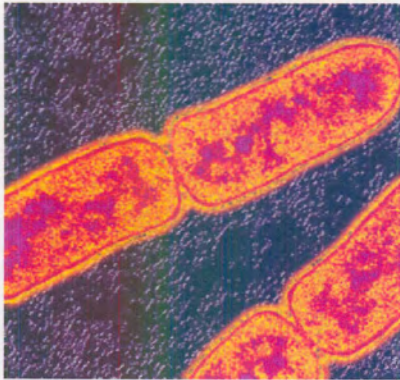

THE MODULATING PROPERTIES OF MYCOBACTERIAL MYCOLIC ACIDS ON MURINE MACROPHAGE FUNCTION

by

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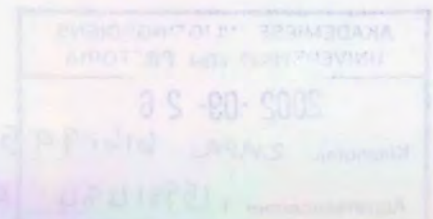

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ABBREVIATIONS

ACAT	acyl coenzyme A: cholesterol acyltranferase
AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cell
ATP	adenosine triphosphate
BCG	Bacillus Calmette-Guerin
BMF	(5-bromo) methylfluorescein
CD	cluster of designation
CR	complement receptor
DNA	deoxyribonucleic acid
DMF	dimethylformamide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorter
FcR	receptor for the constant fragment of immunoglobulin
FCS	fetal calf serum
FITC	fluorescein-iso-thiocyanate
GM-CSF	granulocyte monocyte colonizing factor
GPL	glycopeptidolipids
GTP	guanosine triphosphate
HA	haemagglutinin
HIV	human immunodeficiency virus
HLA-DM	human leukocyte antigen alleles
HPLC	high performance liquid chromatography
HTAB	hexadecyltrimethylammonium bromide
IFN	interferon
IL	interleukin
iMPO	enzymatically inactive myeloperoxidase
iNOS	inducible nitric oxide synthase
LAM	lipoarabinomannan

LAMP	lysosome associated membrane protein
LDL	low density lipoprotein
LM	lipomannan
LPS	lipopolysaccharide
MA	mycolic acids
MDR	multi drug resistance
MHC	major histocompatibility complex
MIIC	MHC class II compartment
MMR	macrophage mannose receptor
MOI	multiplicity of infection
MPO	myeloperoxidase
nCEH	neutral cholesterol ester hydrolase
NF-κβ	nuclear factor κβ
NK	natural killer
NOS	nitric oxide synthase
Ox-LDL	oxidized low density lipoportein
PBS	phosphate buffered saline
PE	phycoerythrin
PEC	peritoneal exudate cell
PDGF	platelet-derived growth factor
PDIM	phthioceroldimycocerasate
PI	propidium iodide
PIM	phosphomannosides
Rab7	member of the small GTPase family
RNA	ribonucleic acid
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
RT-PCR	reverse transcriptase polymerase chain reaction
TACO	tryptophane aspartate-containing coat protein
TDM	trehalose dimicolate
TdR	[methyl-^{3}H] thymidine

TGF	transforming growth factor
Th1/2	T-helper 1 or 2 cell
TLR	toll like recpertor
TNF	tumour necrosis factor
V-H^+ ATPase	vacuolar ATP-dependent proton pump
WHO	World Health Organization

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CHAPTER 1

1.1 GENERAL INTRODUCTION

1.1.1 Tuberculosis, the ongoing epidemic

Tuberculosis has plagued mankind since the beginning of human history, although its impact on morbidity and mortality has varied widely during the centuries and in different regions, often influenced by manmade factors. Urban crowding and poverty have been responsible for the tuberculosis epidemic originating in Europe in the early 1600s [1]. During this epidemic and subsequent spread to other continents, tuberculosis has caused more human deaths than any other disease [2]. In recent decades, the declining incidence rates of tuberculosis and the ability to treat tuberculosis effectively with drugs have changed the perception of tuberculosis as a fatal disease in the general population as well as in the medical community of industrialized countries. In the view of many people tuberculosis is no longer to be a disease of significance.

