response to Th1 and IL12 production.

Table 1.2 Factors that regulate the polarization of Th1 or Th2 immune responses.

Factors involved	Th1	Th2
APC	Dendritic cell, Macrophage	B cell
Cytokines	IL12, IFNγ	IL4
Ag dose	Low	Very low or very high
Co-stimulation	B7-1	B7-2
Steroid	DHEA	Glucocorticosteroid

1.14 Factors that determine resistance or susceptibility to tuberculosis

The search for an effective immunotherapy against tuberculosis requires that cognisance is taken of genetic factors that influence resistance or susceptibility to disease. The following is known to critically determine the outcome of tuberculosis infection in humans:

IFNγ receptor (IFNγ-R)

Levin and Newport (1999) described 6 children having disseminated infection with atypical *Mycobacterium* species in 1996. All were living on Malta. Four were from the same village, of which three were from the same family. This suggested a common immunological genetic disorder. Comprehensive testing revealed impairment in IFNγ production in response to mycobacterial infection. All the children lacked an IFNγ receptor on their leucocytes. When children with impaired IFNγ-R were vaccinated with BCG, they developed disseminated infection. The frequency of occurrence of this phenomenon was found at 0.59 cases per million vaccinated children, making IFNγ -R deficiency very rare.

Natural resistance-associated macrophage protein (*Nramp*) –

In the mouse model, resistance/susceptibility to infection could be related to a single autosomal gene. This gene, bcg, is located on chromosome 1 and is not mycobacterium specific. The gene product determined by cloning and sequencing, was called natural resistance–associated macrophage protein, abbreviated Nramp. This protein, expressed by macrophages and other APC's, was found to modulate the susceptibility to mycobacterial infection. Human Nramp has also been cloned, and the cell distribution is the same as in mice. It is present on chromosome 2q35. It leads to 4 times less tuberculosis infections compared to the Nramp defective-genotype. This demonstrates that Nramp is an important gene in the variability in susceptibility to tuberculosis (Arias *et al.*, 1997). Nramp has two related actions:

1. activation of macrophages to kill intracellular organisms without the help of T cells, through activation of nitric oxide (NO) (Arias *et al.*, 1997).
2. influencing the antigen presentation to $CD4^+$ T cells to modulate the body into a Th1 response (Arias *et al.*, 1997).

The method by which the Nramp induces the Th1 response is not yet known, but IL12 could be a mediator.

IL12 receptor –

IL12 binds to high-affinity β1/ β2 IL12 receptor complexes on the T- and NK cells. Mutations of these receptors lead to severe mycobacterial infections. Both IFNγ and IL12 receptor deficiencies predispose to mycobacterial infections and impair the formations of mature granulomas (Altare *et al.*, 1998). IL12 mRNA and protein are high at the infection site in patients with TB pleuritis. The pleural fluid cells produce IL12 in response to *M. tuberculosis* challenge (Zhang *et al.*, 1994b). Thus, IL12 participates in a resistant immune response against *M. tuberculosis.* IL12 has the capacity to induce proliferation of cytolytic T cells only upon co-stimulation of the T cell receptor with antigen or anti-CD3 (Bertagnolli *et al.,* 1992).

Antigen presenting molecules – The susceptibility to disease is often genetically determined by alleles or genes that code for membrane proteins involved in presenting antigens to the immune cells. The most important of these are coded for by the Major Histocompatibility Complex (MHC) of genes.

MHC class 1 molecules consist of one α-globulin subunit in association with β2 microglobulin. It binds endogenous peptides and present them to $CD8^+$ T cells. Cytosol derived peptides are from endogenous intracellularly digested proteins. Endogenous proteins are known to be degraded within the cytoplasm by the large (26S) LMP-containing proteosome complex. These peptides are transported into the endoplasmic reticulum by an ATP binding transporter, TAP. Here they interact with calnexin associated MHC class 1 molecules and calnexin is released. Finally the MHC class 1 molecule-peptide complex is transported from the ER through the Golgi complex to the plasma membrane.

A substantial role for MHC1 molecules in mycobacterial infection is demonstrated by β2-microglobulin deficient mice. These mice rapidly die from *M. tuberculosis,* but not BCG infection (Kaufmann and Ladel, 1994).

However, it must be kept in mind that β2-microglobulin deficiency not only leads to absence of active $CD8^+$ cells. The observed effect can be due to other effects, such as deficient CD1 presentation (Kaufmann and Ladel, 1994).

MHC class II (MHCII) molecules on macrophages and B cells present antigenic peptides to $CD4^+$ T helper (Th) cells. Most of these peptides are exogenous proteolytic degradation products loaded onto newly synthesized MHCII molecules. MHCII molecules are synthesized in the endoplasmic reticulum (ER) as a transmembrane glycoprotein consisting of homodimerized α and β-chains, of which the C luminal domains comprise the peptide binding groove. In the ER, the α and β -homodimers associate with the invariant chain (Ii), a non-MHC-encoded integral membrane glycoprotein. Interaction between a luminal domain of Ii known as class II associated Ii peptide (CLIP), and the putative peptide–binding groove of MHC II, prevents peptide binding in the ER. As soon as the MHC-Ii complex is formed, it is transported to the Golgi complex. After glycosylation, the MHC-Ii is transported to the trans-Golgi (TGN) network and targeted to the endocytic network.

Using immunoelectron micrography, it was shown that a majority of intracellular MHC class II are found in late endosome structures, with numerous internal membrane vesicles and sheets typical for late endosomes (LE) and lysosomes [MHC class II compartments (MIICs)]. These MIICs structures also contain lysosomal membrane proteins and enzymes and little or no transferrin receptor. In macrophages, MIICs represent the primary site for peptide loading. Some class II molecules that bind peptide are recycled from the surface of the cell.

Mutant mice with MHCII deficiency, and thus non-functional $CD4^+$ T cells, die of *M. tuberculosis* and BCG infection. This points to an essential role of CD4+ and MHC II molecules (Kuby, 1997).

1.15 Mycobacterial mycolic acids (MA) in resistance or susceptibility to tuberculosis

Whereas much is known on the role of protein antigens from *Mycobacterium tuberculosis* in prototype vaccines against tuberculosis, much less is known on the effect that biolipid antigens have. The most abundant cell wall biolipid of *M. tuberculosis* is mycolic acids (MA). Mycolic acids (MAs) are a class of long-chain fatty acids present in mycobacteria and some other related genera, i.e. *Nocardia*, *Rhodococcus* and *Corynebacterium* (Minnikin and Goodfellow, 1980). MAs are located within the bacterial cell wall, covalently esterified to peptido-glycan-linked arabinogalactan polysaccharides, and also non-covalently, hydrophobically associated in the form of 6,6'-trehalose dimycolate (Brennan and Nikaido, 1995). Although uniform in their α-branched, β-hydroxy structure, mycolic acids are heterogeneous with regard to chain length, number of double bonds, cyclopropane groups and side groups (keto-and methoxy-groups) (Minnikin and Goodfellow, 1980, Brennan and Nikaido, 1995). The C_{60}-C_{90} MAs are characteristic for mycobacteria (McNeil *et al.*, 1991). The first evidence for immunoregulatory properties of MA was obtained by the observation that presentation of MA to T cells occurred by professional antigen-presenting cells (APC) in a MHC-independent manner through CD1b molecules (Beckman *et al.*, 1994). These experiments revealed a unique role for MA in stimulating the unconventional subset of $CD4^-CD8^-$ T cells.

1.15.1 CD1 as MA presenting membrane protein

CD1 molecules, unlike the MHC molecules, are capable of presenting non-peptide foreign lipids and glycolipid-microbial antigens to specific T- cells. CD1 molecules are surface glycoproteins (50 000 dalton) expressed as glycosylated heavy chains, non-covalently associated to β2 microglobulin. CD1 is related to MHC, but equally distant from MHC class I and II. It probably originated from a primordial antigen presenting molecule at an equal evolutionary time as the MHC class I and II molecules. CD1 glycoproteins differ from MHC class molecules by being:

- encoded by genes outside the MHC
- non-polymorphic
- independent of the transporter associated with antigen-presentation (TAP) or the invariant chain, required for trafficking to MHC class I and II respectively (Behar *et al.*, 1999)

There are five non-polymorphic CD1 genes in the human: CD1a, -b, -c, -d and e, compared to the rodents' CD1d1 and CD1d2. The β2-microglobulin-associated protein products from these genes are grouped in:

Group 1 CD1 (consisting of human CD1 a, -b and –c) and

Group 2 CD1 (consisting of human CD1d and -e and murine CD1d1 and CD1d2 (Brossay *et al.*, 1997).

The group 1 molecules are abundantly expressed on professional antigen presenting cells such as dermal- and lymph node dendritic cells, Langerhans cells and mantle zone B cells, whereas group 2 CD1 proteins are expressed on the epithelium of the intestine. CD1 homologues are found in all mammalian species examined to date (Porcelli and Modlin, 1999). There are several hints that the unique functions of the CD1 may be related to the special properties of the species of T cells that interact with the molecule. Table 1.3 shows the CD1 molecules found in various mammals.

Table 1.3 Size and complexity of the CD1 gene in various mammals (Porcelli and Modlin, 1999).

Species	Total CD1 genes	Number of genes for indicated isoform				
		CD1a	CD1b	CD1c	CD1d	CD1e
Human	5	1	1	1	1	1
Mouse	2	0	0	0	2	0
Rat	1	0	0	0	1	0
Guinea Pig	≅10	?	>5	>3	?	>1
Rabbit	≅8	?	>1	?	>1	?
Sheep	≅7	?	>3	?	?	?

In addition to mycolic acids and phosphoglycolipids, glucose monomycolate (GMM) is also presented on hCD1b. hCD1a, b and c present acyl-chain-containing polar antigens. hCD1a and hCD1c present lipids recognized by $CD8^+$ cells. CD1 molecules have also been shown to present *Haemophilus influenza* type b glycolipids (Fairhurst *et al.*, 1998). In table 1.4 the major differences between Group 1 CD1 (humans only) and CD1d (mouse and human) are listed.

Table 1.4 Comparison of antigen recognition mediated by group1 CD1 and CD1d molecules (Burdin *et al.*, 2000).

Property	Group1(CD1)	CD1d
Stimulation of autoreactive T cells	Yes	Yes
Stimulation of NK T cells	No	Yes
Presentation of α-GalCer	No	Yes
Presentation of LAM	Yes	No
Presentation of GMM	Yes	?
Reactivity with microbes	*M. tuberculosis* *H. influenza*	*P. falciparum* *T. brucei*
Requirement of acidic pH for binding to lipid antigen	Yes	No
TCR usage	Variable	Invariant or restricted

In mice, two types of T lymphocytes reactive to CD1d have been described. The $CD8^+TCR\alpha\beta^+$ cytotoxic T lymphocytes are specific for foreign, hydrophobic peptides, whereas the CD4 single positive or $CD4^-/CD8^-$ double negative T cells recognise the CD1d without foreign antigen, *i.e.* they are auto-reactive. It is currently not clear whether a self ligand should be bound to mCD1 in order to effect recognition by T cells. The auto-reactive anti-mCD1d cells can be sub-divided depending on their expression of an invariant TCR α-chain and the cell surface phenotype for NK1.1. If both are present, these cells are called natural

killer T cells or NK T cells. The heterogeneity of the mCD1d auto-reactive T cells is seen by the number of reactivity patterns (almost the same as the amount of T cells tested), and is not related to the level of expression of mCD1d on APC. It is speculated that the heterogeneity might reflect the requirement of the mCD1d T cells to be able to recognize members of a diverse set of autologous ligands such as lipoglycans. It is still undefined what the nature of CD1d bound lipoglycans, required to stimulate CD1d auto-reactive T cells, must or should be.

Brossay *et al.* (1998) demonstrated that some of the CD1 auto-reactive T cells require wild type CD1 that trafficks to endosomes. This suggests that CD1 might bind to a self-ligand derived from the endosome. Metabolic radioactive labelling and mass spectrometry helped to identify glycosylphosphatidylinositol as a natural ligand of mCD1d (Joyce *et al.,* 1998). Using a synthetic glycolypid α-galactosylceramide (α-GalCer), it was shown that both hCD1d and mCD1d can bind this ligand and that either can stimulate NK T cells in both mice and humans. This emphasizes the conservation of the CD1 system.

Crystal structure analysis of the CD1d molecule (Figure 1.2) showed an MHC class 1-like fold with a narrow, deep electrostatically neutral and highly hydrophobic antigen-binding groove (Zeng *et al.*, 1997). CD1d binding models place the hydrophobic acyl portion of the lipid molecule into two large hydrophobic pockets. Hydrophilic or polar groups are on the top of the groove. Compared to CD1b, CD1d does not need acidic conditions to induce the conformational changes required for loading the antigen into the hydrophobic pocket of the groove. The nature and the length of the acyl chains buried in the antigen-binding groove do not significantly affect the presentation capability of CD1d (Porcelli *et al.,* 1998). It seems that the CD1d binding groove has less demanding structural requirements in presentation to NK T cells than Group I CD1 (CD1b).

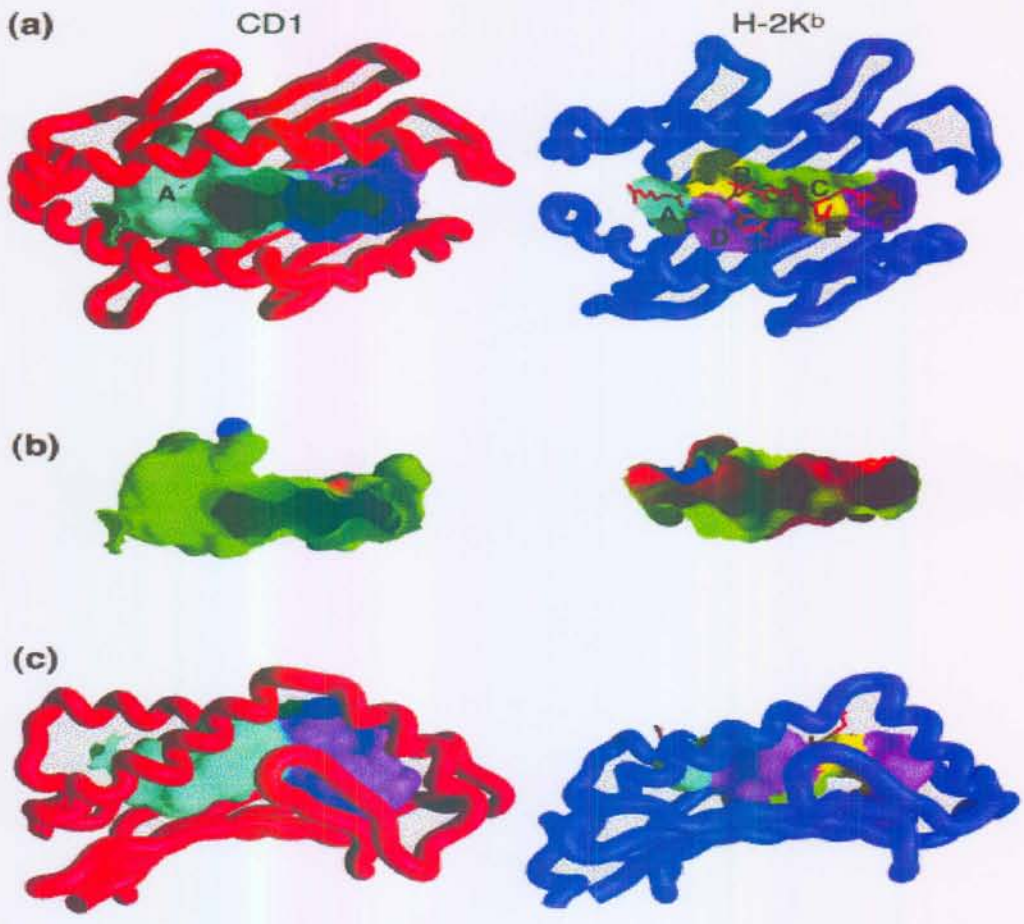


Figure 1.2 Comparison of the ligand binding grooves of CD1 and MHC class I(a) The molecular surface of the groove is shown superimposed on a worm diagram of the backbone of the α1 and α2 domains for CD1d (left) and H-2k ^{b}(right). The groove pockets (A ´and F´) for CD1d and A–F for H-2k^b are differentiated by colour. (b) Electrostatic potential of the groove surface of CD1d and H-2k ^{b}. Areas with a negative charge are coloured red and areas with a positive charge are blue. Green represents various areas of neutral charge. (c) Side view of the α1 and α2 domains with the groove surface superimposed on the backbone as in (a) and (b). (Zeng *et al.*, 1997).

A critical aspect that still has to be resolved, is the loading of the CD1 groove. Are the lipids processed before loading, and how are they inserted into the groove of the CD1 protein? The human CD1b protein is found in a variety of endosomic compartments, also in those in which the MHC class II is found to load peptides. These MIICs are lipid rich late endosomes. The lipid rich composition of the MIICs is an ideal place to concentrate the foreign lipid antigens, such as mycolic acid. In addition, MIIC contains a wide array of degradative enzymes, which may be involved in the trimming of large glycan components of some CD1 presented antigens, such as those found in LAM.

Sieling *et al.* (1999), showed that CD1 is abundantly expressed in the tuberculoid form of *Mycobacteria leprae* infection compared to lepromatous leprosy. In the tuberculoid or self-healing form of leprosy, INFγ secreting Th1 cells are recruited, in comparison to the lepromatous form where Th2 immunity prevails. This suggests that the CD1 molecule expression may determine the predominance of Th1 or Th2 response.

CHAPTER 2

Aims of this study

The appropriate immune response to bacterial invasion is related to the cell wall structure of the invading organism. There are four main bacterial cell wall types:

- Gram-positive
- Gram-negative
- Mycobacteria
- Spirochaetes

Different immunological mechanisms have evolved to destroy and remove the different groups of bacteria. One example is demonstrated by LPS micelles from gram-negative bacteria that modulate the immune system. By deploying a complex pathway, LPS is neutralized by binding to lipoprotein particles or binding to soluble CD14 (sCD14), thereby passing it on to membrane receptors. The above mechanism is accomplished by a lipid binding protein that assist in the transfer, starting a cascade of reactions leading to cytokine release and immune modulation (Roitt *et al.*, 2000). Another example is provided by isolated *Chlamydia pneumonia* lipopolysaccharide (cLPS), which induces a cholesterol ester accumulation and foam cell formation in macrophages (Kalayoglu and Byrne, 1998).

Thus, macrophages that are chronically exposed to cLPS may accumulate cholesterol and contribute to atheroma development in pursuit of destroying the microbial pathogen.

As MA comprises 40% of the dry weight of *Mycobacteria tuberculosis,* it may be anticipated to have at least some effect on the invaded cell and host. The complexity of the mycobacterial cell wall makes it difficult to relate single components to the effect they have on the immune system. It may be argued that the effect of the cell wall as a unit may be quite different than the sum of the effects of each component.

In Chapter 1, the importance of tuberculosis as a disease, the mechanics of the mycobacterial infection and the response of the immune system to the infection were discussed, giving special reference to the newly discovered importance of mycolic acids as immunoregulatory substance. Previous work at the University of Pretoria indicated that some important immunoregulatory cytokines were secreted when MA was introduced in mice, providing increased survival upon challenge with living *Mycobacterium tuberculosis*. This dissertation concerns itself with shedding new light on the role that mycolic acids may play in the progression of tuberculosis or immune resistance to it.

Giving the chemical and physical nature of MA, the first obstacle was to get MA into a soluble form to be introduced into the body. The isolated MA was tested for the effects it may have on mouse peritoneal macrophages. The mouse model was used, as the immune system of the mouse is well known and frequently used in mycobacterial research. Macrophages from the mouse peritoneal cavity were chosen, due to their abundance and ease of isolation. Also, immune markers are readily available.

Mycobacterium tuberculosis escapes the innate immune system and evades destruction inside the macrophage after phagocytosis by various systems. Some of these systems prevent the phagosome to mature into late stage endosomes. In chapter 3, the MA and MA-carriers were studied to learn how they were phagocytosed, and how the phagosomes subsequently developed into late stage endosomes by using fluorescently labelled MA.

The effects of MA on mouse macrophages were found to involve differences in activation status as well as morphology. Chapter 4 deals with the activation and survival of macrophages and characterization of the phenotype of the cells that develops after loading with MA.

In Chapter 5, the effect of MA on the immune system and the killing of *Mycobacterium tuberculosis* are examined. Evidence is presented for a prominent role for MA in the innate immune system. The role of reactive oxygen and nitrogen intermediates are examined in the activation of macrophages with subsequent foam cell formation and killing of the bacteria.

Chapter 6 summarizes the evidence to support a role for MA on the innate immune system. As with the LPS in *Chlamydia pneumonia,* this isolated part of the mycobacterial cell wall seems able to activate macrophages into foam cell formation. The activation status of these foam like cells are discussed.

The complete genomic sequence of *M. tuberculosis* suggests that it contains sterol biosynthetic enzymes as well as two putative cholesterol degrading enzymes (Cole and Barrell, 1998, Cole, 1998, Bellamine *et al.*, 1999). This gives us ground to speculate on *M. tuberculosis* induced foam cells as a possible carbon and energy source for latent intracellular Mycobacteria.
In addition, a possible effect that *Mycobacterium tuberculosis* infection might have on cardiovascular diseases through immune modulation by MA, is proposed.

CHAPTER 3

Evaluation of carriers for mycolic acids

3.1 Introduction

3.1.1 Mycolic acids: properties and solubility

Mycolic acids are high molecular weight, α-branched, β-hydroxy fatty acids, present in the bacterial genera Mycobacterium, Rhodococcus, Nocardia and Corynebacterium. Mycobacterial mycolic acids are covalently bound to arabinogalactan via ester bonds (McNeil *et al.*, 1991). Mycolic acids also occur as components of soluble trehalose dimycolate (cord factor) and trehalose monomycolate in the cellular matrix (Besra *et al.*, 1994).

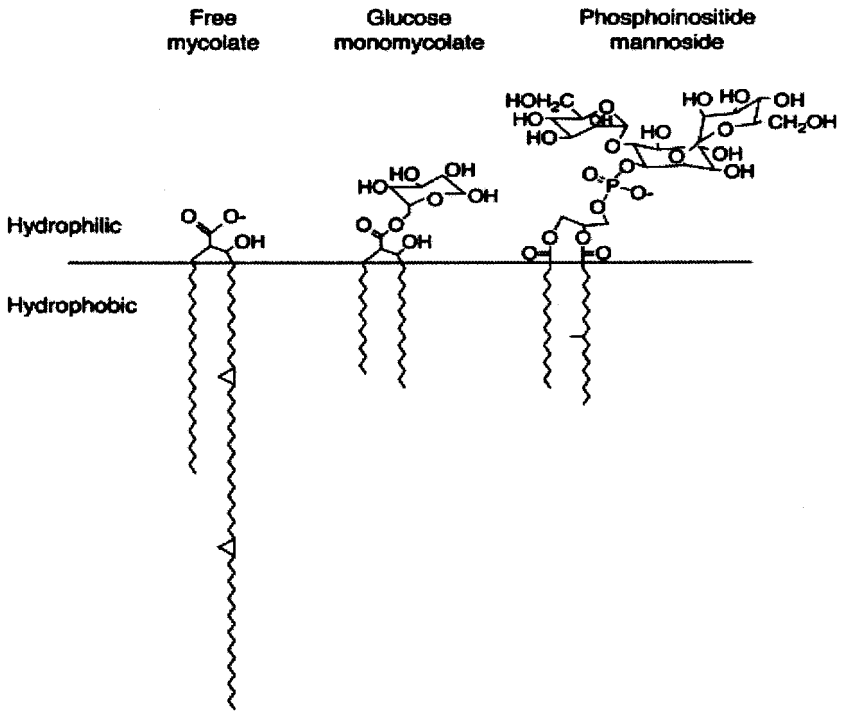


Figure 3.1 Structure of mycolic acid, glucose monomycolate and phosphoinositide mannoside.

Mycobacterial mycolic acids can be distinguished from those of other genera by

- being the largest mycolic acids (C60-C90)
- having the longest side chains (≥ C20-C24)
- containing one or two unsaturated groups, existing as double bonds or cyclopropane rings
- containing functional groups with oxygen in addition to the β-hydroxy group
- being methyl branched in the main carbon backbone (Liu *et al.*, 1996).

Mycolic acids can be divided in three subclasses namely α-mycolates, ketomycolates and methoxymycolates, according to the chemical groups associated with the carbon backbones.

The extracted and purified mixture of mycolic acids appears white and waxy with a melting point of approximately 40°C. It is insoluble in water, but soluble in non-polar organic substances such as chloroform, dichloromethane and tetrahydrofurane.

3.1.2 Dissolving hydrophobic substances in water

To enhance the water solubility of a drug, organic solvents are used which are miscible with water. Alternatively, surface–active agents (surfactants) are used which orientate into concentrated polar and non-polar layers (micelles) in solution. This has been used to solubilize substances such as vitamins, oils and resins (Florence and Rogers, 1971).

For a substance to dissolve, the solvent must overcome the forces of intermolecular attraction of the solute. According to the principle of like dissolves like, ionic compounds dissolve more readily in polar water by virtue of ion-dipole interactions, whereas non-polar substances dissolve more easily in organic solvents as a result of dipole or induced dipole interactions (Van der Waals, London or Debey forces). The solubility of a drug substance is due mainly to the polarity of the solvent, often expressed as the dipole moment, referred to as the

dielectric constant. Solvents with high dielectric constants dissolve ionic substances and are water soluble, whereas solvents with low dielectric constants do not dissolve ionic substances. The former are polar solvents such as water and methanol, while the latter are non-polar substances such as chloroform and oils (Lawrence, 1989).

3.1.2.1 Emulsions

Emulsification is the heterogenous dispersion of one immiscible liquid in another. An emulsion is formed when two immiscible liquids are agitated together and become dispersed in one another. Water coalesces much faster than oil and two phases are formed if the emulsion is not stable. If a stabilising agent (emulsifier) is added to the two mixed, immiscible liquids, one phase may become translucent, but the other will remain in emulsified form. Emulsifiers are simple additives that usually consist of a non-polar hydrocarbon chain with a polar group at the end. The hydrocarbon chain is soluble in oil and the polar head in the water. Thus, emulsifiers selectively locate at the interface between aqueous and oil phases or at the surface defined by the aqueous and air boundaries (Lawrence, 1989). Their presence cause a reduction in the surface tension of water at the interface with the oil, the degree of which determines the stability of an emulsion when the phases are mixed.

3.1.2.2 Micelles

Micelles come about when amphiphillic molecules in water segregate their hydrophobic portions from the solvent by self-aggregation. Micelles can be spheres, discs, oblate or prolate ellipsoids, or long cylinders and can be ionic or non-ionic. Amphiphiles must have a certain concentration to form or produce a structure to shield the tails from the water. This concentration is called the critical micellar concentration (CMC).

At ultra–low interfacial tension, a micro-emulsion forms spontaneously, which has the appearance of a clear solution. By increasing the concentrations of the

emulsifying agent, a change is induced in the micro-emulsion at the so-called critical micellar concentration (CMC), when mostly spherical structures are formed with the hydrocarbon chains projected to the inner part and the polar groups to the surface contacting the aqueous phase (Rosoff *et al.*, 1988). These are called micelles and have a diameter of approximately 5 nm. Inverse micelles can also be produced by amphiphiles in non-aqueous solutions (Lawrence, 1989).

Micelles cannot be detected optically and appear as translucent solutions. Solutes with poor water solubility, such as fatty acids, will dissolve in the inner part of these micelles and thus become solubilized. In general, micellar solubilisation is saturable at approximately 10 % (m/v) solute concentration, depending on parameters such as the chemical properties of the solute and the type of emulsifying agent used. The maximum amount of solute that can be incorporated into a given emulsion system at a fixed emulsification agent concentration, is termed the maximum additive concentration (MAC). Higher solubilisation is achieved when the micellar solution is changed to a micro-emulsion. Micro-emulsions are also called swelled micelles, and consist of a homogenous transparent system of low viscosity and contains a high percentage of both oil and water as well as emulsifier. Micro-emulsions form spontaneously when the components are mixed in the appropriate ratios, and are thermodynamically stable.

The difference between micro- and macro-emulsions is the size of the particles. In the case of macro-emulsions, the size is usually above 0.15 μm and the solution is not transparent (Attwood *et al.*, 1989).

3.1.3 Carriers to solubilize mycolic acids

The carrier used to mobilise MA in aqueous solution or suspension for administration to cells or animals, can influence the absorption, processing and presentation by the macrophages or antigen presenting cells (APC). The carrier

must therefore be chosen in a way that takes into account the effects on the immune system.

3.1.3.1 Cyclodextrins

Cyclodextrins (CD) are a family of cyclic oligosaccharides produced by enzymatic modification of starches that can be applied as alternatives to micellar solubilisation. They are crystalline, homogenous, non-hygroscopic substances which are of a torus-like macro-ring shape, built up of glycopyranose units (Szejtli, 1988). Szejtli (1988) discussed the physical and chemical properties of cyclodextrins in depth. The most important characteristics of the α-, ß- and γ-cyclodextrins used in this investigation, are summarised in Table 3.1.

Table 3.1 Properties of cyclodextrins

	α	ß	γ
Molecular weight	973	1135	1297
Glucose monomers	6	7	8
Internal cavity diameter (A)	5	6	8
Water solubility (g/100 cm^3)	14.2	1.85	23.2
Melting range (°C)	255-260	255-260	240-245

Cyclodextrins are consumed by humans or animals as oral drug-carriers or as food–additives. On entering the gastric area, they undergo rapid dissolution and dissociation-association equilibrium is reached. Only insignificant amounts of CD are absorbed from the intestinal tract into the circulation. Because the bulk of the cyclodextrins is metabolized in the colon by the microflora, their effect on passive diffusion (Attwood *et al.*, 1989) need not be considered.

According to Szejtli, the acute toxicity of cyclodextrins, defined as LD 50 values, could not be accurately determined because high doses given orally did not result in any deaths of the animals. Similarly, sub-acute toxicity studies did not reveal any abnormalities in the experimental animals (Szejtli, 1988). Intravenous injections at a dose of 100 mg/kg for α-cyclodextrin and 788 mg/kg for ß-cyclodextrin, showed signs of nephrotoxicity characterized by proximal tubule

alterations accompanied by cytoplasmic vacuolation, cell disintegration and amorphous mineralisation (Szejtli, 1988). Subcutaneous administration of approximately 400 mg/kg of ß-cyclodextrin to male and female rats, produced signs of intoxication consisting of sleepiness and respiratory problems, which generally disappeared after 24 hours. Intramuscular administration led to the ulceration of the injection site after 32 daily injections into the same area with a dose corresponding to 80 mg/kg, but no mortality was recorded. No kidney damage could be seen after 12 days injecting a daily dose corresponding to 20 mg/kg. At a dose of 50 mg/kg, alteration was recorded in the kidneys and irreversible nephrotoxicity resulted. No mutagenic or teratogenic changes were recorded (Szejtli, 1988).

Fatty acids are generally easily soluble in cyclodextrins (Szejtli, 1988). The solubility of members of the homologous series of aliphatic fatty acids in an aqueous cyclodextrin solution, increases from caproic acid onwards. On average, five CH groups are co-ordinated to one cyclodextrin molecule. [The available chain length is almost identical to the actual length of the fatty acid molecule (Attwood *et al.*, 1989).]

3.1.3.2 Polyethylene glycol (PEG)

Polyethylene glycols are molecules of the general formula: $HO\text{-}(\text{-}CH_2CH_2O)_n\text{-}H$. The type nomenclature *i.e.* 300, 400, 6000 *etc.*, indicates the molecular mass (MW) of a particular compound.

The PEGs with low molecular mass such as 300 MW and 400 MW are clear liquids, almost tasteless and odourless. PEGs with higher molecular mass have a soft and waxy appearance at room temperature. A solution of 50% m/v PEG in water is a clear liquid with low viscosity. PEGs are soluble in water, chloroform and acetone. They are insoluble in ethers, fats, fatty oils and paraffins (Blume *et al.*, 1993).

PEGs 1500 to 6000 are used as carriers for active molecules, which are insoluble or sparingly soluble in water. They also act as lubricants and binders for the production of tablets (Attwood *et al.*, 1989). PEGs are substances with very low toxicity and good compatibility with the skin.

PEGs with low molecular weight, such as PEG 300 and 400, are good solvents for numerous substances that are not soluble in water. The solvent power is ascribed to a complex formation between the active substance and PEG. Some of these complexes are loose and reversible, but sometimes PEG complexes can inactivate a molecule by binding too strongly, eg penicillin G. Consequently, the influence of the PEG on the efficiency and uptake of the active substance needs to be tested in each particular case.

3.1.3.3 Sesame seed oil

Sesame oil is also known as benne or teel oil from the seeds of the cultivated *Sesamum indicum*. The oil consists of olein, stearin, palmitin, myristin, linolein, sesamin and sesamolin. It is a pale yellow oil and odourless with a bland taste. It solidifies at – 5°C. Sesame oil is soluble in chloroform, ether, petroleum ether and carbon disulphide, while only slightly soluble in ethanol and insoluble in water (Ten Wolde *et al.*, 1997).

Sesame oil is a non-toxic, edible oil, which finds frequent application in the food and cosmetic industries. Sesame oil was also found not to be toxic when in an injectable form. It has been used as a carrier for gold for injections in patients with rheumatoid arthritis (Ten Wolde *et al.*, 1997).

3.1.3.4 Solutol HS 15

Solutol HS 15 is also known as polyethylene glycol 660 hydroxystearate, and is known as a non-ionic solubilizer for injection solutions. It is a white paste at room temperature and becomes liquid at 30°C.

Solutol HS 15 consists of a polyglycolester of 12-hydroxystearic acid (70%) as hydrophobic component and polyethylene glycol 660 (30%) as hydrophillic component. The main fatty acid component is 12-hydroxystearic acid, but stearic and palmitic acid are also present in detectable amounts (Fromming *et al.*, 1990).

Solutol HS 15 is very stable. Prolonged exposure of the Solutol/water mixture to heat, may induce physical separation into two phases which can be reversed by homogenisation. Aqueous solutions of Solutol can be sterilised by autoclaving at 120°C. The solubility of Solutol decreases with increasing temperature. Solutol is soluble in chloroform, water, ethanol and 2-propanol. In these solvents it forms clear solutions (Information sheath, Pharmaceutical specialities, BASF, 1997).

The following LD_{50} figures are based on the Technical Information for Solutol HS 15 (BASF, 1997).

LD_{50}	mouse intraperitonealy	> 8,5 g/kg
LD_{50}	mouse intravenously	> 3,16 g/kg
LD_{50}	rabbit intravenously	> 1,0 < 1,4 g/kg

3.1.3.5 Microbeads

In the development of single-shot vaccines, biodegradable, non-toxic microspheres have been used to introduce immunogens to elicit a protective immune response. Polymeric microbeads of poly (lactic-coglycolic) acid (PLGA) and other polymer types, were being used which released the immunogen structurally and chemically intact (Cleland *et al.*, 1994).

Fluorescent microspheres are currently used in a variety of diagnostic tests and blood flow experiments. The microspheres used in these assays are hydrophobic particles that will passively absorb a number of molecules (Powell *et al.*, 1996).

Macrophages and other cells take up particles and micro-organisms via phagocytosis. By loading immunogen on the non-toxic, non-degradable microsphere vehicle, the phagocytosis of these microspheres by macrophages and the effect of the immunogen on the immune system can be studied. By using non-degradable microspheres, the secondary immunological influence of the antigen carrier can be minimized. This system would allow the study of the MA as immunogen mainly to pave the way to a more complex biodegradable vehicle in the future (Eldridge *et al.*, 1991).

3.1.3.6 Liposomes

Charged lipids and neutral lipids doped with a charged amphiphile, spontaneously form dispersions of unilamellar vesicles with diameters ranging from 0.1 to 2μm. Phosholipids, a main constituent of biological membranes, have a high tendency to aggregate in bilayers. Upon sonication, these transform into closed, self-sealing, solvent–filled vesicles called liposomes. These liquid crystals, when first formed, consist of several bimolecular lipid lamellae, separated by aqueous layers. Sonication causes the transformation of these units into unilamellar liposomes. Water-soluble drugs can be entrapped in liposomes in the aqueous layers, while lipid-soluble drugs can be intercalated within the hydrocarbon layers of the lipid interior (Farhood *et al.*, 1995, Bangham, 1983).

Important considerations in the preparation of lipid vesicles are lipid surface charge, vesicle size and aqueous volume of the liposomes. Large uni- or multilamellar liposomes are cleared rapidly from the circulation on intravenous injection, and are targeted to mainly the reticulo-endothelial system. An addition of cholesterol to the bilayer is beneficial for maintaining the physical structure (Deamer and Gavino, 1983, Bakouche and Gerlier, 1986). The net charge of liposomes can be varied by incorporation of negatively or positively charged molecules such as diacetylphosphate or stearylamine respectively. Sending the

liposome solution through a polycarbonate sieve of precise pore diameter, can control the homogeneity of size of the liposomes.

A disadvantage of liposomes is that phospholipids are susceptible to oxidation. This requires special care during storage, such as maintaining them in a nitrogen atmosphere (Florence and Rogers, 1971).

Liposomes can be administered intravenously, orally or intramuscularly. Direct administration to the nose or the lungs as an aerosol of liposomes looks promising as a mode of delivery.

3.1.4 The route of administration

The nature of the product to be administered and the desired pharmacological actions are factors determining the route of administration to be employed. Each route of administration is associated with certain limitations and provisos. One of the most important considerations is the volume of the drug which must be administered. In humans, large volumes (*i.e.* greater than 10 ml), can only be introduced via the intravenous route, although the rate of infusion is critical. Volumes up to 10 ml can be introduced intraspinely, while the intramuscular route is normally limited to 3 ml, subcutaneous to 2 ml and intradermal to 0.2 ml.

The choice of the solvent is critical to the intended route of administration. Intravenous and intraspinal injections are restricted to dilute aqueous solutions, whereas oily preparations, solutions, suspensions and emulsions can be injected intramuscularly and subcutaneously (Florence and Rogers, 1971).

As most infections of TB is found in the lung, an inhalant route seems appropriate. This will place the drug directly into the biosphere where it elicits its function.

3.1.5 Isotonic/iso-osmotic nature of injectables

A solution is iso-osmotic to host tissue fluid into which it is introduced, when the amount of dissolved solutes is equal in both systems. Injectable fluids are

formulated to be iso-osmotic to reduce the irritation that can result from osmotic incompatibility with the body tissues or, if introduced intravenously, to reduce the damage to erythrocytes. If an iso-osmotic preparation is injected into the body, there is no net fluid flux from or to the injection site, and hence no hindrance on passive diffusion (Duma and Akers, 1984, Florence and Rogers, 1971).

Isotonic and iso-osmotic are only synonymous if the dissolved solute cannot pass through membranes. If membranes are permeable to solutes (as is the case with aqueous solutions of glucose, urea and alcohol), the solution acts like pure water and both solute and solvent pass through the membrane into cells, causing them to swell and burst (Duma and Akers, 1984).

3.2 Aims and objectives

The experiments carried out in the course of this investigation were aimed at exploring the suitability of the following carriers for administration of mycolic acids:

1. Cyclodextrins α, ß and γ;
2. Polyethylene glycols 300 and 6000;
3. Sesame seed oil;
4. Solutol HS 15;
5. Polystyrene microspheres and
6. Liposomes using phosphatidylcholine and cholesterol.

3.3 Experimental

3.3.1 Materials

3.3.1.1 General reagents

Cyclodextrin α was obtained from Sigma Chemical Co (St Louis, MO).

Cyclodextrins ß and γ were a gift from the Department of Chemistry at the University of Pretoria.

Chloroform was from Saarchem (Analytical Grade, RSA).

5-Bromomethylfluorescein (5-BMF) and propidium iodide were purchased from Molecular Probes (Leiden, The Netherlands).

Potassium carbonate and 18-crown-6 ether were obtained from Sigma Chemical Co (St Louis, MO).

Methanol (HPLC grade) was obtained from Merck (Darmstadt, Germany).

Silica gel GHL plates were from Merck.

PEG 300 and PEG 6000 were obtained from Merck (Darmstadt, Germany).

Sesame seed oil at 100% purity was obtained from Pick & Pay, a South African general retail store.

Saline (0,9% NaCl) in infusion bags was purchased from Adcock Ingram Critical Care, Johannesburg, South Africa.

Fluorescent microspheres (1 micron diameter) were obtained from Molecular Probes (Leiden, The Netherlands).

Solutol HS 15 was a gift from BASF Pharmaceuticals (Ludwigshafen, Germany).

Phosphatidylcholine and cholesterol were from Sigma Chemical Co (St Louis, MO).

Macrophage cell lines from C57BL/J6 (4.4) and Balb/C (2C11/12) mice were gifts from Prof. Johan Grooten (University of Gent) and Prof. Patrick de Baetselier (University of Brussels) respectively.

3.3.1.2 Reagents for HPLC

For the preparation of reagents used in mycolic acid separation on HPLC, double distilled deionized water and HPLC grade methanol (BDH, Poole, UK) were used.

Reagent A: Potassium hydroxide (25%) Analytical Grade (Saarchem, South Africa) dissolved in methanol-water (1:1), *i.e.* 62,5 g potassium hydroxide was dissolved in 125 ml water and 125 ml methanol HPLC Grade (BDH, Poole, UK) was added.

Reagent B: Concentrated hydrochloric acid Analytical Grade (Saarchem, South Africa) diluted 1:1 with water.

Reagent C: Potassium bicarbonate (2%) Analytical Grade (BDH, Poole, UK) dissolved in methanol-water (1:1). Potassium bicarbonate (10 g) was dissolved in 250 ml water and 250 ml methanol was added.

Reagent D: Para-bromophenacylbromide dissolved in acetonitrile and crown ether (Pierce Chemical Co., Rockford, IL, Cat. No 48891) were dispensed in 500 µl quantities into small amber-coloured screw cap vials with Teflon-coated septa. The caps were tightened and the vials were wrapped with Parafilm™. Reagent D was stored at 4°C.

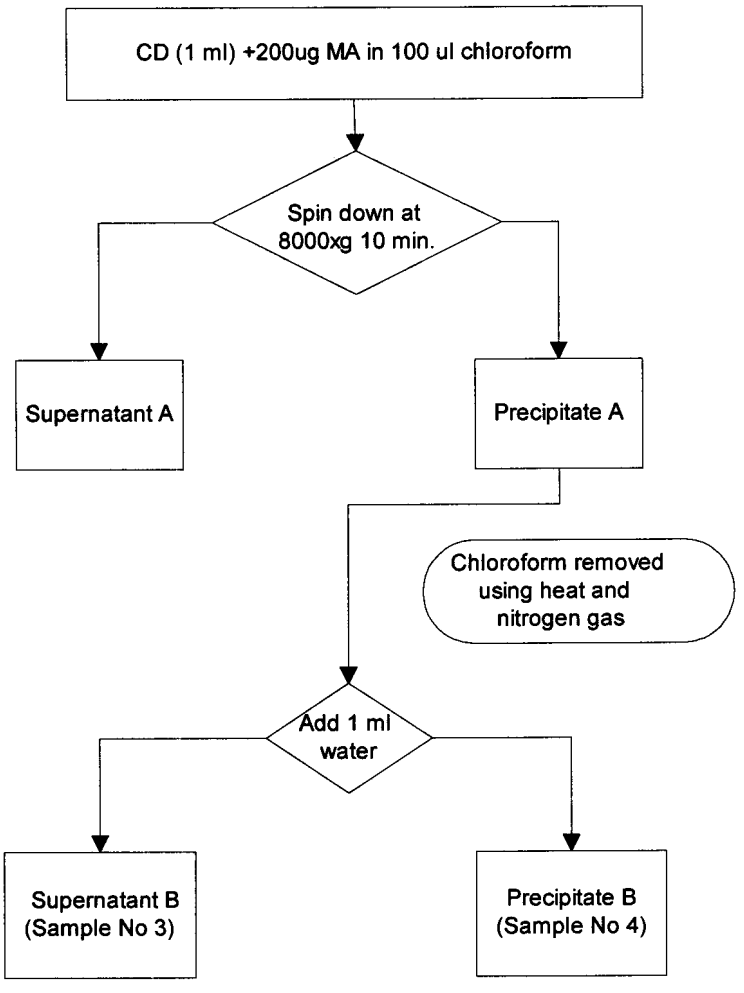
Reagent E: Reagent E was prepared by mixing reagent B 1:1 with methanol.

HPLC Standard: High molecular weight internal standard (C-100) from Ribi ImmunoChem Research Company (Hamilton, MT, Cat No R-50). The standard, 1 mg, was dissolved in 20 ml chloroform HPLC Grade (BDH, Poole, UK) at 4°C and aliquots of 100 µl were dispensed into 4 ml amber WISP vials, dried, capped with Teflon-coated septa and stored at 4°C.

3.4 Methods

3.4.1 Preparation of cyclodextrins α, ß and γ - mycolic acid complexes

Saturated solutions of α, ß and γ cyclodextrins were prepared in double distilled water. The solutions were heated to 80°C and cooled down, which resulted in the formation of a clear solution. In each vial of a set of three, 1,0 ml of saturated solutions of cyclodextrins (CD) α, ß and γ were introduced. Mycolic acids were added as chloroform solutions according to the scheme in Fig. 3.2. As high temperature does not influence the structure of cyclodextrins, chloroform was removed by heating the samples at 80°C under a stream of nitrogen. Samples from all the preparations were extracted using chloroform, weighed and analysed by HPLC for quality control.



Negative control: saturated CD solution 1 ml + 100µl chloroform
Sample 1 and 2 – equivalent to sample 3 and 4

Figure 3.2. Lay-out of the procedure to prepare cyclodextrin-mycolic acid complexes and sampling for quality control.

3.4.2 Solubility of mycolic acids in aqueous solutions of PEG 300 and 6000

3.4. 2.1 PEG 300

In a first vial containing 100 μg MA, 200 μl of a 100% solution of PEG 300 was added. In a second and third vial containing 200 μg MA each, 200 μl 50% (v/v) solution of PEG 300 in water were added. The solutions were heated to 80°C for 30 min to melt the MA into the PEG 300. During this time, the vial was vortexed and heated again. After the mixtures were allowed to cool down to 20°C, 50 μl samples were withdrawn from each vial and analysed for the presence of MA by HPLC. The empty vials were also rinsed with chloroform and sampled for MA determination by HPLC.

3.4. 2.2 PEG 6000

Into a vial containing 490 μg MA, 200 μl of a 20% (v/v) solution PEG 6000 in water was added. The solution was heated to 80°C, vortexed for 5 minutes and allowed to cool down to 20°C. Between each step the solubility was evaluated visually. The volume of the solution was then increased to 1 ml by adding 200 μl aliquots of a 20% solution of PEG 6000. After the introduction of each aliquot, the content of the vial was evaluated visually. The sample was heated and vortexed as described for the first step. The concentration of PEG 6000 in the solution was then increased to 40% by introducing solid PEG 6000. Heating and vortexing of the sample was done in between. The stability of the resulting homogenous suspension was then determined by leaving it at room temperature for 3 days, taking care not to disturb the suspension. Undissolved MA was observed floating on the surface.