This attitude comes in conflict with recent figures from the World Health Organization (WHO), documenting that every minute, more than 10 individuals develop tuberculosis, amounting to 8 million new cases annually. Two to 2.5 million of these tuberculosis sufferers will die of the disease (see also Fig. 1.1). These appalling figures put tuberculosis in the list of the top major killers, together with AIDS and malaria [3]. The situation is worsened by the increasing incidence of multidrug resistant strains (MDR), and the deadly combination of tuberculosis with AIDS [4]. Co-infections with HIV and *M. tuberculosis* increase the risk of developing tuberculosis 30-fold. These statistics are daunting, considering that effective chemotherapy is available.

Two obstacles are in the way of effective chemotherapy of tuberculosis: first, tuberculosis drugs (isoniazid, rifampicin, pyrazinamide, streptomycin and ethambutol) must be taken for long periods of time (usually 6 months or longer) and second, a combination of three specific drugs is required to avoid the development of drug resistance [3]. These obstacles make compliance to the therapy regimen difficult to achieve. With more than 1 billion bacteria in the lung of a patient

suffering from active tuberculosis, poor compliance can lead to the development of resistant strains. Therefore, development of new drugs active against *M. tuberculosis*, particularly drugs that would allow shorter courses of therapy, is a major priority of tuberculosis research.

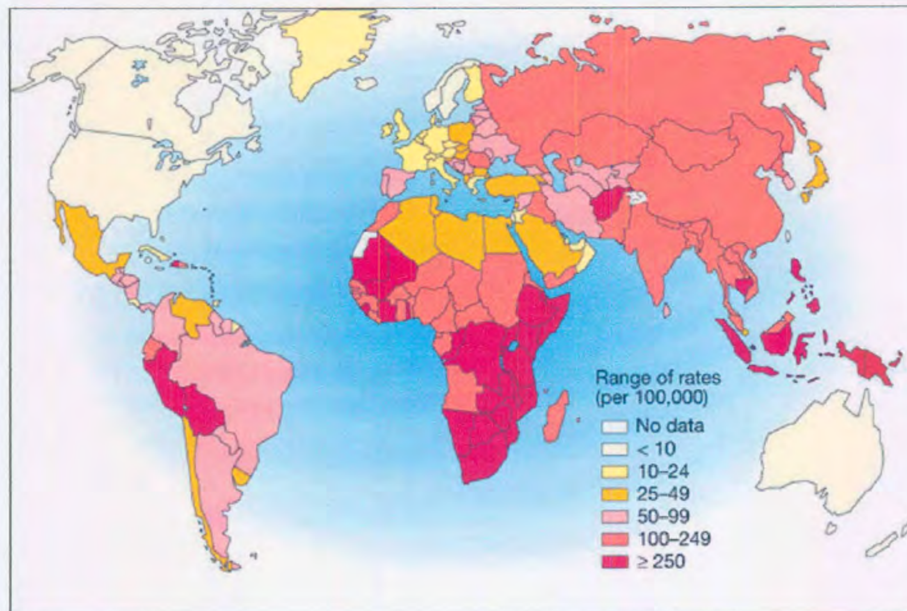

Figure 1.1. Estimated tuberculosis incidence cases per 100 000 of the population [5].

The prevention of tuberculosis is also complex. BCG, the widely administered, live attenuated vaccine against tuberculosis, is inconsistently effective in adults [6]. Understanding the pathogenic strategies of *M. tuberculosis,* in particular the interaction of cell wall components with host targets, will support both the development of new drugs and the design of either live attenuated or subunit vaccine candidates to combat this global health emergency.

1.1.2 Clinical manifestation of *M. tuberculosis*

Tuberculosis is a bacterial infectious disease caused by the obligate human pathogen, *Mycobacterium tuberculosis*. Mycobacteria are distinctive rod shaped bacteria characterized by a lipid-rich cell wall that avidly retains Carbol fuchsin dye even in the presence of acidic alcohol (acid fast staining). In order to understand the pathogenesis of *M. tuberculosis* infection, it is essential to understand the clinical manifestation of this pathogen in its natural host.

Although tuberculosis may occasionally be contracted via the gastro-intestinal route, it is almost exclusively transmitted by aerosolized droplets containing infectious *M. tuberculosis*. These droplets are generated by the cough of a person with *M. tuberculosis* infection of the lung and are inhaled by an uninfected person. The inhaled bacilli lodge in the terminal air spaces of the lung where they are phagocytosed by alveolar macrophages in which they proliferate. This leads to the induction of a localized pro-inflammatory response which leads to recruitment of mononuclear cells from neighbouring blood vessels to the site of infection. These cells wall off the site of the infection, forming a granuloma, or tubercle, that defines the disease. The granuloma consists of a kernel of infected macrophages, surrounded by foamy giant cells and macrophages with a mantle of lymphocytes delineating the periphery of the structure. This tissue response typifies the 'containment' or primary phase of the infection (Fig. 1.2). In some cases, presumably because of low immune resistance or a large infectious inoculum, the disease progresses. Therefore, the disease often shows a chronic, cyclic pattern characterized by healing and progressing lesions co-existing within the same individual. The primary lesions become larger, liquefy and release the mycobacteria, which may cause the disease to spread for example to the bronchi. Cavities are left in the lung, typical of mycobacterial pathology. Most hosts have a well functioning immune system and the primary infection is rapidly controlled, with most bacteria being killed or rendered 'dormant'. Dormancy is a latent state of tuberculosis that is well contained. Despite this successful initial parasitization of the human host, the primary infection is almost invariably asymptomatic in adults.

The state of dormancy is not well characterized, and is thought to result from oxygen depletion causing the bacilli to shut down many metabolic pathways temporarily until more favourable conditions for bacterial growth occur. The only clinical evidence of *M. tuberculosis* infection during latency is delayed type hypersensitivity against mycobacterial antigens, demonstrated by a tuberculin skin test.