In order to establish the concentration of dissolved MA, a 200 μl sample was carefully withdrawn from the bottom of the vial, the solution shaken and a second sample of 200 μl, containing the suspended MA, was also withdrawn. This allowed the determination of the non-soluble MA fraction. The remaining suspension was then removed, the vial extracted three times with 500 μl chloroform and these combined rinses analysed by HPLC.

3.4.3 Sesame seed oil as carrier for mycolic acids

A sample of 430 μg mycolic acid was dissolved in 200 μl chloroform and 200 μl of sesame oil added. The vial was vortexed and heated to 80°C. Nitrogen gas was blown over the oil surface to remove chloroform. After the removal of chloroform, the remaining 200 μl of the MA mixture in sesame oil was divided into two identical parts:

A) Duplicate samples of 50 μl of the sesame oil/MA preparation were transferred to vials containing 200 μl chloroform and the HPLC standard.
B) To the remaining part of the sesame oil and MA preparation, chloroform (100 μl) was added and vortexed. Duplicate samples of 100 μl were withdrawn and analysed by HPLC.

The first set of duplicate samples were to represent the solubilized MA in sesame oil, while the second set were to represent the solubilized MA as well as the insoluble MA left in the vial. The control consisted of 50 μl sesame oil and 200 μl chloroform.

3.4.4 Solutol HS 15 as carrier for mycolic acids

3.4.4.1 MA introduced from a chloroform solution

A stock solution of MA in chloroform was prepared at a concentration of 5 mg/ml. Three concentrations (5%, 10% and 25% v/v) of Solutol HS 15 were prepared in water. To 200 μl aliquots MA stock solution, aliquots of 200 μl of the different concentrations of Solutol HS 15 were added. Control solutions of MA in chloroform and Solutol HS 15 alone were used as positive and negative controls respectively.

3.4.4.2 MA introduced as a dried solid

To prepare an unsaturated MA solution, 10% Solutol in 0,9% saline (1 ml) was added to 250 μg dried MA in a sterile Eppendorf tube. The sample was heated

for 10 min at 80°C, vortexed for 10 seconds and left to cool down to room temperature. A 100 µl sample was used for HPLC analysis.

To prepare a saturated MA solution, 10% Solutol in 0,9% saline (200 µl) was added to 3 mg dried MA in a sterile Eppendorf tube. The sample was heated for 10 min at 80°C, vortexed for 10 seconds and left to cool down to room temperature. Three 50 µl samples, taken from the bottom of the vial, were prepared for HPLC.

3.4.4.3 Toxicity testing of Solutol HS 15

3.4.4.3.1 *In vitro* toxicity testing

Three concentrations of Solutol HS 15 (100 µl each of 1, 0,5 and 0.1% v/v in PBS) were added to C57BL/J6 macrophage cultures (1ml) containing $2X10^5$ cells and incubated for 10, 30 and 60 minutes. The macrophage cultures contained either 10% or 20% (v/v) foetal calf serum. The Solutol HS 15 was removed by washing four times with complete medium (2ml) before the cells were put back in the incubator. After 24 hours the cells were dissociated from the flask and tested for survival by staining with 5 µl propidium iodide per 1ml cell suspension and analysing on the FACS.

3.4.4.3.2 *In vivo* toxicity testing

Ten mice were injected intravenously with 100 µl Solutol HS 15 (10% v/v saline) with and without mycolic acids (25 µg). Daily weighing and examination was done for 30 days to record any signs of toxicity.

Two mice were injected intraperitoneally with 100 µl Solutol HS 15 (10% v/v) with and without MA (25 µg). After 2 hours the peritoneal macrophages were removed, one half tested for viability using propidium iodide on the FACS and the other put into culture and assessed for viability after 24 hours using the trypan blue exclusion test. As negative control, peritoneal exudate macrophage cells obtained from non-injected mice were used.

3.4.4.4 Effect of Solutol HS 15 on haemolysis

The turbidity of fresh human erythrocyte suspensions in different concentrations of Solutol HS 15 was measured at 690 nm to determine the latter's isotonicity. The decrease in turbidity is proportional to the degree of lysis of the erythrocytes.

Human red blood cells were suspended in Iso M after collection in EDTA tubes.
Iso M was made up by taking 200 ml of Tris/NaCl buffer pH 7.4 Tris 24.2 g, NaCl 163.0g made up to one litre, adjusted with HCl and diluted to 2 litres), adding 6.7 ml of 0,15 M $MgSO_4$ and 3 ml of 0,1 M $CaCl_2$ and diluting to 2 litres.
Iso BSA was made up by dissolving BSA (1 g) in 1 litre of Iso M.
All cells were washed in Iso BSA and used as the standard in which no lysis occurs.

Haemolysis of erythrocytes was tested by exposing the cells to the following samples listed below:

Experimental samples:

1. Solutol-saline (10% v/v) containing MA introduced as a dry solid (250 μg/ml) and autoclaved for 20 minutes at 121°C
2. Solutol-saline (10% v/v)
3. Saline (positive control)
4. Water (negative control)
5. Solutol-water 10% v/v MA 250 μg/ml

Solutions of MA were prepared by placing an amount of Solutol into a vial with MA, heating it up to 80°C for 5 min and vortexing it for one minute. These solutions were then left to cool down to 20°C.
A standard curve indicating the percentage haemolysis expressed as change in turbidity at 690 nm was prepared as follows: EDTA-blood (2 ml) suspended in Iso M (5 ml) was centrifuged at 750g and the buffy coat removed. An aliquot consisting of 2% erythrocytes in Iso M (named as suspension A) was used as the sample comprising 100% intact erythrocytes, *i.e.* 0% haemolysis. From this 1.5, 1.0 and 0.5 % erythrocyte suspensions were prepared in triplicate.

All test suspensions (1-5) were added to a 96-well flat-bottomed plate (90µl) and a background value was obtained on a Titertek Multiscan colourimeter. The 96 well plate was then cooled on ice. After 10 min on ice, 10 µl of uniformly suspended erythrocytes (suspension A) was added to all the wells. The 96-well plate was then incubated at 37°C. After 15 min the erythrocytes were resuspended and the turbidity recorded as absorbance at 690 nm in a Titertek Multiscan colourimeter.

3.4.4.5 Determination of the particle size of Solutol HS 15 / MA

Different concentrations of Solutol HS 15 (10% v/v) were prepared in water or saline. Mycolic acids (250 µg), saponified or not saponified, were placed into 4 ml glass vials. To each of these, 1ml of the prepared concentrations of Solutol HS 15 was added. The samples were heated for 10min at 80°C and vortexed for 10 seconds. Solutions of water alone, Solutol alone and saline alone were also used. Autoclaving of some samples was done at 121°C for 20 minutes. The solutions were left to cool down to room temperature and particle sizes were evaluated by light scattering in a flow cytometer. Effects of autoclaving on the micelle size, size distribution and pH of the suspensions were also evaluated.

3.4.5 Microsphere carriers for mycolic acids

3.4.5.1 Preparation of the microspheres

Insoluble polystyrene microspheres were used as a transport vehicle to load MA into the macrophages. Uptake via phagocytosis was tested using these fluorescent microspheres with MA melted onto the surface. This was never intended as an injectable, but only as a method of testing the immunological presentation by APC.

Saponified MA (125 µg) in chloroform was put into an Eppendorf tube that was silanated and dried at 100°C under a stream of nitrogen gas. Microspheres were diluted in PBS to 2 X 10^7 per ml, heated to 100°C on a heat block and 500 µl hot

suspension added to the melted MA at 100 °C. The suspension was then sonicated with a Branson B30 sonifier (at 50% power output for 1 minute), allowed to cool down and again sonicated at room temperature for 10 seconds. The microsphere-mycolic acids suspension was prepared just prior to use.

3.4.5.2 Toxicity testing of microspheres

Uncoated or MA-coated microspheres were added to a C57BL/J6 macrophage cell line culture for phagocytosis. After 24 hours the macrophages were dissociated from the flasks and tested for cell death using trypan blue.

3.4.6 Liposomes as carriers for MA

The original methods of Bangham, (1983) for the preparation of liposomes were followed. This involved the deposition of a thin lipid film from an organic solvent medium on the walls of a container, followed by agitation with an aqueous solution of the material to be encapsulated.

3.4.6.1 Solubility of MA in liposomes

Phosphatidylcholine and cholesterol were both made up to 100mg/ml in chloroform. A mixture of phosphatidylcholine (161 μl) and cholesterol (97 μl) was put into a 5ml new sterile glass vial. MA (500 μg) was added and the chloroform removed by heat (80°C) under a stream of nitrogen gas. PBS (2 ml) was added. A liposome suspension was obtained by sonicating the heated (80°C) phosphatidylcholine / cholesterol / MA mixture. An MA-free liposome suspension was made and used as negative control. Suspensions of 200 μl were sampled for analysis by HPLC.

3.4.6.2 Toxicity of liposomes

To C57BL/6J macrophage cultures of 2 $x10^5$ cells in 3ml medium, 100μl of the liposome suspensions with and without MA were added. After 24 hours cell survival was recorded by using the trypan blue exclusion method.

3.4.6.3 Effect of liposomes on haemolysis

To one millilitre of a 1% packed cell volume per volume (pcv/v) erythrocyte suspension in RPMI 1640 medium, the following volumes of liposomes were added:

1. 0 μl
2. 200 μl liposomes only
3. 200 μl liposomes containing 50 μg MA

The suspensions were incubated at 37°C for 1 hour before the supernatants were tested for the presence of lactate dehydrogenase on an auto-analyzer at Dept of Chemical Pathology at University of Pretoria. The positive control consisted of a (1% v/v) erythrocyte suspension in which all cells were lysed by sonication.

3.4.7 Macrophage cultures

Macrophage cultures C57BL/J6 (4.4) and BALB/C (2C11/12) were maintained in RPMI 1640 (Life Technologies, Paisley, U.K.), supplemented with 10% foetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100U/ml), streptomycin (100U/ml), sodium pyrovate (1 mM) and β mercaptoethanol ($5x10^{-5}$ M).

3.5 Results

3.5.1 Cyclodextrin as carrier for mycolic acids

When adding chloroform aliquots with or without mycolic acids to the saturated solutions of cyclodextrins, precipitates were observed in all the CD preparations tested. Testing the supernatant fractions after centrifuging down the precipitates (Sample 3 in Fig 3.2) for the presence of mycolic acids, gave negative results, similar to the negative controls (Samples 1 and 2). Upon attempting to redissolve the precipitated CD in water, only those precipitates that did not contain MA dissolved. This confirmed that a specific interaction occurred between the dextrins and the MA. When analysing the precipitate fractions (Sample 4, Fig 3.3) very little mycolic acids were recovered. Fig 3.3 shows the yields of mycolic acids recovered in chloroform extracts of the CD precipitates, indicating that mycolic

acids were only recovered from the γ-CD precipitate complexes. The low yields recovered probably reflect poor extractability of MA from CD-MA complexes using chloroform. This serves as circumstantial evidence that the MA were indeed complexed to, rather than adsorbed onto the cyclodextrins, since adsorbed mycolic acids would have been recovered equally well from all three types of CD. Moreover, the probability that the mycolic acids were adsorbed onto the surface of the CD molecules are small, as the outer parts of the molecules are hydrophilic and the inner parts hydrophobic. The difficulty of extracting MA from the CD using chloroform may also pose a problem for the release of mycolic acids from the MA-CD complexes in the gastro-intestinal tract when taken orally. As the majority of cyclodextrins can be toxic in the injectable form, and the mycolic acids absorption from the gastric tract may pose a problem, it is unlikely that this method of mycolic acid administration will be suitable.

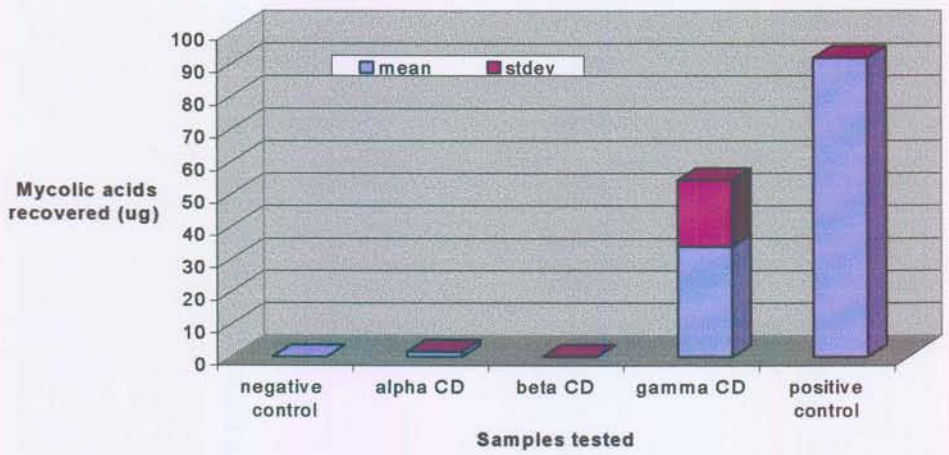


Figure 3.3 Recovery of mycolic acids from the three types of cyclodextrins used. Blue bars represent the average of triplicate values.

3.5.2 PEG as carrier of mycolic acids

When testing the solubility of MA in PEG as such or in aqueous PEG solutions, it was almost impossible to assess the degree of solubilisation by eye. The results of HPLC in Table 3.2 indicate that PEG 300 (undiluted or 50% v/v in water) does not solubilize mycolic acids. Upon using aqueous PEG 6000 solutions for the

solubilization of MA, a suspension was obtained which could not be dissolved by adding more of the PEG solution or by adding solid PEG. When 40% (m/v) PEG 6000 in water was used to solubilize MA, a suspension was obtained. Although 77% of the expected mycolic acids were recovered in the suspension (Table 3.3), the mycolic acids were not in solution. After leaving the mixture undisturbed for 3 days, the mycolic acids could be observed floating on the surface, while the clear solution drawn from the bottom of the tube did not contain any significant amount of dissolved mycolic acids.

Although PEG solutions heated to a temperature of 80°C appeared to solubilize MA, an unstable suspension resulted upon leaving the mixtures undisturbed to cool to room temperature. This cooling resulted in oil droplets floating on the surface of the solution. PEG was therefore considered to be an unsuitable carrier for mycolic acids.

Table 3.2 HPLC analysis of samples from attempted MA solubilisation in PEG 300.

SAMPLES	VOLUME WITHDRAWN	AMOUNT MA EXPECTED	AMOUNT MA RECOVERED	PERCENTAGE RECOVERY
Undiluted PEG 300/MA	200 µl	100 µg	4,1 µg	4,1%
50% PEG 300/ MA Sample 1	50 µl	50 µg	0.00 µg	0%
50% PEG 300/ MA Sample 2	50 µl	50 µg	1.01 µg	2.0%
MA (control)	200 µl	100 µg	92,0 µg	92,0%
PEG 300 alone	50 µl	0 µg	0.00 µg	-
MA recovered from the vial	200 µl	0 µg	0.00 µg	-

Table 3.3 HPLC analysis of samples from attempted MA solubilization with a 40% (m/v) aqueous PEG 6000 solution.

Samples	Volume taken	MA expected	MA recovered	Percentage recovery
PEG 600/MA *	200 µl	98 µg	0 µg	0%
PEG 600/ MA#	200 µl	98 µg	75,5 µg	77.0%

* This sample was not shaken and 200 µl was taken from the bottom of the vial.

\# This sample was shaken and the concentration determined on 200µl of the suspension.

3.5.3 Sesame oil as carrier for mycolic acids

Mixing the oil with chloroform solutions of mycolic acids tested the solubility of mycolic acids in sesame oil. After the mycolic acids were brought into solution in a vial containing the mycolic acids/chloroform and sesame seed oil, the chloroform was evaporated off, leaving the mycolic acids to either remain in oil solution, or to stick to the sides of the tube. The first duplicate sample that was taken (Experiment A), was for determining the amount of MA dissolved in the sesame oil and the second set (Experiment B),to determine both the solubilized MA, and insoluble MA left in the vial.

From the results it appears as if mycolic acids are soluble in sesame oil. About 60% mycolic acids could be recovered from the amount of oil that was spiked. The high degree of variation and the low yields could have been due to erroneous pipetting of the viscous oil. It is clear from Table 3.4 however that the mycolic acids were dissolved in the oils and were not left sticking to the wall of the tube.

Table 3.4 Concentration of MA dissolved in Sesame oil, as detected by HPLC

Samples	Volume taken	MA recovered	MA expected	Percentage recovery
Experiment A				
1.MA /Sesame oil	50 µl	48,0 µg	112,5 µg	42,6%
2.MA /Sesame oil	50 µl	77,0 µg	112,5 µg	68,4%
Total amount	100 µl	125,0 µg	225 µg	58,1%
Experiment B				
1.Sesame oil/vial	50 µl	88,0 µg	112,5 µg	78,2%
2.Sesame oil/vial	50 µl	28,0 µg	112,5 µg	24,8%
Total amount	100 µl	125,0 µg	225 µg	53,9%
Sesame oil alone	100 µl	0 µg	0 µg	-
Positive control	100 µl	100 µl	92 µl	92%

3.5.4 Solutol as carrier for MA

3.5.4.1 Solubility of mycolic acids in aqueous Solutol

To determine the optimal concentration of Solutol to solubilize mycolic acids for administration into animals, mycolic acids were aliquoted in 1 mg quantities and 200 µl of Solutol/water solutions added in concentrations of 5, 10 and 25% (v/v). Dissolution was effected by heating to 80°C. After cooling, the amount of mycolic acids dissolved in each sample was measured with HPLC. The results are presented in Fig. 3.4.

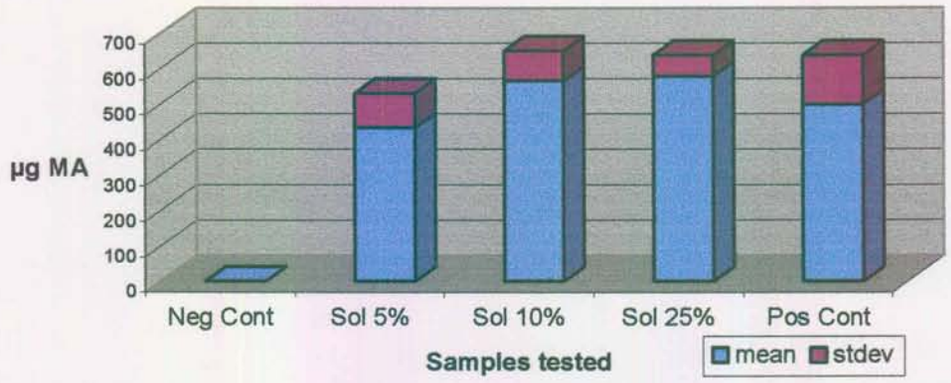


Figure 3.4 Concentration mycolic acids recovered from micelles using different concentrations of Solutol HS 15. The positive control consists of mycolic acids in chloroform. The negative control is Solutol alone. These solutions were obtained by using mycolic acids in chloroform and then removing the chloroform with heat and nitrogen gas. All determinations were done in triplicates.

Saturation of the solution of mycolic acids in Solutol/water was achieved between 5 and 10% (m/v) Solutol. As nothing was gained by increasing the concentration of Solutol to 25%, a 10% Solutol suspension was taken to be optimal.

Using 10% v/v Solutol HS 15 in saline (200 µl), adding it to 3 mg dried MA in an Eppendorf tube, heating it to 80°C, cooling it down to room temperature and releasing a sample for HPLC, it was shown that up to 9.7 mg/ml mycolic acids could be accommodated in 10% Solutol. This solution was not clear and consisted of a macro emulsion. No improvement could be gained by increasing the Solutol concentration to 25%. Based on this observation, Solutol at 10% was taken to be the optimal concentration for this carrier. It has a capacity to carry in excess of 9 mg/ml of mycolic acids.

3.5.4.2 *In vitro* testing of toxicity of Solutol HS 15

To determine the toxicity of Solutol, *in vitro* cultures of a C57BL/J6 derived cell line of macrophages were exposed to three concentrations of Solutol at three different times as indicated in Fig 3.5.

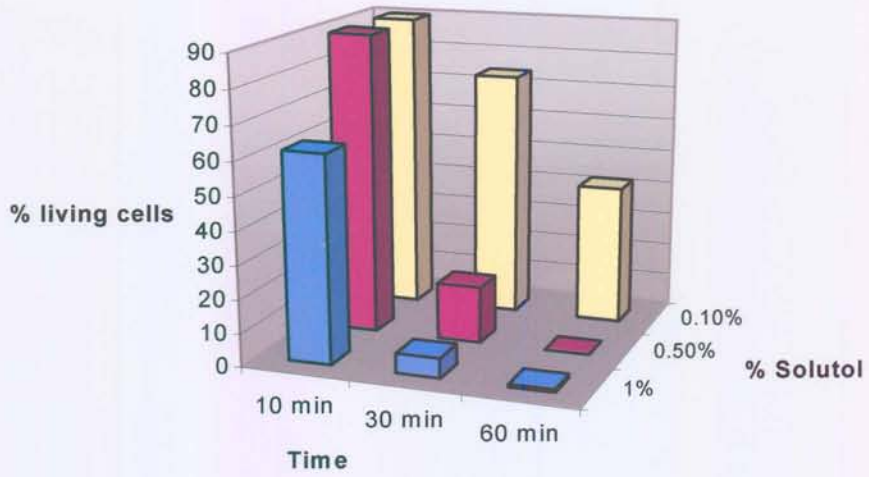


Figure 3.5 *In vitro* toxicity of Solutol tested on a mouse macrophage cell line using variable time and concentrations of exposure. Surviving cells were counted via flow cytometry using propidium iodide. Each bar represents the average of triplicate cell cultures.

The results from Fig 3.5 show that Solutol is toxic to the cells at concentrations as low as 0.1%. Exposure to Solutol concentrations higher than 0.1% practically killed all the cells after 30 to 60 minutes. Increasing the serum in the *in vitro* cell growth medium from 10 to 20%, decreased the toxicity somewhat (results not shown).

3.5.4.3 *In vivo* toxicity of Solutol HS 15 and mycolic acids

To determine whether Solutol HS 15 at 10% is toxic to animals, ten mice were injected intravenously with 100 μl of Solutol solution with and without MA. No weight change or other macroscopic change were observed which could imply toxicity. In addition, no cell death could be detected among peritoneal macrophages two hours after Solutol HS 15 injection. In Figure 3.6 the number of living macrophages obtained from mouse peritoneal exudate after intraperitoneal injection of 10% Solutol (100μl) could be seen after 24 hours in culture.

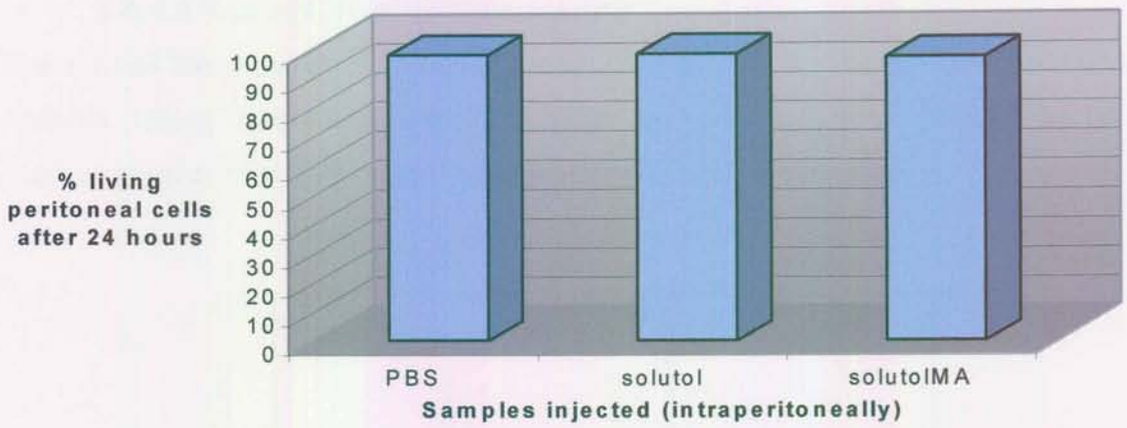


Figure 3.6 *In vivo* toxicity of solutol after two hours of intraperitoneal exposure. Percentage living macrophages were measured after 24 hours in culture. Solutol with or without mycolic acids (25 μg) was injected IP and after 2 hours peritoneal macrophages were removed and put into culture for 24hours before survival was assessed by trypan blue exclusion.