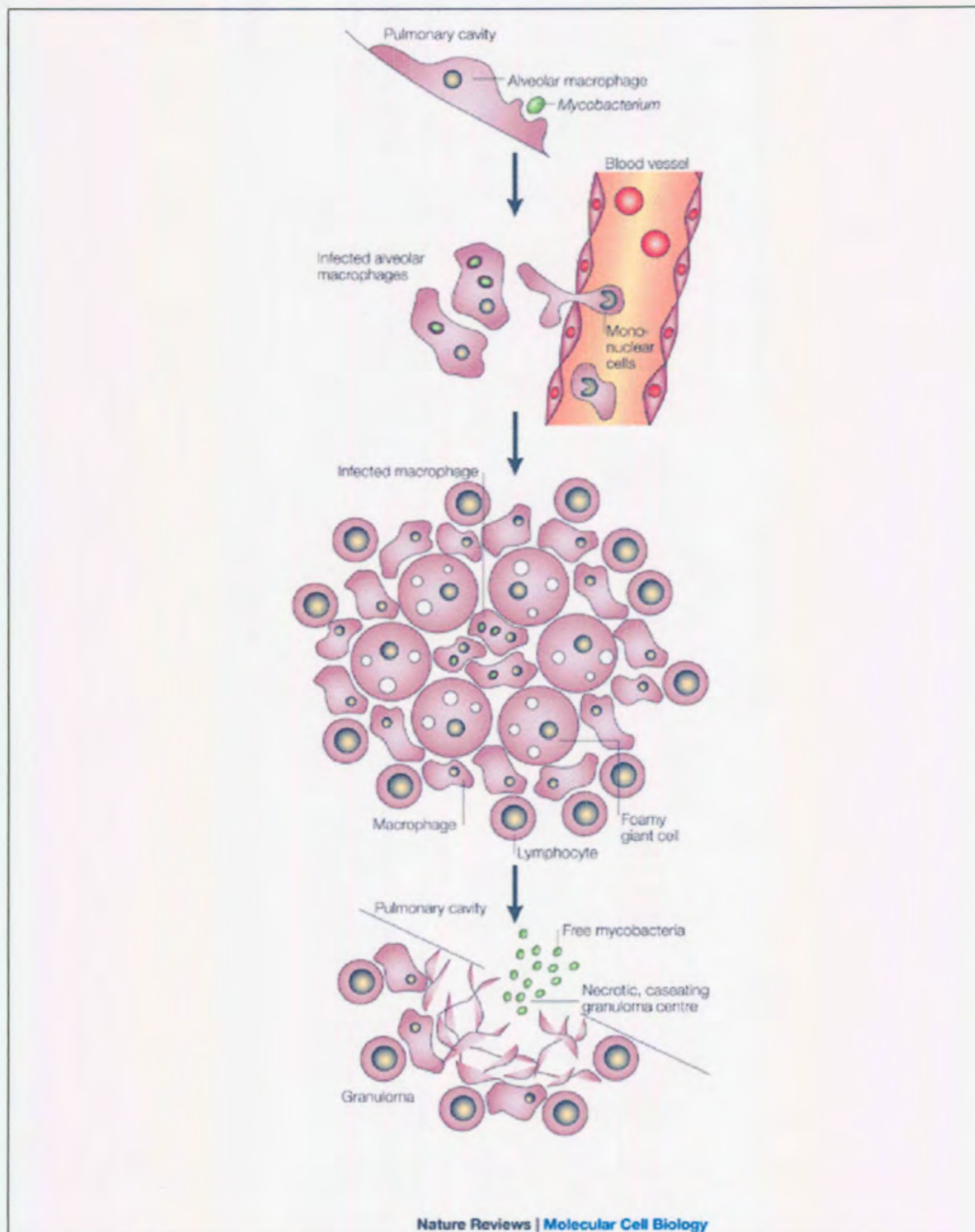

Figure 1.2 Cellular events following infection with *M. tuberculosis* [7].

The development of tuberculosis happens when the so-called dormant foci that are left in the host after a primary infection become reactivated. Reactivation occurs when the host immune system fails to control bacterial growth or when the immune system is deteriorating, for example, as a result of malnutrition, old age or co-infection with HIV. Under such circumstances, the mycobacteria start to proliferate rapidly and the center of the granuloma undergoes caseation and

spills viable infectious bacilli into the airways and vascular system (Fig. 1.2). This leads to development of a productive cough that facilitates aerosol spread of infectious bacilli. In addition these 'spilled' bacilli are then able to spread to and colonize virtually every organ in the host.

The disease is a chronic wasting illness characterized by fever, weight loss, and in the case of pulmonary reactivation, cough. The cough of tuberculosis is a symptom of chronic pulmonary inflammation and the mechanism by which the organism disseminates to new hosts. Many of the symptoms of tuberculosis, including the tissue destruction that eventually liquefies infected portions of the lung, is mediated by the host immune response against *Mycobacterium tuberculosis* rather than a direct toxicity of the bacterium itself.

1.1.3 Pathogenesis of *M. tuberculosis*

1.1.3.1 *M. tuberculosis* and the macrophage

1.1.3.1.1 Route of entry

M. tuberculosis primarily infects and survives within macrophages, the very cells that are out to provide an effective initial barrier to contain the bacterial infection (Fig. 1.3). In order to ensure successful parasitization and survival in the host macrophage, *M. tuberculosis* employs several strategies already at the point of entry into the target cell [8].

Firstly, the bacilli are able to render specific host-invasion strategies dispensable [9,10]. In contrast to other bacterial pathogens that avoid phagocytosis as a specific pathogenic strategy, *M. tuberculosis* uses any of a number of different cell surface receptors to gain entry into macrophages including complement receptors (for both opsonized and non-opsonized entry), mannose receptors, Toll-like receptors (TLRs), Fc receptors (that can internalize IgG-opsonized bacteria), scavenger receptors and CD14 [9].

Secondly, recent work showed that, in addition to binding to one or more specific receptor molecules, mycobacteria interact with the plasma membrane steroid cholesterol. When macrophages are depleted of cholesterol by pharmacological treatment, mycobacteria can no longer enter the macrophage [11]. This inhibition of uptake is specific for mycobacteria, as other microorganismic parasites of the macrophage can still enter cholesterol-depleted macrophages.

Presumably, the glycolipid-rich mycobacterial cell wall contains components that interact directly with cholesterol. In accordance with this, mycobacteria specifically bind to cholesterol, indicating the presence of a high-affinity cholesterol binding site at the mycobacterial cell surface [11]. The identification of the mycobacterial cell wall constituents responsible for cholesterol binding might lead to strategies to interfere with cholesterol-mediated mycobacterial entry. Depleting the macrophages of cholesterol does not compromise the function of CR3-mediated internalization. Rather, cholesterol may function as a direct 'docking' site for mycobacteria to stabilize their interaction with the macrophage membrane, after which the mycobacteria can be efficiently internalized [11].