Having established that Solutol appeared non-toxic to mice when injected intraperitoneally, mycolic acids (25 μg in 100 μl of 10% Solutol) was injected intraperitoneally and the macrophages obtained two hours later from the peritoneal exudate. After 24 hours in culture, the cells appeared healthy and unaffected by the exposure to Solutol/mycolic acids (Fig 3.6).

3.5.4.4 Haemolytic activity of Solutol HS 15

In order to assess the potential risk of intravenous injection of mycolic acids/Solutol, the haemolytic activity of several preparations were tested in a quantitative haemolysis assay. The effects of MA saponification and water/saline medium were tested on the pH and haemolytic properties of the various Solutol preparations. In Table 3.5 the haemolysis results are shown and the pH values listed for the samples treated in various ways.

Table 3.5 Haemolytic activity and pH of Solutol and Solutol/Mycolic acids preparations.

SAMPLE	%HAEMOLYSIS	PH
Solutol-saline 10% v/v MA 250 μg/ml	6 %	6.02
Solutol-saline 10% v/v	6 %	5.50
Saline	0 %	5.58
Water	100 %	6.21
Solutol water 10% v/v	50 %	5.52
Solutol water 10 % v/v MA 250 μg/ml	50%	5.62

As expected, saline alone and water alone gave 0 and 100% lysis, respectively. If Solutol (10% v/v water) was used with and without mycolic acids, there was still 50% haemolysis of the erythrocytes added. If saline and Solutol were combined, then 6% haemolysis was obtained, indicating that Solutol micelles can haemolyse small amounts of erythrocytes. By adding mycolic acids to the Solutol saline, no change in the haemolysis was seen. From these results it seems that micelles of mycolic acids do not increase haemolysis of erythrocytes. The pH of the 10% v/v Solutol/saline is affected only moderately by adding 250μg/ml mycolic acids. Moreover, it appears not to be necessary to buffer the solution, as a pH of 6 is acceptable for an injectable.

3.5.4.5 Results from light scattering

The size of the micelles is of significance, as it will be injected intravenously into animals. Using 1µm beads as size reference, micelles were analysed by light scattering on a flow cytometer. In Figure 3.7 the results are shown.

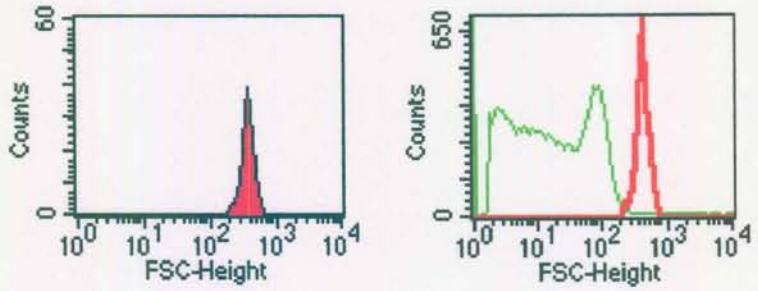


Figure 3.7 Histogram plots of micelles using 10% Solutol and mycolic acids at 250µg/ml. Beads that are 1µm in diameter were used as a size reference. Liposomes were all smaller than 1 µm in size. The beads (size1 µm) are shown in red.

It was concluded that the final product (Solutol 10% v/v saline and 250 µg mycolic acids sterilised by autoclaving) consists of micelles smaller than 1 µm in diameter.

3.5.5 Microspheres as carriers for MA

3.5.5.1 Loading of the microspheres

The method of coating the microspheres by melting the mycolic acids onto the microspheres was successful, as seen on the high resolution scanning electron microscope. Recovery of mycolic acids in organic solvents (chloroform or tetra-hydrofurane), from the polystyrene microspheres, was hampered due to the incompatibility of chloroform and polystyrene. The solubilized polystyrene mixture could not be separated on an HPLC column. The ideal microsphere system would comprise of biodegradable polymer microspheres, that are compatible with organic solvents. In Figure 3.8 the uncoated and coated microspheres are shown.

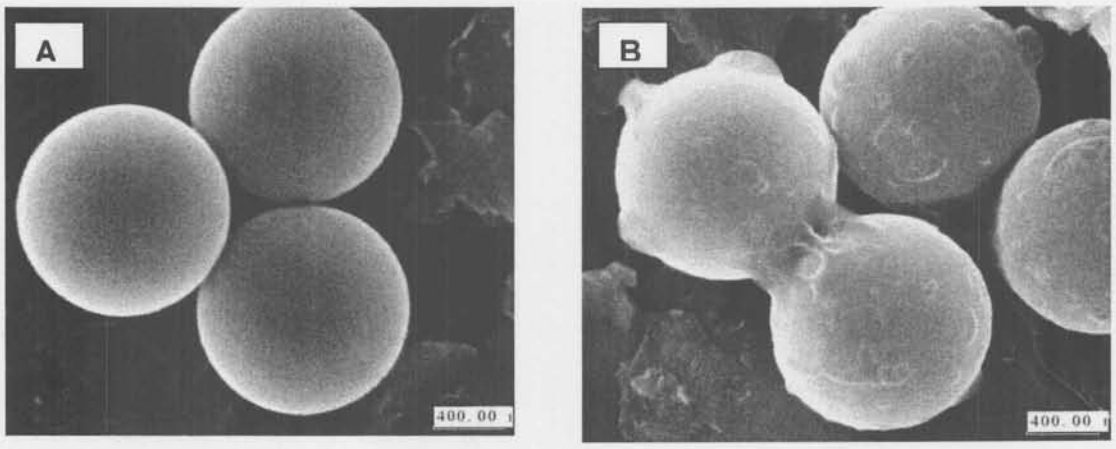


Figure 3.8 Polystyrene microspheres. (A) Microspheres without MA and (B) MA melted onto the microspheres .

3.5.5.2 Toxicity testing of beads

No macrophage cell death occurred with any concentration microspheres (beads) or microspheres-MA that was given to the macrophage cultures. Overloading the macrophages with more than 20 microspheres per cell didn't seem to have an effect on the growth of the macrophages.

Microspheres have previously been successfully applied as carrier for protein vaccines and here microspheres were used as a delivery system for mycolic acids to peritoneal macrophages *in vivo* and *in vitro.* In both cases the need for biodegradable microspheres is not important. Using non-degradable microspheres, the effects of the product can be isolated from the effects of the microspheres. Moreover, polystyrene microspheres have hydrophobic surfaces and mycolic acids stick well to these surfaces. If this delivery system has good immunomodulating effects, biodegradable microspheres can in principle be considered for delivery of mycolic acids into humans.

3.5.6 Liposomes as carriers for mycolic acids

3.5.6.1 Solubility of mycolic acids in liposomes.

Liposomes made with phosphatidyl choline and cholestrol accommodate the mycolic acids into solution. The amount of mycolic acids recovered from liposomes ranged between 70 and 110 % of the expected value.

3.5.6.2 Toxicity of liposomes and mycolic acids

The pH of the liposome solution was buffered at pH 7.4 by the PBS in preparing the liposomes for toxicity determination. No toxicity could be detected in adding liposomes or the liposomes loaded with mycolic acids to macrophage cultures (results not shown).

3.5.6.3 Haemolysis of erythrocytes using liposomes

The presence of lactate dehydrogenase released when erythrocytes lyse gives an indication of the ability of the liposomes to haemolyse cells.

Haemolysis of erythrocytes, tested by measuring lactate dehydrogenase activity amounted to none in all liposome-added samples. In Figure 3.9 the effect of liposomes on haemolysis is seen.

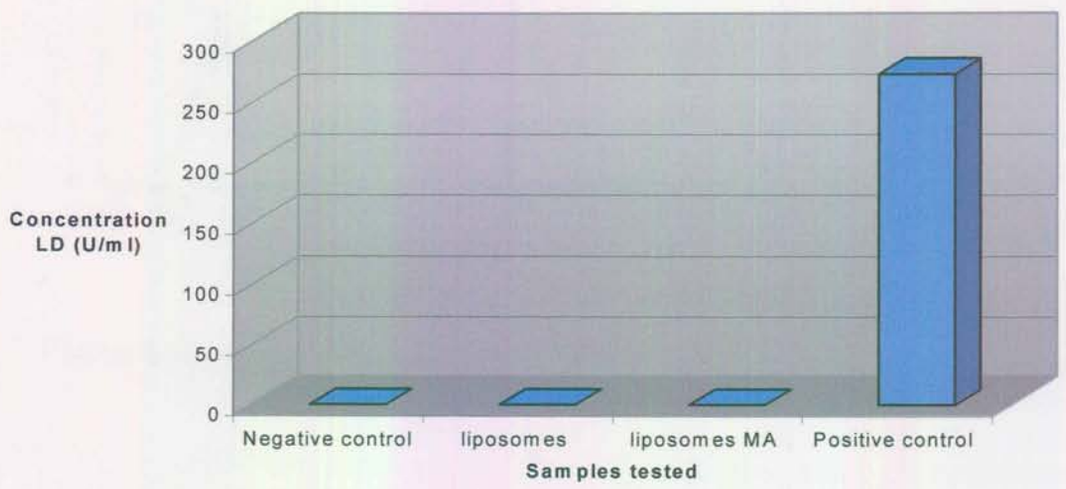


Figure 3.9 Haemolysis of erythrocytes using lactate dehydrogenase release as indicator. The negative control is 1% erythrocytes without liposomes. The positive control is 1% erythrocytes lysed by sonication. Samples were incubated at 37°C and 4°C, with no difference in haemolysis rate.

3.5.6.4 Sizing of liposomes

Using the flow cytometer, the size of the liposomes were determined in comparison to a 1 μm polystyrene bead (Figure 3.10).

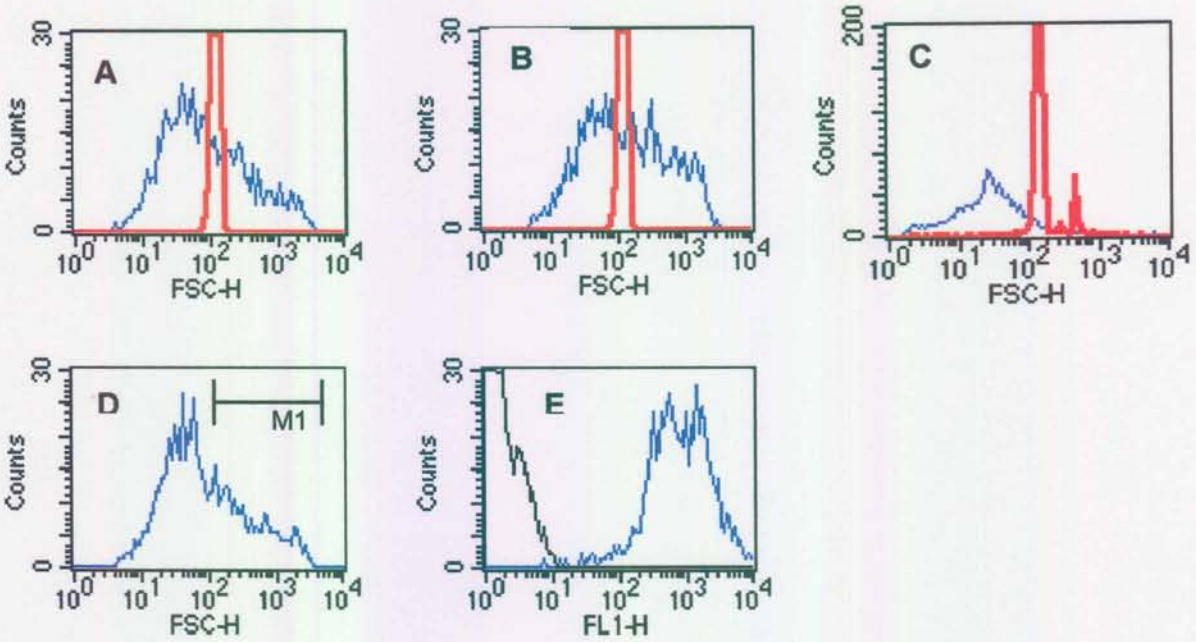


Figure 3.10. Sizing of liposomes with and without MA in the flow cytometer. Red represents beads (1μm in size) and blue the liposomes with and without MA (A) liposomes alone (B) liposomes-MA (C) liposomes-MA sent through a 0.8μm polycarbonate sieve (D) liposomes-MA in the sediment after centrifugation at 13 000g for 5 minutes (E) difference in fluorescence of liposomes after labelling with 5-bromo-methyl fluorescein.

Liposomes varied in size from around 3μm to very small. The average size is around 0,8 μm. In liposomes alone, 37% of the particles are bigger than 1μm and in MA-containing liposomes 47% are bigger than 1μm. Liposomes were then sent through a polycarbonate sieve to obtain a more homogenous liposome suspension. This excluded the larger population, leaving liposomes of mainly 0,8μm size. If liposomes were centrifuged at high speed, less of the bigger

liposomes were found in the precipitate. The size profile after nebulizing the liposomes-MA showed that the liposomes withstand the process well.

3.6 Discussion

Pharmaceutical formulations should display high availability, ease of administration and chemical and biological stability. The carrier must be immunologically inactive, or should augment the effects of immunity towards the antigen.

Mycolic acids form a solid wax at room temperature and need to be forced into a dispersion of acceptable size using heat and/or organic solvents. The extremely hydrophobic, large sized properties of mycolic acids demand their introduction in an aqueous environment on an inert carrier, while still enabling release and effector activity. Such preparations may consist of emulsions, micelles or liposomes. These are rarely used for parenteral adinistration, because lipid particles must usually be smaller than 1 μm to prevent emboli in blood vessels (Duma and Akers, 1984). The size range of the particles are of importance, as this may determine the method of uptake (pinocytosis or phagocytosis), which can influence the intracellular trafficking leading to presentation of the antigen. In liposomes, the size of the vesicles influences the Th1 or Th2 outcome of the immune response to the protein antigen content (Brewer *et al.*, 1998). The different carriers for MA were first evaluated for their ability to solubilize MA and then for the effect they had on the tissues and cells. Functionality of the solubilized MA was assessed separately.

Inclusion of a solute into a cyclodextrin (CD) cavity, substitutes its water content. This may provide the driving force to complexation of CD and the solute. Fatty acid solubility can increase up to 30 fold by complexing with cyclodextrins, depending on the aliphatic chain length. On average, five CH_2 groups are co-ordinated to one cyclodextrin molecule. If αCD is used, it seems that long chain fatty acids have to be stretched to be accommodated into the available cavity. Thus, depending on the

type of CD, formation of complexes brings about differences in the stretching of the aliphatic chain of various fatty acids. As the mycolic acids are not soluble in a water solution, the effect of using chloroform as solubilizer may influence the availability of the CD cavity. Chloroform enters the cyclodextrin cavity and must be released before the mycolic acid can enter. The dissolution of mycolic acids in chloroform and added to a preheated cyclodextrin solution, did not negatively affect the incorporation of mycolic acids. Although the α-, β- and γ-cyclodextrins appeared to interact with mycolic acids in water, the complexes precipitated out of solution. This implies that the complexes are not water soluble and that a big part of the mycolic acid molecule is still in contact with the water. Extraction of mycolic acids from these complexes appeared to be problematic, especially with the smaller α- and β-CD types. If stretching of the mycolic acids occured, it might have influenced the release, biological activity and solubility of the complexes. Cyclodextrins were therefore not considered to be likely candidates as carriers for mycolic acids in pharmaceutical preparations destined for oral administration or intramuscular injection.

Various sparingly water-soluble substances have been accommodated in solution by using polyethylene glycol. The power to dissolve the active substance is ascribed to a complex formation with PEG. PEG has been used in formulas to make submicron lipid emulsions (Lindberg, 1999), liposomes (Ng *et al.*, 2000), and other forms of injectables (Lee *et al.,* 1999). PEG is a suitable gellant for simple aliphatic hydrocarbon liquids but lack compatibility with many oils. PEG is often derivatized to contain groups that enable solubilization of the different oils and fats that are usually not soluble in standard PEG (Miller *et al.*, 1998). PEG polymers as the low molecular weight liquids or as aqueous solutions of the higher molecular weight solids, were unable to solubilize any significant amount of mycolic acids. The results showed clearly that mycolic acids cannot be solubilized by PEG as such.

In contrast to PEG, sesame oil was able to solubilize mycolic acids, although the recovery by HPLC showed a high degree of variability. Sesame oil has been used as carrier in injectables using gold as the product. No anti-inflammatory effects were seen in supplementing the diet of volunteers with high levels of sesame oil (Ten Wolde *et al.*, 1997). Its complexity as a mixture of natural oils, however, detracts from its solubilization properties, especially with mycolic acids. As the effect of mycolic acid as an immunomodulating agent is to be determined, complex mixtures of this kind are not desirable.

Solutol HS 15 was developed as carrier system for non-watersoluble products. It contains PEG linked to a mixture of mainly 12- hydroxystearic acid as mono- and di-esters, and thirty percent pure poly-ethylene glycol (660 MW). Non-water soluble substances such as vitamin A, D, E and K are solubilized in these systems making use of its micellar nature. When using Solutol as a drug carrier, the reversal of the multi-drug resistance (MDR) of tumour cells to anti-cancer drugs was found to be a side-effect (Coon *et al.*, 1991). Solutol easily accommodated mycolic acids in an aqueous environment in the form of micelles.

The Solutol micelles appeared to be toxic to mouse macrophage cell cultures at very low concentrations. This toxic effect was hardly noticeable after *in vivo* injection and could be decreased *in vitro* by working in 20% foetal calf serum (FCS). Because the immune cells *in vivo* were not affected by the Solutol, this can still be a good option for a carrier of mycolic acids.

In contrast to Solutol, no toxicity could be detected in using polystyrene microspheres in macrophage cultures or by *in vivo* loading of peritoneal macrophages. High uptake of beads by both cultured and peritoneal derived macrophages was observed. Using the beads, it was found that their consumption by macrophages was erratic. Some macrophages were crammed with beads and others had none. Moreover, it was also observed that a high amount of mycolic acids melted onto the polystyrene beads, made them float to

the surface if suspended in aqueous medium. The highest MA-containing beads, were thus not taken up if added to *in vitro* macrophage cultures.

The process of phagocytosis of beads tends to release IL12 depending on the size of the particle (Fulton *et al.*, 1996). That might influence the ensuing immune response. MA on biodegradable beads might therefore provide a useful method of MA-induced immunomodulation. The inconsistent phagocytosis of beads and the fact that beads are not biodegradable, necessitated investigation into alternative methods of loading MA into macrophages.

Solubilization of MA by using liposomes was investigated. The liposome is a biodegradable system that can carry hydrophilic drugs in its enclosed aqueous environment and the hydrophobic drugs in the lipid bilayers. Liposomes were first characterised by Bangham *et al.* (1965). Although liposomes were initially designed as models for biological membranes, their potential for drug delivery was soon realised.

There are several reasons why liposomes are preferred as carriers for drugs: Liposomes obtained from natural phospholipids are biocompatible, immunologically inert and biodegradable. A large variety of molecules can be incorporated into the liquid or the lipid phase. Drugs are shielded from the external environment, thereby limiting the side effects. Drugs can be delivered by fusion and endo- or phagocytosis. Antibodies can confer active targeting of liposomes.

Liposomes injected intramuscularly or subcutaneously are released from the injection site and are taken up by the reticulo-endothelial system (RES) at a rate determined mostly by the size of the liposomes. The bigger the size, the slower the release from the injection site. Retention of liposomes in the draining lymph nodes, is both size- and charge dependent. Very small liposomes rapidly appear in the circulation after subcutaneous injection (Dams *et al.*, 2000, Oussoren *et al.*, 1999). Clearance of liposomes after intravenous injection also depends on size and

surface charge of the liposomes (Gabizon and Papahadjopoulos, 1992). Rigid, small size (100-200 nm) liposomes tend to be retained in the blood without degradation. These RES-avoiding, long-circulating liposomes passively accumulate in tumours due to the permeable vasculature in these tissues. Therefore these long-circulating liposomes are useful tools in tumour imaging and therapy. By adding PEG or glucuronide derivatives into the liposomes, the circulation time could be manipulated. (Dams *et al.*, 2000). PEG containing liposomes are sterically stabilised and are thus considered promising tools for delivery of therapeutic agents. Repeated injection of PEG liposomes dramatically enhanced the clearance of subsequently injected PEG liposomes and should be taken into consideration when repeated administration of PEG liposome-carried therapeutic drugs is required (Dams *et al.*, 2000).

The formulation of liposomes requires careful consideration of procedures and materials to ensure liposome stability. For example, phosphatidylcholine-cholesterol liposomes carrying the drug carboplatin, showed no degradation or size change during six months of refrigerated storage, but hydrolysis of the phosphatidylcholine was increased when an antioxidant, ascorbyl palmitate, was added (Pietzyk and Henschke, 2000). Free radical peroxidative damage against phospholipid membranes *in vivo* can be reduced by adding reducing agents such as alpha carotene to the liposomes (Farombi and Britton, 1999). Surfactants should preferably be avoided, but up to 1% can be tolerated.

Immunogenicity of a protein antigen, encapsulated inside liposomes, could be manipulated by alternating the neutral lipids/cholesterol/negatively charged phospholipids ratio. Other factors included the PC acyl chain length. Increasing fatty acid unsaturation decreased the immunogenicity of the liposome encapsulated antigen (Bakouche and Gerlier, 1986). In contrast, Yasuda *et al.* (1979) showed that immunogenicity in mice of a liposomal membrane-embedded hapten, was not influenced by cholesterol concentration or degree of saturation of the fatty acids. Membrane bound antigens on liposomes with phospholipids that have a high

transition temperature, such as synthetic distearoyl PC, were more immunogenic than low transition temperature lipids (*i.e.* PC) (Dancey *et al.*, 1978).

The cell wall of *Mycobacterium* spp is composed of an asymmetric lipid bilayer according to model proposed by Minnikin and Goodfellow (1980). The inner leaflet contains MA linked to arabinogalactan, and the outer leaflet contains extractable lipids such as trehalose-containing glycolipids, phenolic glycolipids or glycopeptidolipids. X-ray diffraction studies showed that MA hydrocarbon chains arrange in a tightly packed parallel, quasi-crystalline array, mainly organised in a direction perpendicular to the cell surface. Most of the extractable lipids underwent major thermal transitions between 30°C and 60°C. In contrast to this, MA exhibited a transition phase at a temperature of between 60°C and 70°C. The mycobacterial cell wall therefore appears to consist of a bilayer with an outer leaflet of moderate fluidity and an inner mycolic acid layer of extremely low fluidity (Liu *et al.*,1996). Here the MA-containing liposomes have a mixture of low and high transitional temperature lipids, as well as cholesterol. Liposomes-MA thus constitute a novel form of liposome, probably of low fluidity and low permeability. Immunogenicity is at this stage not predictable as the antigen is also part of the lipid membrane and the immunity mechanism towards MA is unknown.