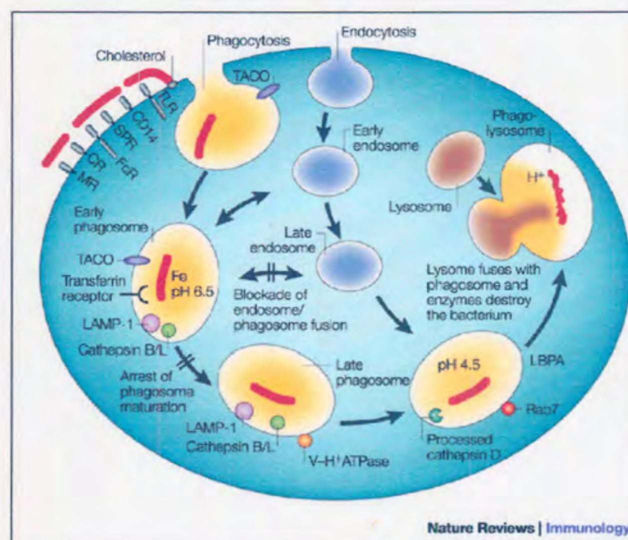

Figure 1.3 The intracellular lifestyle of *Mycobacterium tuberculosis*. CR, complement receptor; FcR, receptor for the constant fragment of immunoglobulin; LAMP-1 lysosomal-associated membrane protein 1; LBPA, lysobiphosphatic acid; MR, mannose receptor; Rab7, member of the small GTPase family; SPR, surfactant protein receptor; TACO, tryptophane, aspartate-containing coat protein; TLR, Toll-like receptor; V–H^+ATPase, vacuolar ATP-dependent proton pump [12].

Thirdly, the cholesterol association may determine the subsequent intracellular events, as cholesterol mediates the phagosomal association of the tryptophane aspartate-containing coat protein (TACO), which prevents the maturation of the phagosome to a phagolysosome [13].

Fourthly, the initial interaction with surface receptors are important, since this determines the subsequent fate of *M. tuberculosis* within the macrophage. For example, interactions with the constant regions of immunoglobulin receptors (FcRs) and Toll-like receptors have been shown to stimulate host-defense mechanisms, whereas those with complement receptors promote mycobacterial survival [14-16]. Much work has focused on the interaction of mycobacteria with the Toll like receptors (TLR). Two TLRs, TLR2 and TLR4, have been implicated in the activation of macrophages by mycobacteria. Viable *M. tuberculosis* could activate an NF-κB reporter gene in Chinese hamster ovary cells transfected with TLR2 and TLR3 via distinct ligands, independent of CD14 expression. Thus a soluble, heat-stable factor mediates TLR2-dependent activation, while a heat-sensitive cell wall-associated factor induced activation via TLR4. The cell wall component, LAM, isolated from fast-growing mycobacteria, stimulated TLR2- but not TLR4-mediated activation, whereas LAM isolated from *M. tuberculosis* or *M. bovis* Bacillus Calmette-Guerin (BCG) failed to activate via either receptor [17]. These data indicate that a variety of mycobacterial cell wall components can activate signaling cascades within the host cell, which direct the subsequent development of an immune response.

1.1.3.1.2 Survival within the macrophage phagosome

i) Arresting phagosome maturation

The second host defense barrier, which requires more sophisticated coping mechanisms by the mycobacterium, is its survival within the phagosome, a harmful environment that is detrimental to many microbes (Fig. 1.3). *M. tuberculosis* solves this obstacle by arresting the phagosome at an early stage of maturation and preventing its fusion with lysosomes [18,19]. Figure 1.3 illustrates phagosome and endosome maturation and the influence of *M. tuberculosis* on this process. Whereas virtually all eukaryotic cells are capable of engulfing small particles and fluids that end up in the endosomal pathway, phagocytosis of larger particles is a unique property of specialized cell types, the 'professional' phagocytes. Both the endosomal and phagosomal pathways undergo interconnected maturation processess that merge at a late stage, prior to fusion with lysosomes. Although phagosome and endosome maturation form a continuum, distinct steps can be distinguished by means of different markers and tracers, some of which are shown in figure 1.3. Once engulfed, *M. tuberculosis* ends up in a phagosome, the maturation of which is arrested at an early stage. This is demonstrated by the observation that mycobacterium-containing