Liposomes as drug vehicles can be used to deliver drugs into various compartments of the body. Liposomal carriers have been successful in enhancing the clinical efficacy of a number of drugs from inhalation into the lungs, to topical application on the skin (Farhood *et al.*, 1995). Pulmonary delivery of glucocorticosteroids and cyclosporine (in liposomes) has been done by liquid aerosol generated nebulizers. The size of the droplets was in the range of 1µm, thereby allowing deep penetration into the lung.

Tuberculosis is mainly a pulmonary disease and the transmission of the bacilli is almost exclusively in and from the lungs. Drug delivery directly into the biophase (the lungs), would perhaps improve efficacy of MA as it is not necessary for the

liposomes to circulate the blood system to reach the target area. Dilution or elimination of the drug will then also not be such a problem.

From the results seen with incorporation of mycolic acids into liposomes, the preparation of a liposome carrier system seems feasible. The low toxicity seen in this system towards cells was encouraging. The liposomes-MA could also be nebulized in an aerosol.

CHAPTER 4

Mycolic acids and macrophages

4.1 Introduction

4.1.1 Uptake of antigens

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

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

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

4.1.2 Receptors involved in phagocytosis

4.1.2.1 Fc receptors

The functions of Fc receptors fall into two categories:

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

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

4.1.2.2 Complement receptors

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

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

4.1.2.3 Mannose receptors

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

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

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

4.1.2.4 Scavenger receptors

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

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

4.1.2.5 Collectins

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

4.1.3 Receptors on macrophages that bind *Mycobacterium tuberculosis*

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

4.1.4 Macrophages and TB

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

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

- The presentation of antigen

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

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

4.1.5 Maturation of phagosomal vacuoles

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

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

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

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

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

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

4.1.6 Macrophages and foam cell formation

4.1.6.1 Definition:

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

4.1.6.2 Introduction to foam cells

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

4.1.6.3 Macrophage scavenger receptors involved in atherogenesis/foam cells.

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

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

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

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

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

The three classes of scavenger receptors namely

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

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

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

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

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

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

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

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

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

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

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

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

4.1.6.5 Metabolism of cholesterol

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

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

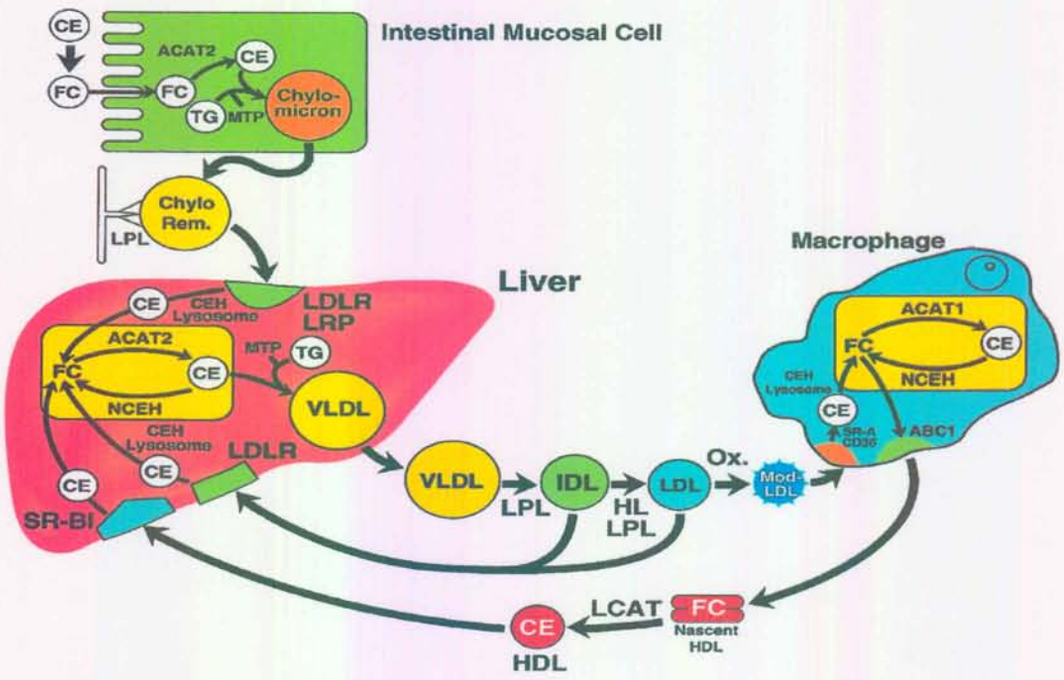


Figure 4.1. Schematic overview of cholesterol metabolism. Details are given below in text. FC–free cholesterol, CE-cholesterol ester, TGs – triglycerides, MTP-microsomal transfer protein, LDL-low density lipoprotein, VLDL-very low-density lipoprotein, CEH-cholesteryl ester hydrolase, NCEH-neutral cholesterol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

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

4.1.6.6 Pathogenesis of foam cell formation

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

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

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

4.1.6.7 Lipoprotein modification

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

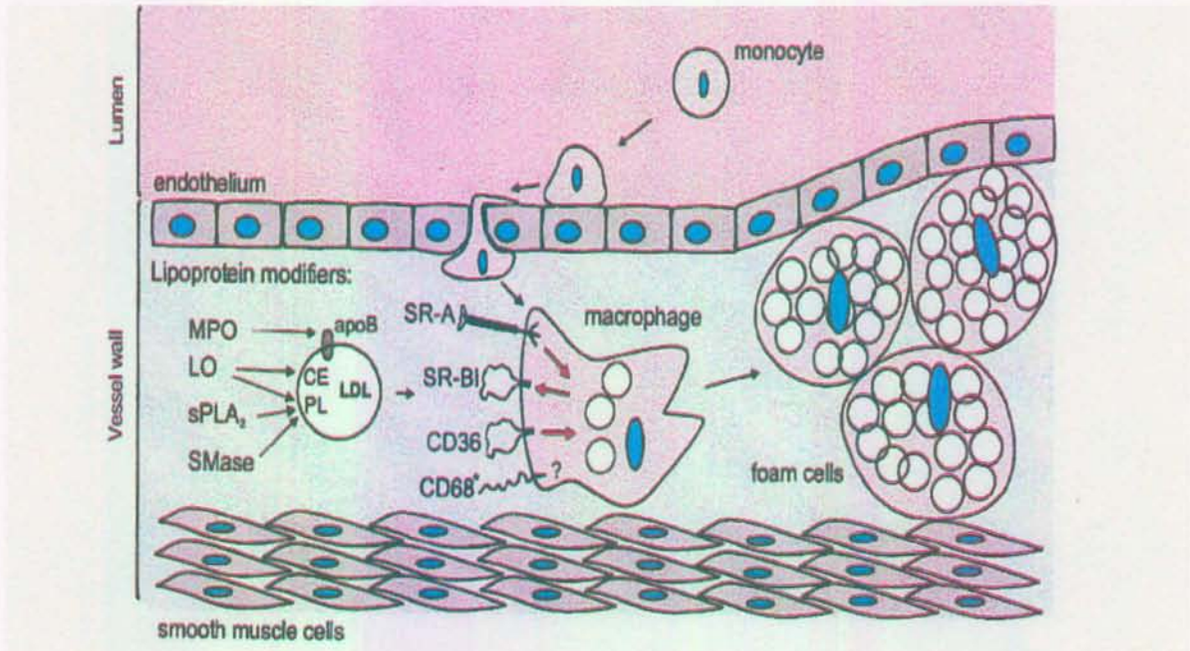


Figure 4.2 Lipid-modifying enzymes and scavenger receptors from macrophages which play a role in foam cell formation. Arrows from the LDL-modifying enzymes indicate on which component it is working. CD68 is included as it binds oxLDL but its role is still not clear. SMase-sphingomyelinase, sPLA2-phospholipase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

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

4.1.6.7 Oxidation of lipoproteins by monocytes-macrophages

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

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

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

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

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

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

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

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

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

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

4.1.6.8 CD1, foam cells and atherosclerosis

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

4.1.6.9 Foam cells and IFNγ

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

4.1.6.10 Surface phenotype of foam cells

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

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

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

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

4.1.7 Hypothesis

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

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

4.2 Materials

4.2.1 Macrophage cultures

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

4.2.2 Animals

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

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

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

4.2.4 Antibodies

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

4.2.5. Lysis buffer

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

4.2.6 DNA stain for flow cytometric determination of DNA synthesis

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

4.2.7 Carrier systems

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

4.2.8 LPS testings

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

4.2.9 LysoTracker

LysoTracker was purchased from Molecular Probes (The Netherlands).

4.2.10 Microscopy

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

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

4.2.11 Lyophilised *Mycobacterium tuberculosis*

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

4.2.12 Reagents for cholesterol determination

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

4.3 Methods

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

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

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

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

4.3.2 Collection of peritoneal exudate macrophage cells

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

4.3.3 Collection of alveolar macrophages

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

4.3.4 Collection of cells from the spleen

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

4.3.5 Collection of blood samples and preparation of mouse serum

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

4.3.6 Preparation of mycolic acid-mouse serum conjugates

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

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

4.3.7 MA uptake and LysoTracker co-localization studies in macrophages

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

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

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

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

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

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

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

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

4.3.8 Phagocytosis of liposomes by PECs

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

4.3.9 Morphological changes of macrophages loaded with MA

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

- PBS
- Liposomes
- Liposome-mycolic acids

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

4.3.10 Culturing and preparation for light microscopy

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

4.3.11 Preparation for electron microscopy

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

4.3.12 MA dose dependency to activate macrophages

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

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

4.3.13 Time dependency of macrophage activation by MA

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

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

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

4.3.15 Neutral lipid staining of macrophages loaded with MA

Peritoneal mouse macrophages were loaded with either

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

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

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

4.3.16 Cholesterol determination in MA-treated PECs

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

- Liposomes
- Liposomes-MA

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

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

4.3.17 Macrophage proliferation after MA loading

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

4.3.17.1 Macrophage proliferation measured by thymidine incorporation

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

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

4.3.17.2 Macrophage proliferation measured by propidium iodide staining and FACS

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

4.3.18 Cell surface labelling of foam-like cells

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

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

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

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

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

4.3.19 CD1d expression on alveolar macrophages after MA immunization

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

4.4 Results

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

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

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

- PBS
- Phospatidyl choline
- Cholesterol
- Mycolic acids

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

4.4.2 Coupling of 5-BMF to mycolic acids

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

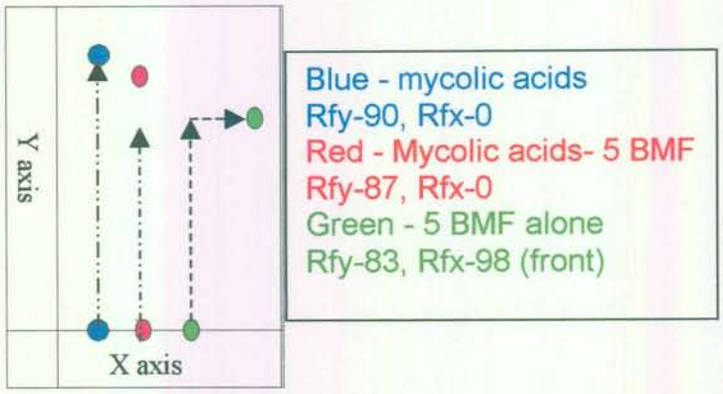


Figure 4.3. Reconstruction of the two-dimensional thin layer chromatographic system to assess the labelling of MA with 5-BMF. The samples tested were mycolic acids, mycolic acids coupled to 5-BMF, and 5-BMF alone. The Rf values for the Y and the X-axis are given. The mobile phase for the Y-axis was chloroform: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

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

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

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

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

4.4.3.1 MA uptake and LysoTracker co-localization studies in macrophages

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

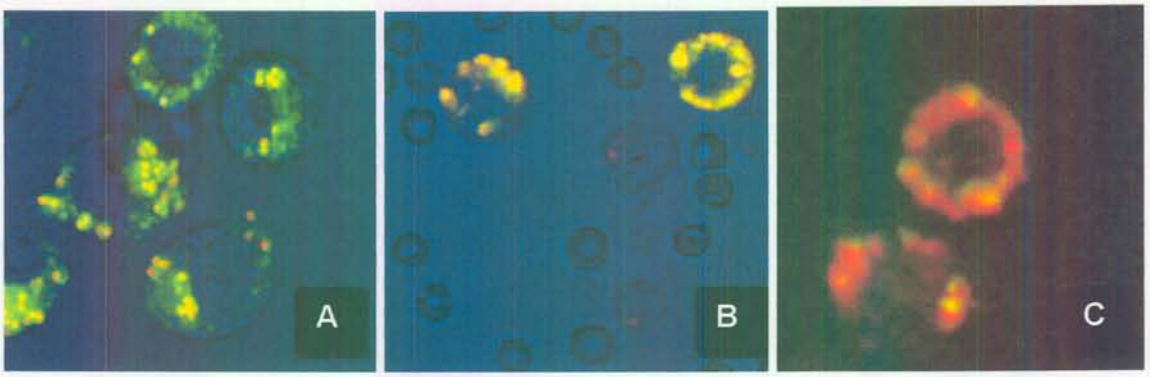


Figure 4.4 Maturation of phagosomes by co-localization with LysoTracker. In (A) LysoTracker green was co-incubated with unlabelled MA coated on 1μm red fluorescent beads. (B) LysoTracker red co-incubated with fluorescent green labelled MA solubilized in serum. (C) LysoTracker red co-incubated with fluorescent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

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

4.4.3.2 Phagocytosis of liposomes by macrophages

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

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

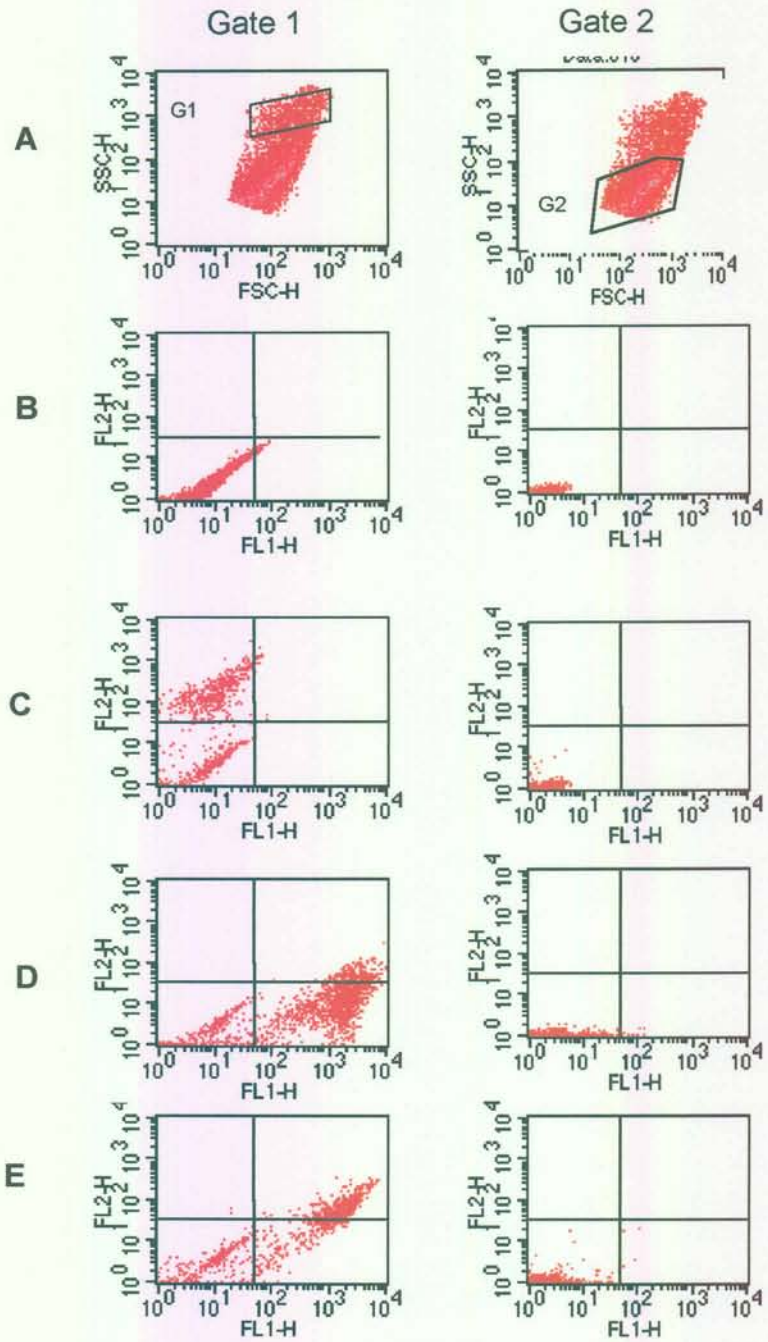


Figure 4.5 FACS data showing uptake of 5-BMF in peritoneal macrophages. (A) Gate selection (B) Unlabelled cells in gate 1and gate 2, (C) Cells in gate 1 and 2 labelled with F4/80 (FL2), (D) Cells in gate 1 and 2 with phagocytosed 5-BMF labelled MA (FL1), (E) Cells in gate 1 and 2 labelled with F4/80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

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

4.4.3.3 Morphological changes of macrophages following MA uptake

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

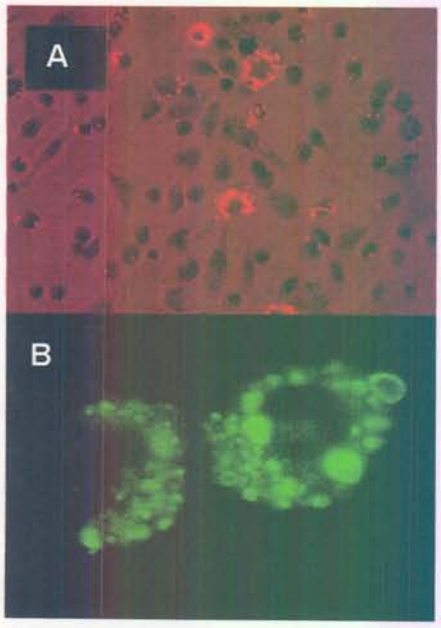


Figure 4.6. Demonstration of (A) bead-MA overloading and (B) liposome-MA.

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

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

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

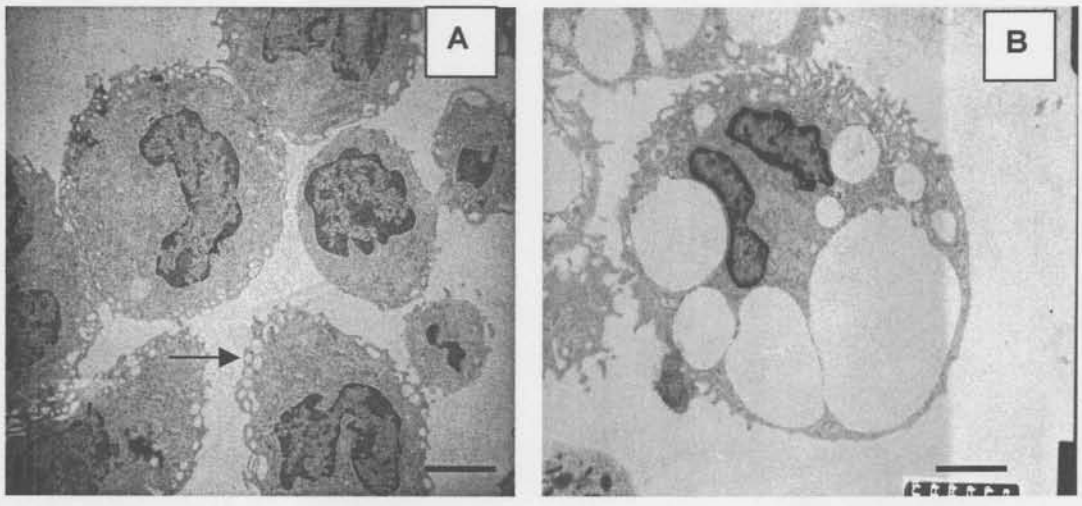


Figure 4.7. Electron microscope images of (A) liposome - and (B) liposome-MA treated mouse peritoneal macrophages. The liposome treated cells contained small vacuoles near the surface of the macrophages (see arrow). In comparison the MA treated cells have large vacuoles.

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

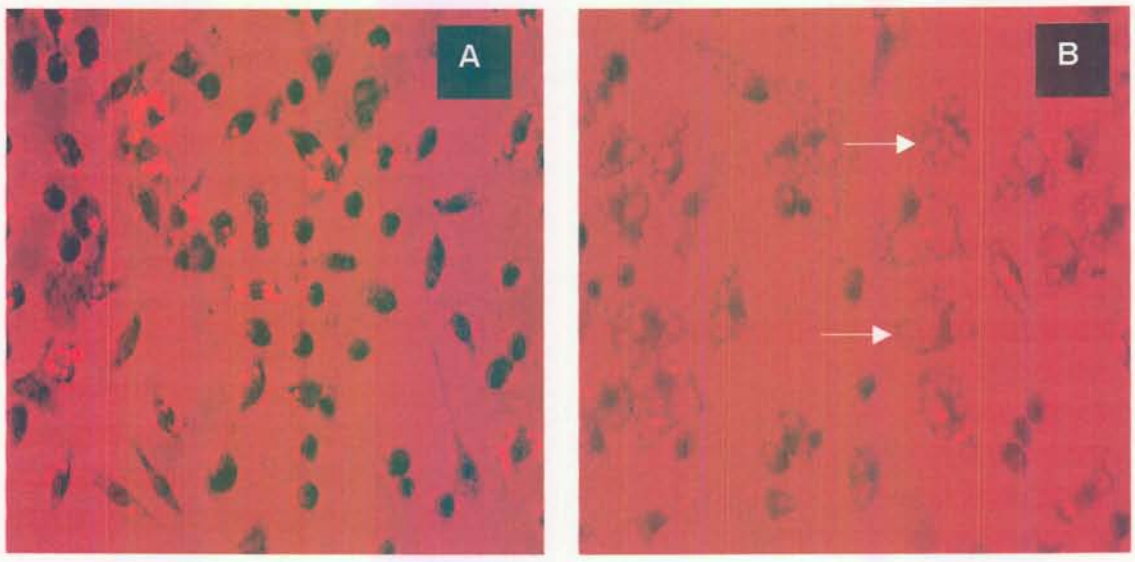


Figure 4.8. Uptake of (A) beads and (B) beads-MA in peritoneal macrophages evaluated by fluorescence microscopy. In (B) the formation of vacuolated cells are seen even when they don't contain any beads (see white arrows).