phagosomes have low levels of the molecules characteristic of lysosomal membranes, including LAMP-1, LAMP-2, and GTP-binding protein rab7, while retaining the transferrin receptor and rab5 [20,21]. The early mycobacteria-harbouring phagosome characteristically retains TACO, which apparently prevents its further maturation. *M. tuberculosis* inhibits the V–H^+ ATPase mediated phagosomal acidification and prevents fusion with the endosomal pathway. Although the lysosomal acid protease cathepsin D might be present in the mycobacterium-containing phagosomes in a small amount, the enzyme remains in an inactive form, which may be due at least in part to the exclusion of the vacuolar proton-ATPase from the membrane. This lack of acidification is probably responsible for the failure of enzymes such as cathepsin D to be activated [22]. The inhibition of phagosome maturation is assumed to be an active process controlled by mycobacteria, since only viable bacilli can accomplish it.

The arrest of phagosomal maturation is not fully effective. Some phagosomes mature to form phagolysosomes. Phagosome maturation is promoted in activated macrophages, particularly after IFN-γ stimulation, which stimulates anti-mycobacterial mechanisms in macrophages, notably reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI). The role of RNI in the control of human tuberculosis remains controversial, despite a body of evidence from animal experiments that supports its role in the control of *M. tuberculosis* [23-25].

ii) Acquisition of essential nutrients

Another obstacle for the bacterium to overcome while residing inside the macrophage phagosome, is obtaining the nutrients essential for survival. Residing in the early recycling endosome, *M. tuberculosis* needs ready access to iron [8,26]. Iron is also required in various host-defense mechanisms. To successfully compete for iron with its host, the pathogen possesses specialized iron scavenging molecules [27].

Persistent microbes proceed to a stage of dormancy with reduced metabolic activity that facilitates their survival under conditions of nutrient and oxygen deprivation. These bacteria can persist without producing disease by switching to lipid catabolism and nitrate respiration to ensure their survival [28,29]. Early studies already demonstrated that *M. tuberculosis* harvested from mouse tissues preferentially metabolize fatty acids as carbon source [30]. Recent evidence

has been provided that *M. tuberculosis* in chronically infected lung tissues, i.e. at late stages of infection, obtains carbon from fatty acids. A pathway that is required for this acquisition is the glyoxylate shunt. One enzyme of this pathway, isocitrate lyase, is up-regulated by *M. tuberculosis* organisms when they are inside macrophages [31]. A mutant *M. tuberculosis* strain with a disruption in the gene encoding isocitrate lyase was unable to sustain acute phase of growth. *In vitro* infection of macrophages revealed that the expression of mycobacterial isocitrate lyase was prolonged in activated macrophages in comparison to resting cells [28,31]. This suggests that the manipulation of nutritional requirements of *M. tuberculosis*, coupled with the immune status of the host, dramatically alters the course of infection and could open up potential avenues for therapeutic intervention.

1.1.3.1.3 Effect on the immune interface

The arrested phagosome maturation could also impair mycobacterial antigen presentation on MHC. In resting macrophages, *M. avium* containing phagosomes contain surface-derived MHC class II heterodimers, but fail to acquire MHC class II from intracellular stores. At late infection time points, this causes a paucity of phagosomal MHC class II molecules. Class II MHC heterodimers and HLA-DM, a chaperone of antigen loading, traffick through specialized late endosomal-like compartments (MIIC) on their way to the plasma membrane. It has been argued that the arrest in phagosome maturation could prevent the fusion of *M. avium* containing phagosomes with late endosomal vesicles like MIIC containing MHC class II molecules [32], as phagosomes formed around inert particles acquire MHC class II molecules and HLA-DM at a late stage during phagosome biogenesis. This is consistent with the model that mycobacteria in resting macrophages shunt their resident vacuoles away from the normal antigen –processing and -presentation machinery of their host cell, explaining some of the apparent immunosuppressive activities of mycobacteria. Activation of the infected macrophages, however, alleviated the seclusion of *M. avium*-harbouring phagosomes from intracellular pools of MHC class II, such that HLA-DM is no longer contained .