4.4.3.4 Dose dependency of liposomes-MA to activate macrophages

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

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

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

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

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

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

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

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

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

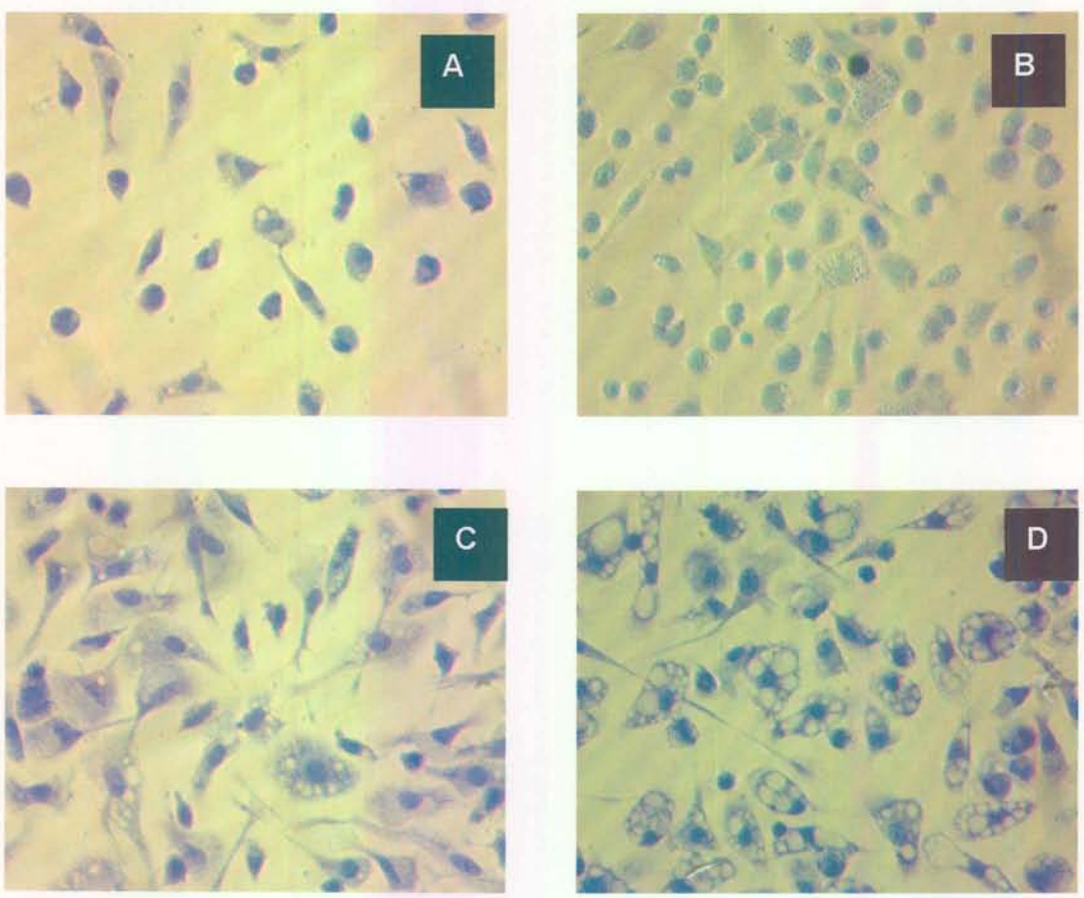


Figure 4.9. Effect of *in vivo* loading of beads, beads-MA and dead *Mycobacterium tuberculosis* on the morphology of mouse peritoneal macrophages. (A) Native macrophages (B) macrophages exposed to beads (C) Macrophages exposed to dead *Mycobacterium tuberculosis* (D) macrophages exposed to beads-MA.

4.4.3.6 Neutral lipid content of macrophages loaded with liposomes-MA

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

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

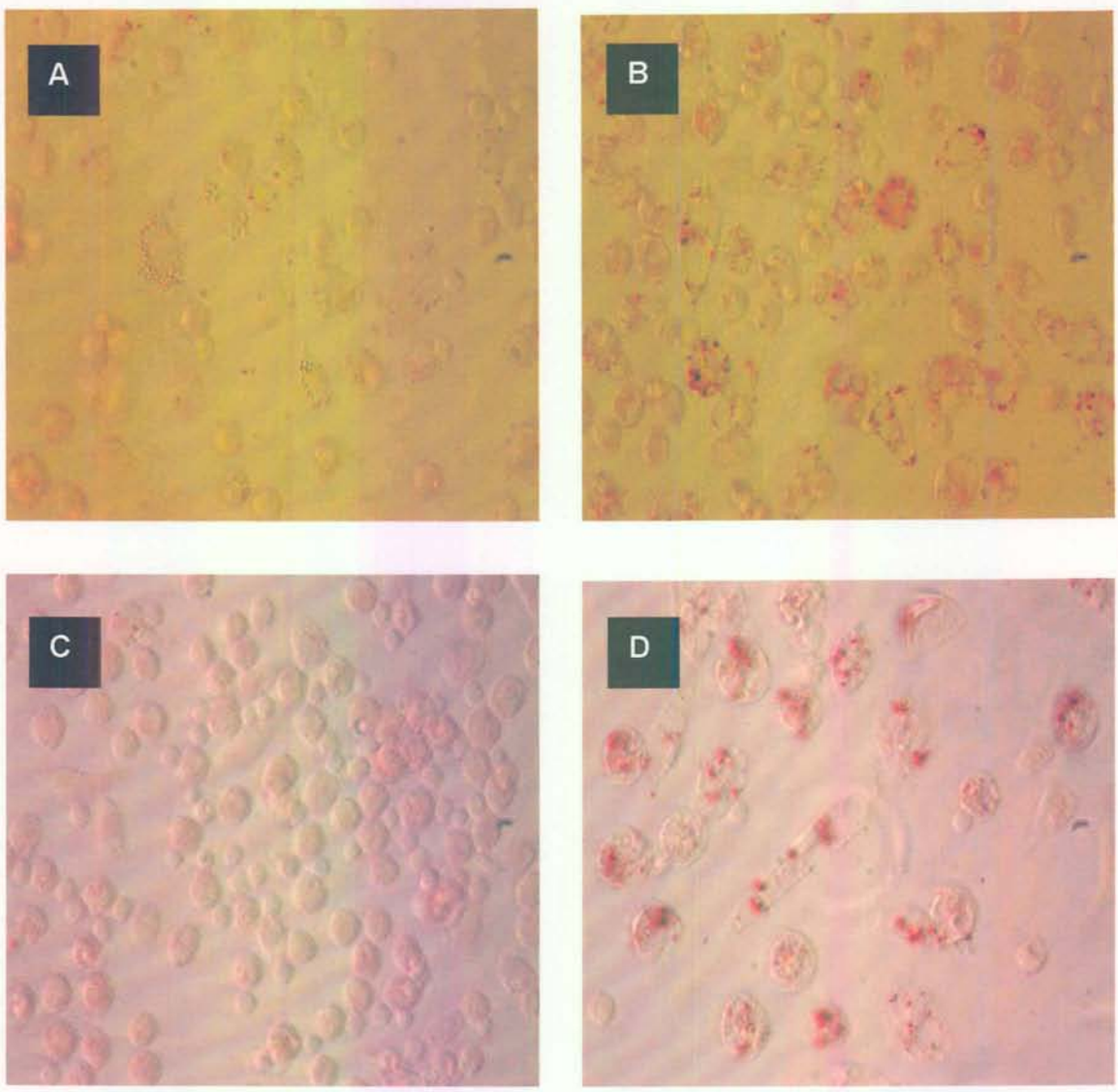


Figure 4.10. Effect of MA on accumulation of neutral lipids inside mouse peritoneal macrophages. (A) beads alone (B) beads with MA (C) liposomes alone (D) liposome containing MA. Note that the red stain is not present in the vacuoles

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

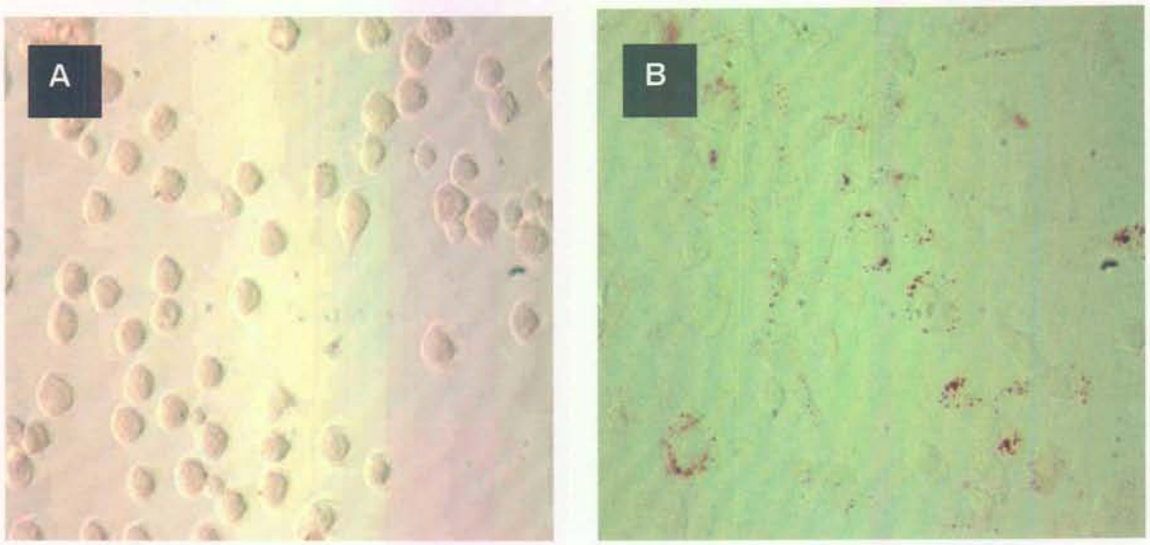


Figure 4.11. Oil Red O staining pattern of mouse peritoneal macrophages exposed to dead mycobacteria (A) Native macrophages and (B) macrophages exposed to dead *Mycobacterium tuberculosis.*

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

4.4.3.7 Cholesterol accumulation in MA-loaded macrophages

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

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

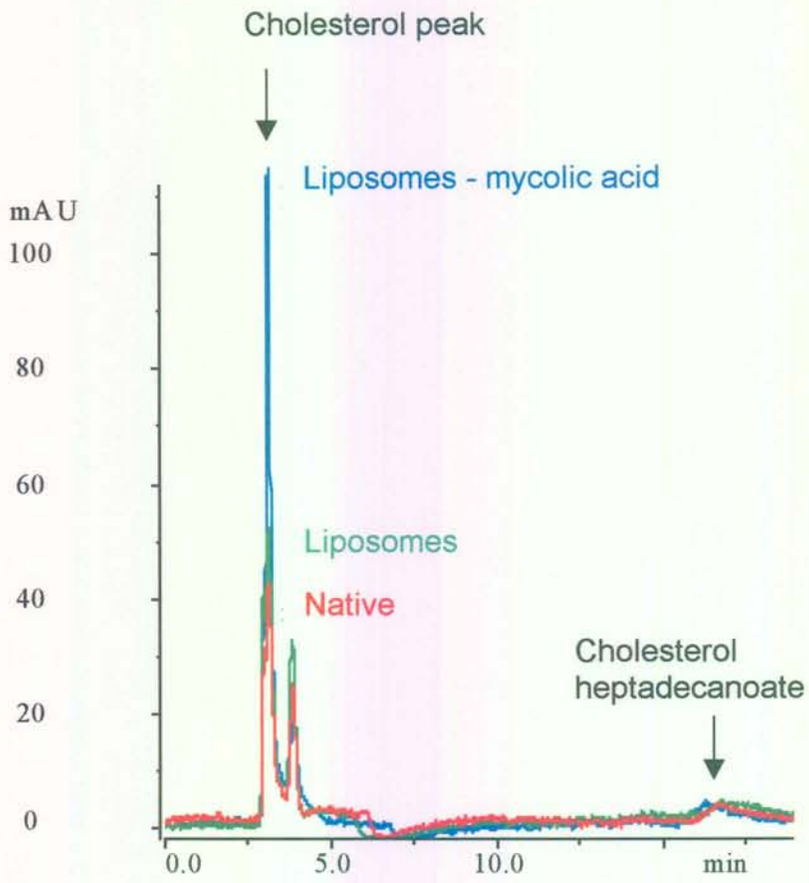


Figure 4.12. Cholesterol elution pattern obtained from neutral lipid extracts from native, liposome and liposome-MA treated macrophages. Cholesterol heptadecanoate was used as internal standard.

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

4.4.3.8 Macrophage proliferation after loading with liposomes-MA

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

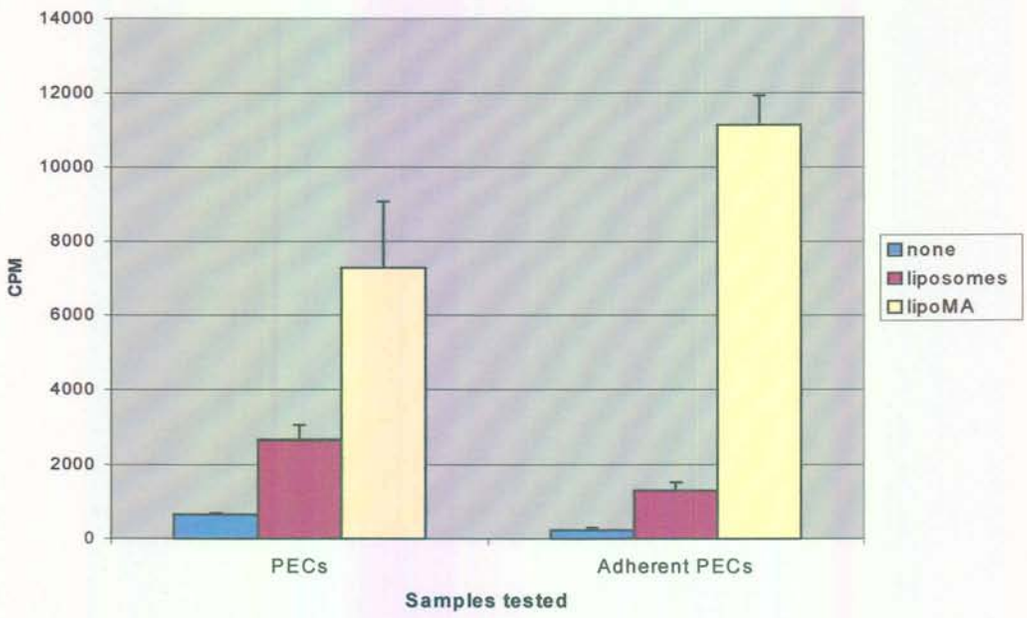


Figure 4.13. Cellular proliferation of PECs after 48 hours of *in vivo* exposure to liposomes or liposomes-MA

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

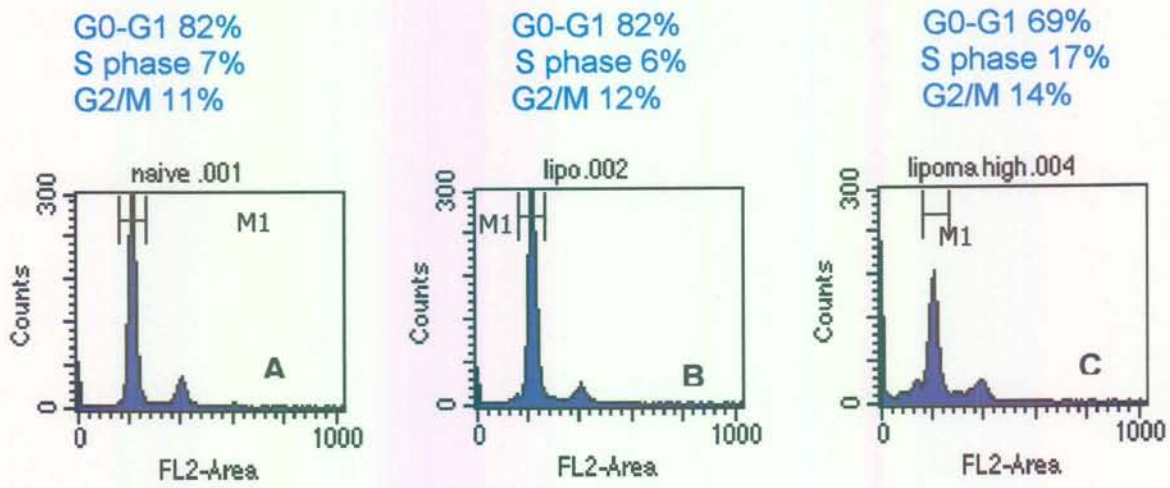


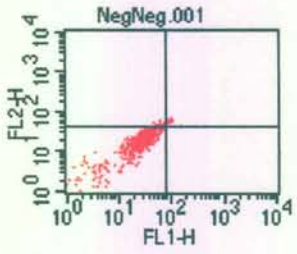
Figure 4.14. FACS analysis of cellular proliferation of PECs upon MA induction. The cells were harvested, stained with PI and analyzed for DNA profile. (A): Native PECs; (B): PECs loaded with liposomes; (C): PECs loaded with 25μg MA-containing liposomes.

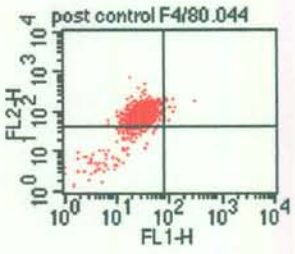
Compared to untreated or liposome treated macrophages that lasted only 9 days in culture, MA induced foam cells survived for up to 30 days in normal macrophage medium conditions.

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

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

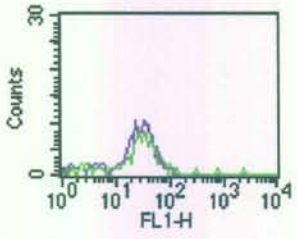
PEC population gated for use - no labelling

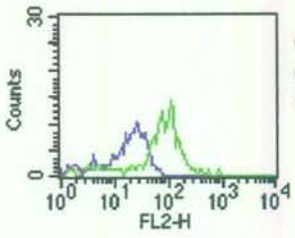




F4/80 - positive cells were used for I-A^d surface labelling

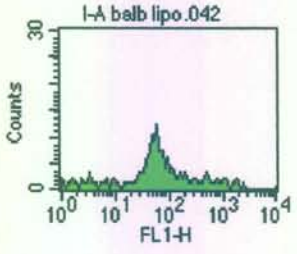
Negative control - overlay of unlabelled (purple) macrophages and isotype control (green)



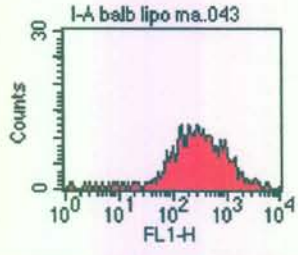


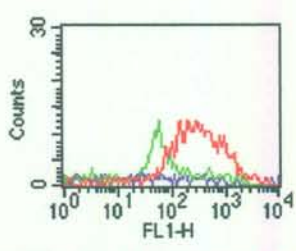
Overlay of unlabelled cells (purple) and F4/80 labelled macrophages (green)

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



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

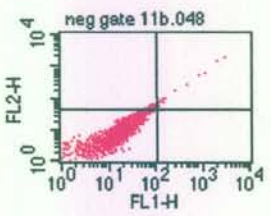


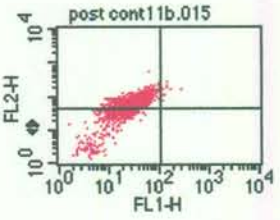


Overlay of I-A^d labelled macrophages (red) – liposome-MA treatment compared to liposome treatment alone (green)

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

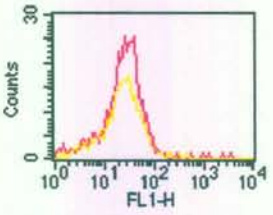
PEC population gated for use-no labelling

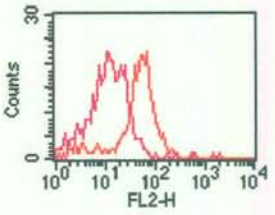




F4/80-positive cells were used for CD11b surface labelling

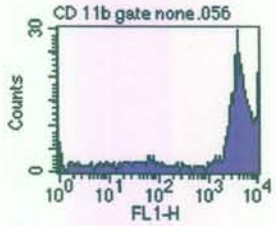
Negative control - overlay of unlabelled macrophages (pink) and isotype control (yellow)

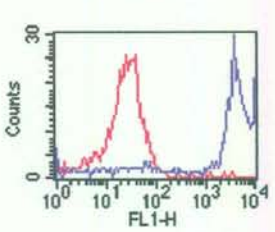




Overlay of unlabelled cells (pink) and F4/80 labelled macrophages (red)

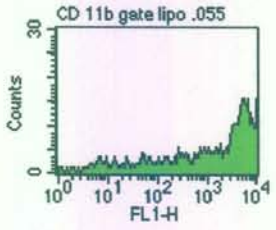
CD11b labelled macrophages– no treatment given

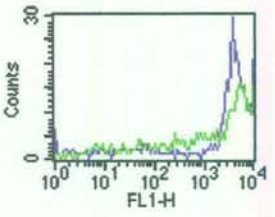




Overlay of isotype control (pink) and CD11b labelled macrophages (purple) – no treatment

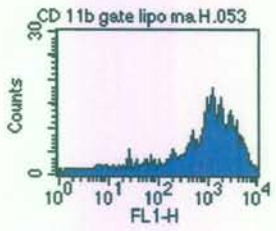
CD11b labelled macrophages – liposome treatment (green)

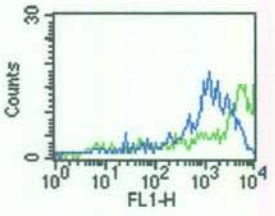




CD11b labelled macrophages. Overlay of liposome treatment (green) to no treatment (purple)

CD11b labelled macrophages– liposome-MA treatment (blue)

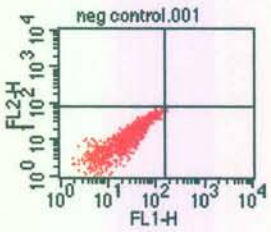


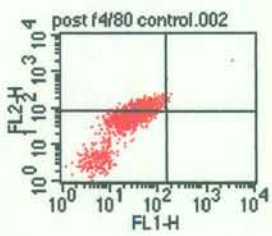


CD11b labelled macrophages – Overlay of liposome-MA treated macrophages (blue) and liposome treated macrophages (green).

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

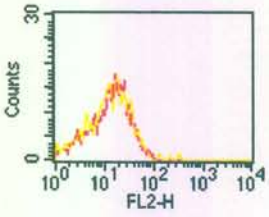
PEC population gated for use - no labelling

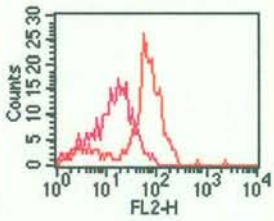




F4/80 - positive cells were used for CD1 surface labelling

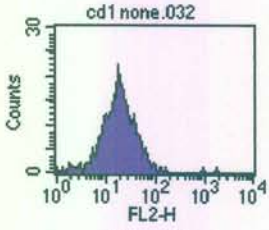
Negative control - overlay of unlabelled macrophages (pink) and isotype control (yellow)

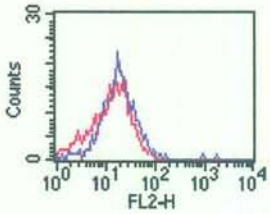




Overlay of unlabelled cells (pink) and F4/80 labelled macrophages (red)

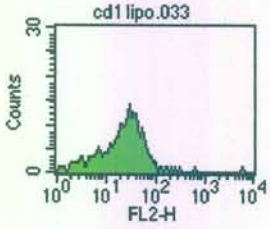
CD1d labelled macrophages (purple) – no treatment

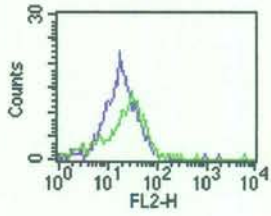




Overlay of isotype control (pink) and CD1d labelled macrophages (purple) – no treatment

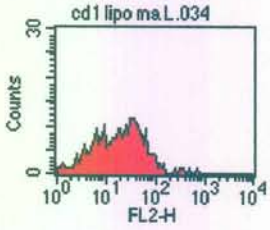
CD1d labelled macrophages (green) – liposome treatment

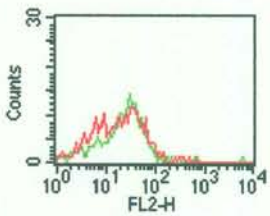




Overlay of CD1d labelled macrophages (purple) – liposome treatment (green) compared to no treatment

CD1d labelled liposome-MA treated macropages (red)





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

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

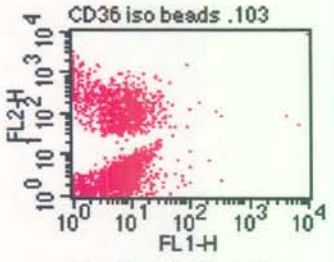
Table 4.7 Changes observed in the surface markers in mouse peritoneal exudate cells upon exposure to MA. (N) shows no change in expression, (+) shows increased expression and (-) shows decreased expression.

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

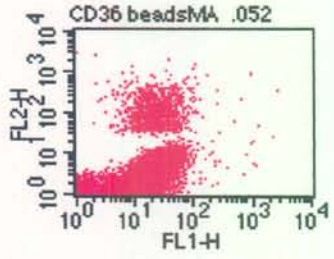
From the above, it is seen that MHCII expression increased, CD11b expression decreased, and the rest stayed the same.

4.4.4 CD36 surface labelling of macrophages loaded with beads-MA

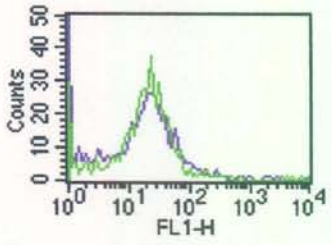
Changes in CD36 expression on bead-MA loaded macrophages was evaluated by flow cytometry. In Figure 4.18 it is shown that no increase in CD36 surface labelling was detected.



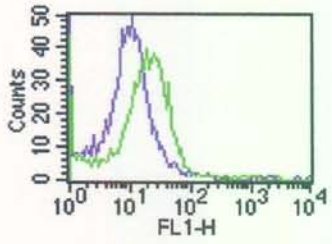
PECs loaded with fluorescent (FL2) beads and incubated with FITC labelled isotype control (FL1)



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



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



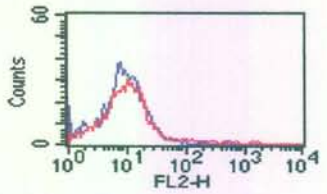
Macrophages loaded with beads isotype control (purple) overlaid with macrophages treated with beads-MA and labelled with FITC labelled anti-CD36 (green)

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

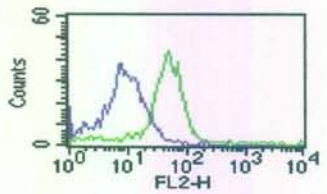
From Figure 4.18 it is evident that CD36 is induced in the macrophages after upake of MA-beads, in comparison to when beads only were taken up.

4.4.5 Effects of intravenous MA administration on alveolar macrophages.

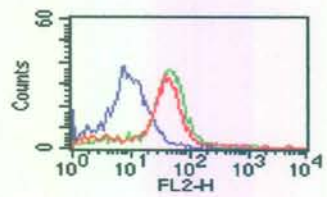
CD1d expression on PECs was found to remain unchanged after beads-MA uptake. In order to assess CD1d expression on alveolar macrophages after intravenous administration of MA on beads, serum or liposomes lung lavages were done on treated animals and cells prepared for FACS analysis, probing CD1. Figure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



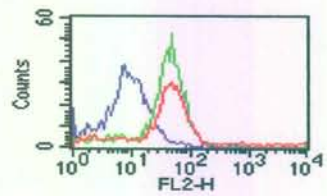
Unlabelled untreated alveolar macrophages (purple) overlaid with isotype control (red)



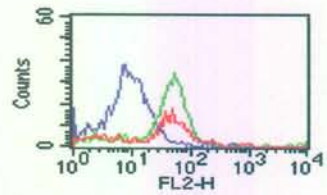
Unlabelled untreated alveolar macrophages (purple) overlaid with CD1d labelled untreated macrophages (green)



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



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



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

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

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

4.5 Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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.

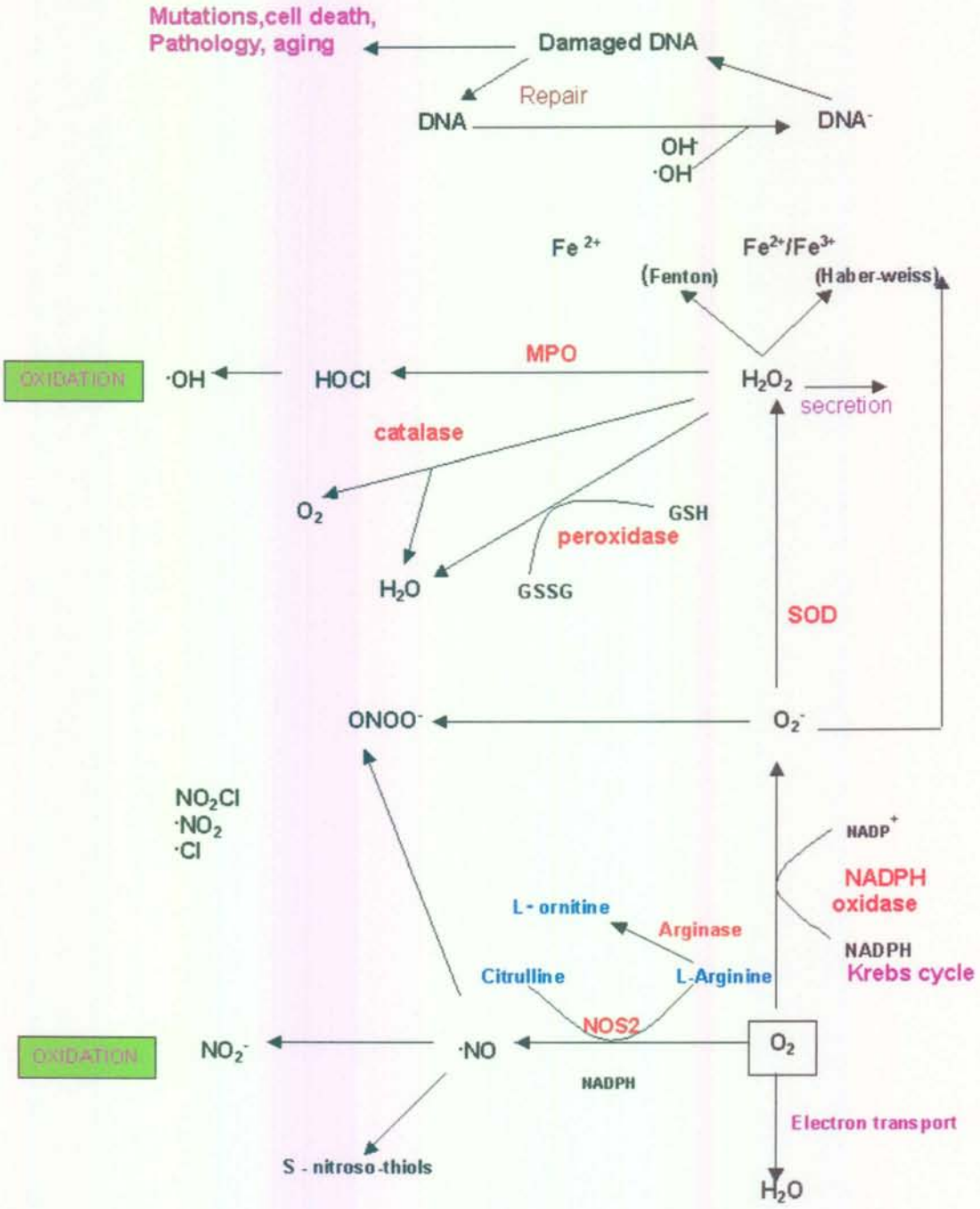


Figure 5.1. Reactive oxygen and nitrogen pathways.

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 thiocianate (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.

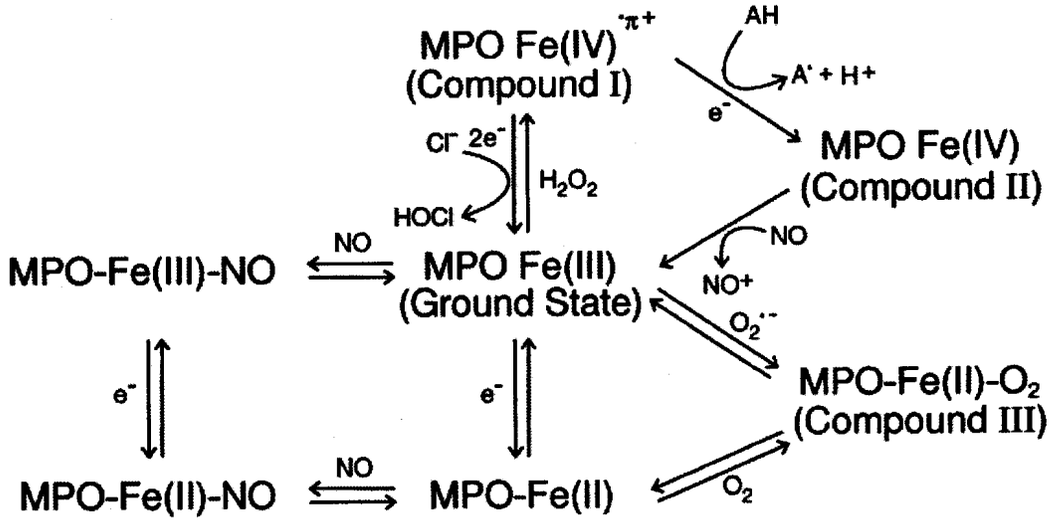


Figure 5.2. Enzyme kinetic model for myeloperoxidase (Taken from Podrez *et al.*, 2000)

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).

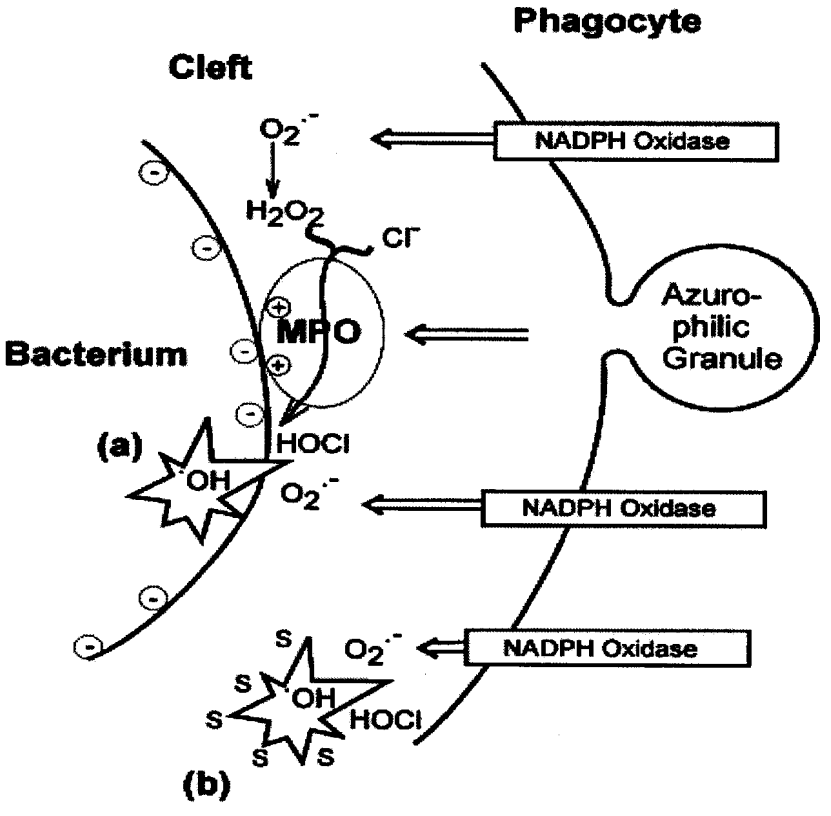


Figure 5.3. The geometry of MPO-mediated bacterial killing within the phagosome. (Taken from Saran *et al.*, 1999).

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 $^{\cdot}OH$ radicals in close vicinity to bacterial targets (a). In case (b), where the $^{\cdot}OH$ 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:

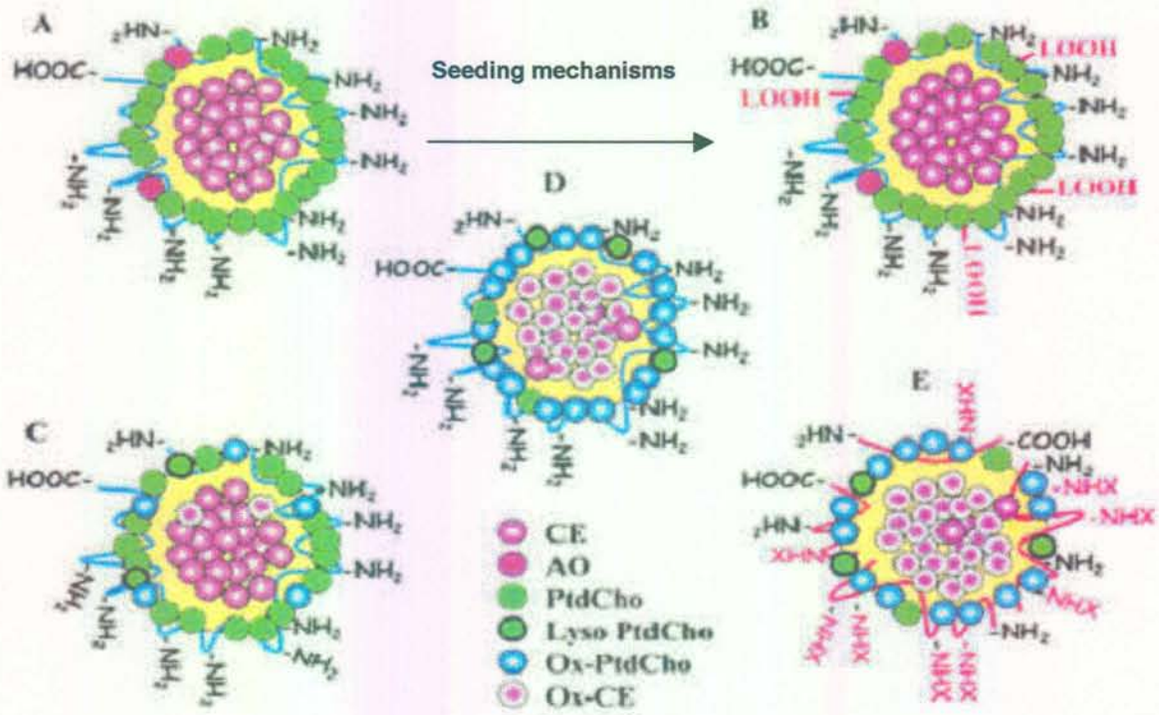


Figure 5.4. Different forms of oxidized LDL: (A) native LDL, (B) seeded LDL, (C) minimally oxidized LDL (D) extensively oxidized LDL (E) oxidatively modified LDL. See detail in text. CE – cholesterol esters, AO - Antioxidants, PtdCho - phosphatidyl choline, Lyso Ptdcho l-lysophosphatidyl choline Ox- 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, Na2HPO$_4$ (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_20; 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_20, 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.

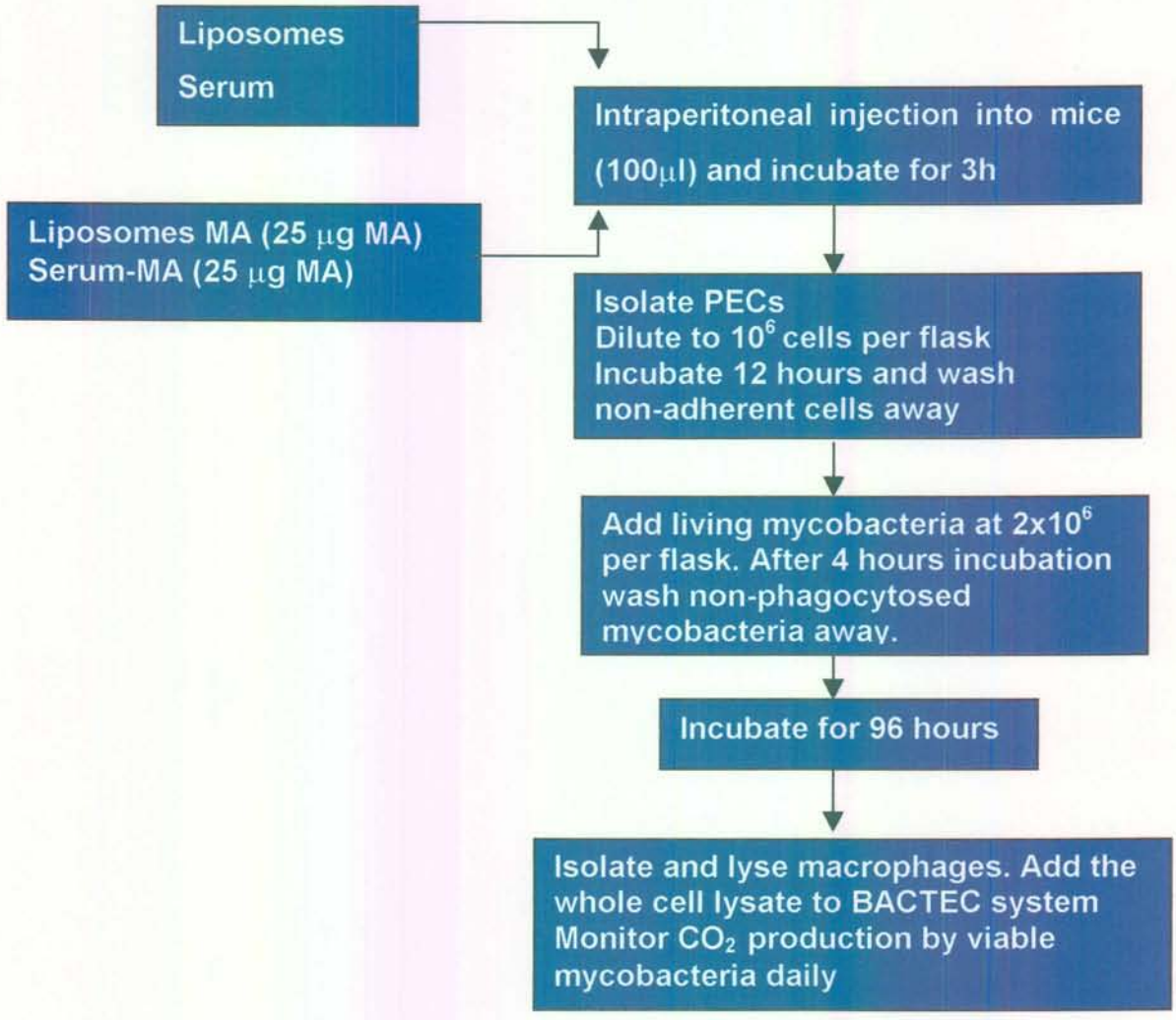
5.3.1 Fluorescein-iso-thiocyanate (FITC) labelling of mycobacteria

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 ($1x10^6$ 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*.



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 x 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 + catachol +H_2O_2 $\xrightarrow{\text{MPO}}$ **Brown black insoluble 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 ($1x10^6$ 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 x 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 $MnCl_2$ 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:

- EA = Enzymic activity (in mU)
- Cu = Concentration of urea in the 416 µl reaction mix (in mM)
- Vt = Total volume of the enzymic extract (i.e. 50 µl)
- Ve = Volume of the extract that was tested (i.e. 17 µl)
- 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.

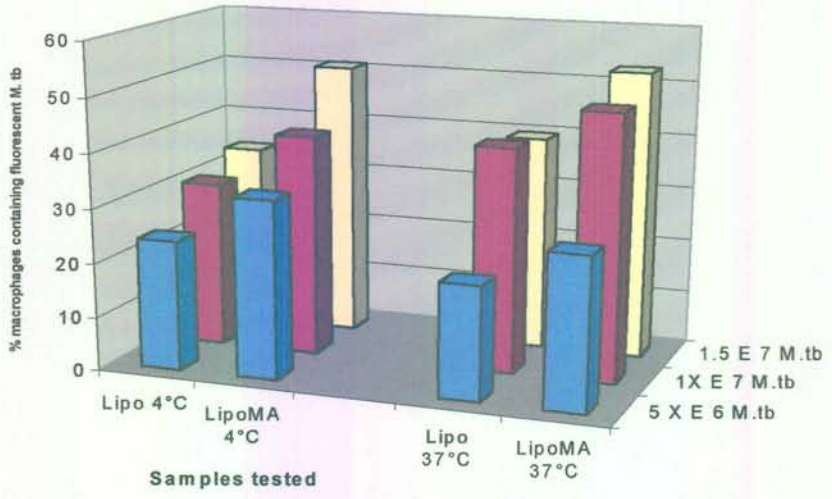


Figure 5.5. Binding (4°C) and uptake (37°C) of FITC labelled MtbH37Ra. No statistical evaluation was done on these samples.

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.

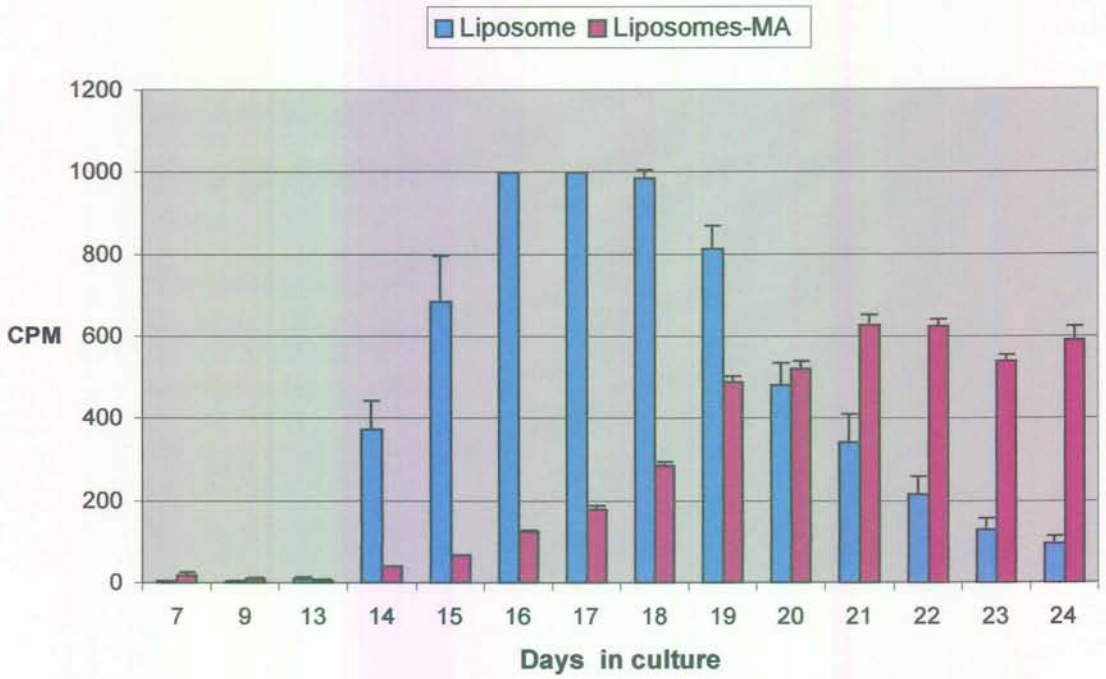


Figure 5.6. Differences in growth potential of *M. tuberculosis* surviving intracellularly in macrophages loaded with liposomes (blue bars) or liposomes-MA (red bars). Each bar represents the mean of 3 samples +/- s.d.

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.

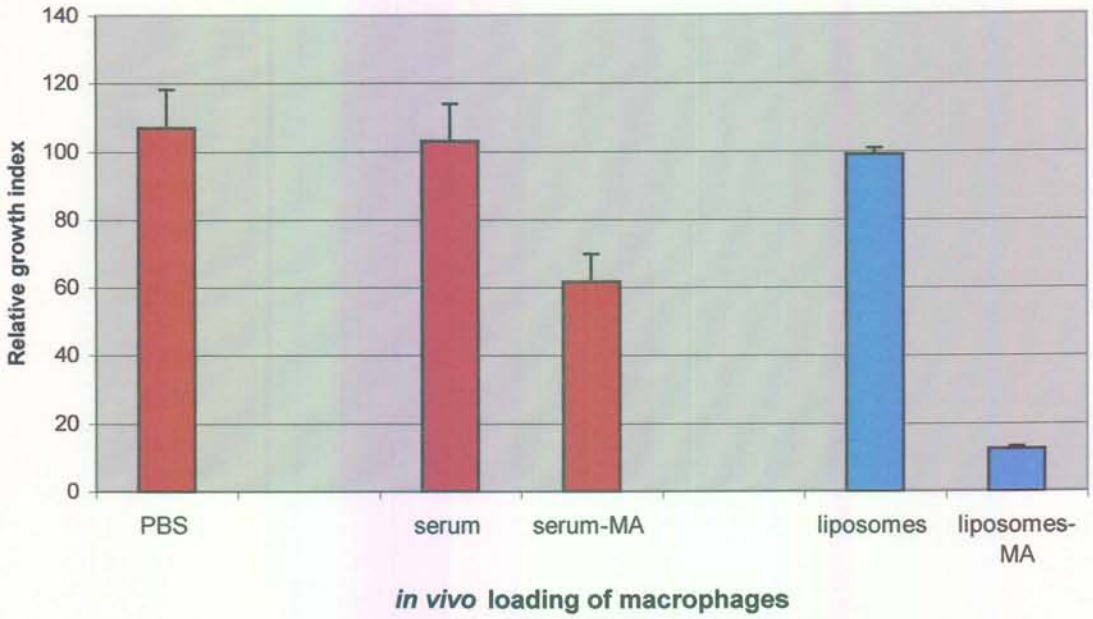


Figure 5.7. Inhibition of growth by *Mycobacterium tuberculosis* surviving intracellularly in macrophages loaded *in vivo* with PBS, serum/serum-MA (day 10) or liposomes/liposomes-MA (day 16) in 2 separate experiments (red vs. blue bars). Each bar shows the mean +/- standard deviation of three to five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

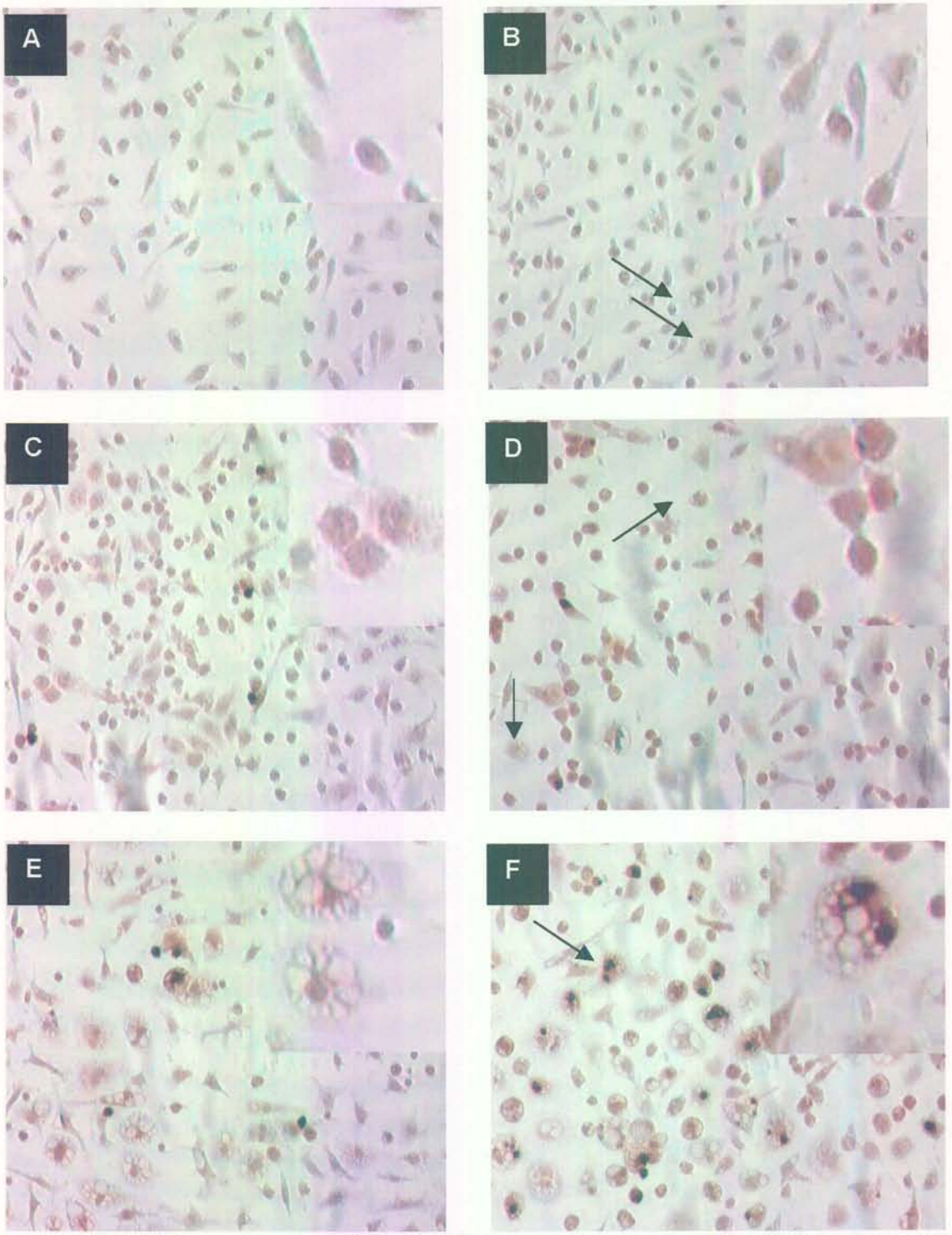


Figure 5.8. Cytochemical staining for MPO of adherent macrophages. (A) native-(PBS) macrophages (B) dead TB added to native (PBS) macrophages (C) Liposome treated macrophages (D) dead TB added to liposome treated macrophages (E) liposome-MA treated macrophages (F) dead TB added to liposome-MA treated macrophages. Arrows refer to remarks in the text.

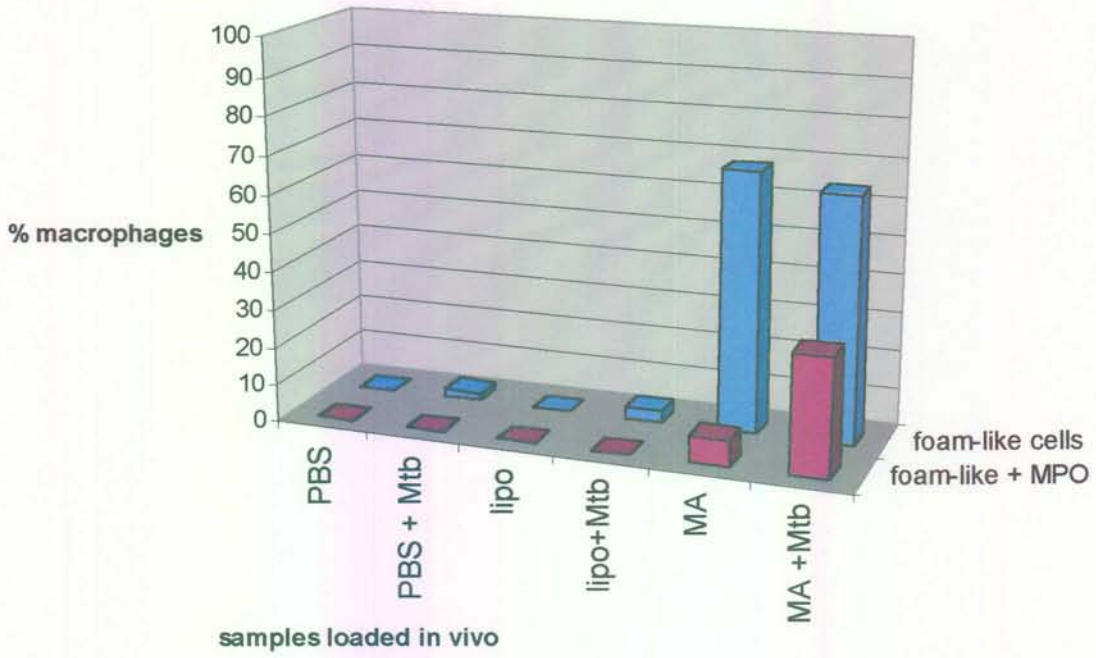


Figure 5.9. Statistics of MPO-staining of macrophages as depicted in Figure 5.8. Three hundred cells for each point were counted under the microscope and plotted.

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).

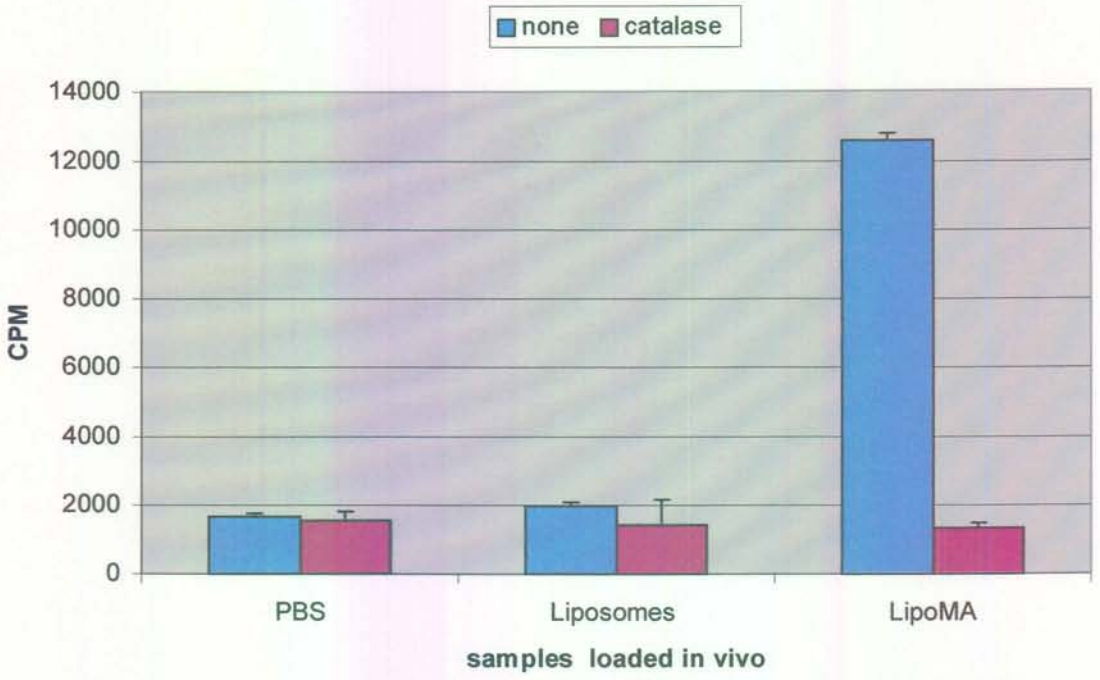


Figure 5.10. Effect of catalase on the uptake of [methyl-^{3}H] thymidine in macrophages loaded with PBS, liposomes or liposomes-MA. Each bar represents the mean (+/- s.d.) of 5 values.

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.

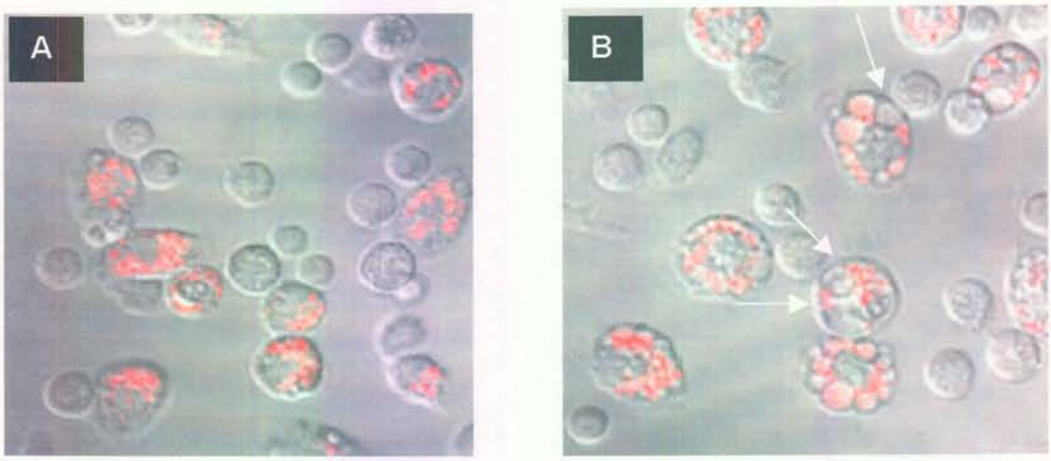


Figure 5.11. Fate of Cy5-labelled catalase in A) liposomes, B) liposomes-MA loaded macrophages. The white arrows indicate vacuoles without any labelled catalase.

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

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.

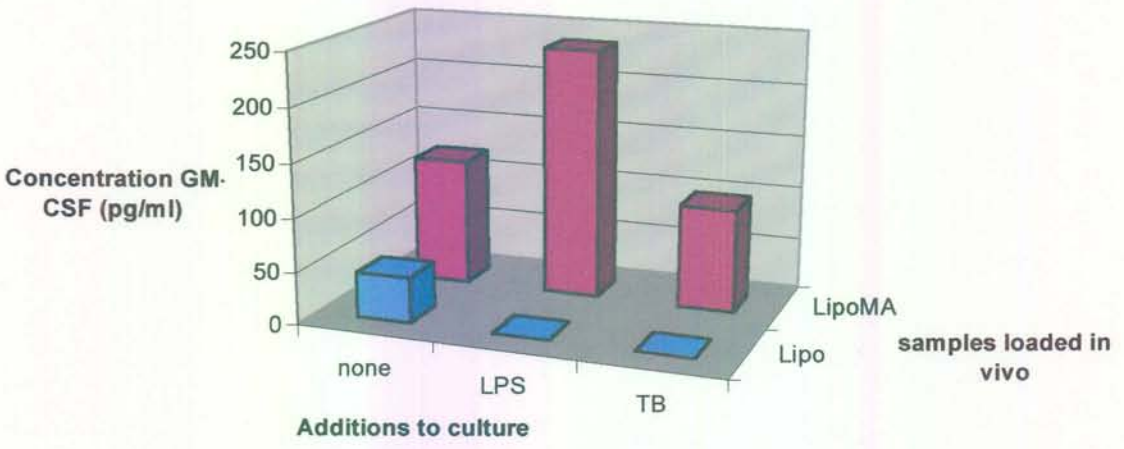


Figure 5.12. GM-CSF values from culture medium of macrophages preloaded *in vivo* with liposomes and liposomes-MA. Cultures were also carried out in the presence of dead MtbH37Ra bacteria or LPS. Results represents single values that were reproducible in at least 2 experiments.

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.

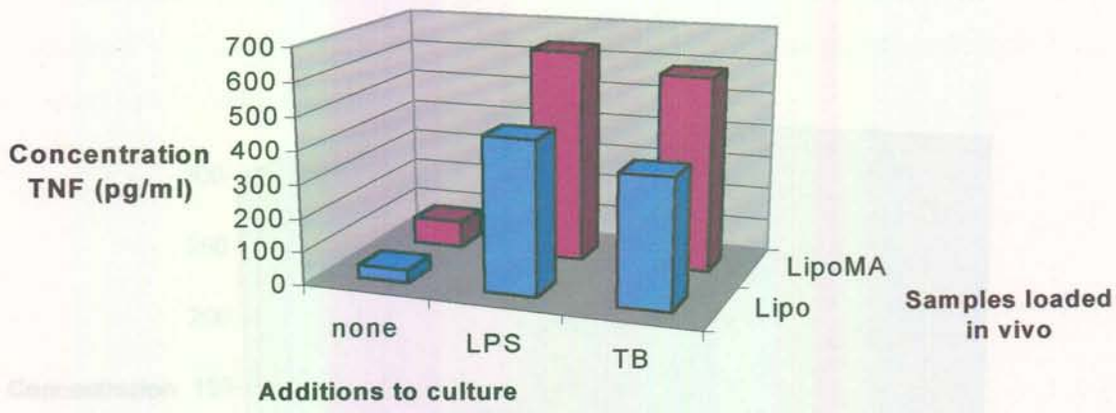


Figure 5.13. TNFα concentration detected in supernatants from macrophage cultures loaded *in vivo* with liposomes or liposomes-MA. Cultures were also carried out in the presence of dead MtbH37Ra or LPS. Results represent single values that were reproducible in at least 2 experiments.

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).

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.

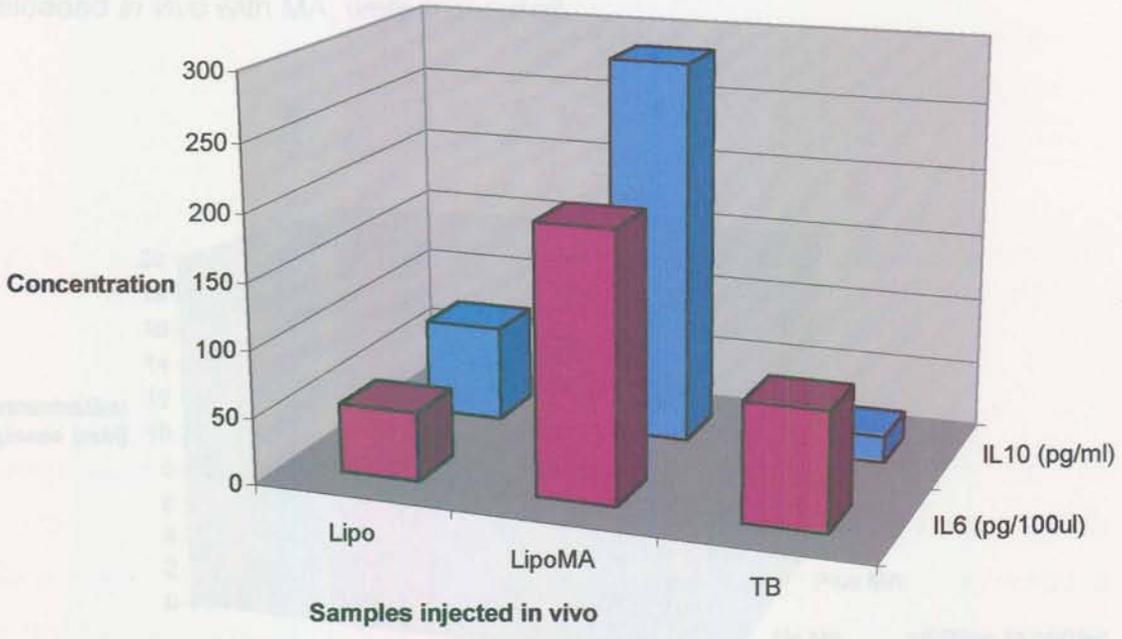


Figure 5.14. IL6 and IL10 concentrations from macrophage cultures loaded *in vivo* with liposomes, liposomes-MA or dead MtbH37Ra bacteria. IL10 is given in pg/ml and IL6 in pg/100 μl. Results represent single values that were reproducible in at least 3 experiments.

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.

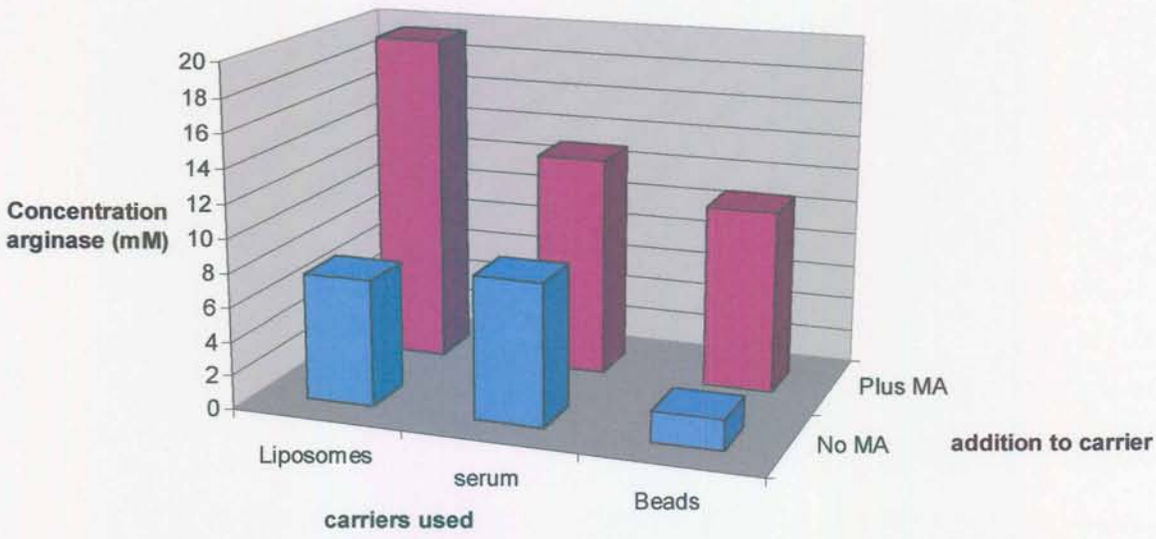


Figure 5.15. Arginase determination from adherent macrophages after MA loading *in vivo,* using different carriers. Results represent the mean of 4 values that were reproducible in at least 2 experiments.

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.

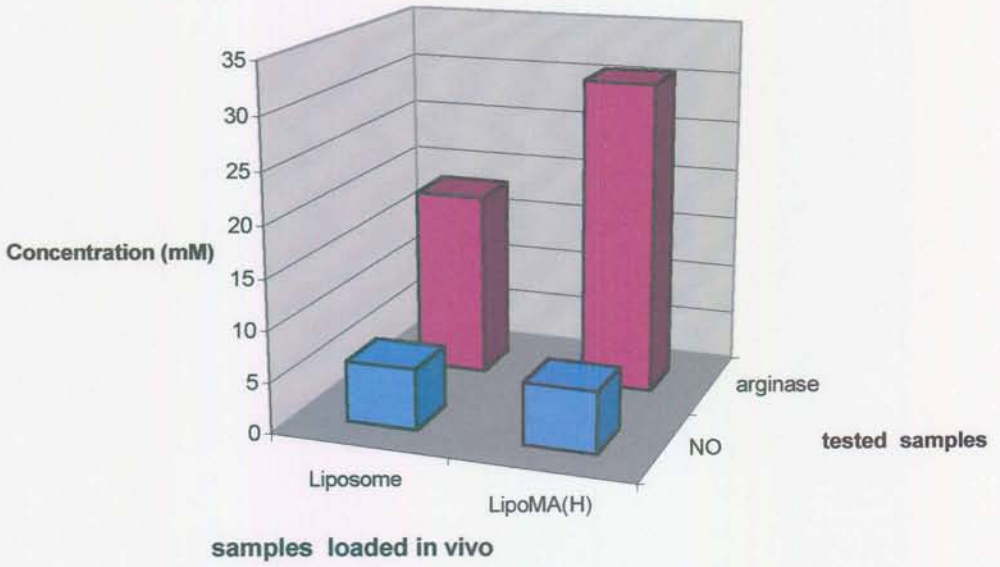


Figure 5.16. NO and arginase production by macrophages that were loaded *in vivo* with liposomes or liposomes-MA. Results represent single values that were reproducible in at least 2 experiments.

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,

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.

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