Mycobacterium spp. also affect the cellular immune response through modulation of the cytokine environment in the vicinity of the infected macrophage. Infected macrophages produce copious amounts of pro-inflammatory cytokines such as interleukin–1 (IL-1), tumour necrosis

factor-α and IL-6. IL-6 mediates B-cell maturation but suppresses T-cell proliferation at concentrations in excess of 1 ng/ml [33]. Although high, this concentration could easily be reached within the confines of a granuloma. Together, these aspects probably minimize the capacity of infected macrophages to stimulate a localized cellular immune response. However, with time, such a response does develop and the bacterium must cope with the consequences through alternative strategies.

Based on this understanding, it becomes clear that *M. tuberculosis* is adapted to its human host by exerting three distinct, sequential pathogenic strategies. First, the organism successfully replicates within host macrophages directly after infection, a property shared with other intracellular bacterial pathogens, but apparently achieved through unique mechanisms. Second, the organism either resists or modifies the host immune response to allow continued controlled bacterial replication without sterilization. Third, *M. tuberculosis* is able to persist within its host in a relatively inactive state, retaining the potential for reactivation. These three stages of infection represent distinct environments to which the organism must respond to survive and which are investigated by the experiments in this thesis. It is assumed that bacterial mediators are involved that influence the host environment to sustain the infected state.

1.1.3.2 What are the bacterial mediators?

Although it is clear that *Mycobacterium tuberculosis* arrest the maturation of phagosomes to maintain them as a habitable environment, it is not fully understood how the bacillus achieves this effect. A number of studies shed light on the anomalous association of host proteins with the bacteria-containing phagosomes to arrest maturation, but the identification of the bacterial mediator that accomplishes this has not yet been uncovered.

Current models of microbial influence on phagosome maturation and trafficking focus on secretion systems that transfer proteinaceous virulence factors into the resident phagosomal membrane or the surrounding cytosol. Mycobacteria lack secretion systems analogous to those of other pathogenic bacteria, and secretion of proteins by mycobacteria is still a largely undefined process. Although no mycobacterial mediators have been demonstrated to modify phagosome maturation, lipids are ideal candidates. A mycobacterial sulpholipid, when coated onto yeast

particles, impaired phagosome fusion with lysosomes [34]. Other mycobacterial glycolipids, including lipoarabinomannans (LAM) and glycopeptidolipids (GPL), exert pleiotropic effects on macrophages [35]. Considering the endotoxin-like activity of these lipids along with extremely high lipid content of the mycobacterial cell wall, it is likely that these constituents affect not only phagosome maturation, but also mediate other pathogenic strategies. The contribution of mycolic acids, the most abundant of these mycobacterial lipids to the interaction of *M. tuberculosis* and host cells and especially the macrophages will therefore constitute the main focus of this study. For this, it is necessary to first focus on host factors involved in the dialogue between the pathogen and its host.

1.1.4 Host factors in *M. tuberculosis* infection

1.1.4.1 Cytokines: central role for IFN-γ

Besides the initial macrophage defenses, other immune cells offer substantial help to the macrophage in controlling growth of mycobacteria. The main features of these interactions are illustrated in figure 1.4. The granuloma is the site of infection, persistence, pathology and protection. Effector T cells participate in the control of tuberculosis by macrophages. These include conventional $CD4^+$ and $CD8^+$ T cells responding to antigen presented to them on MHC by antigen presenting and target cells and unconventional γδ, CD4/CD8 double-negative or single-positive T cells, that recognize antigen on tuberculosis infected and affected macrophages in the context of CD1.

Interaction of macrophages with other effector cells occurs in a milieu of both chemokines and cytokines. These molecules serve both to attract other inflammatory effector cells such as lymphocytes and to activate them. Interferon-γ (IFN-γ) and tumour –necrosis factor-α (TNF-α), produced by T cells, are important macrophage activators. Macrophage activation permits phagosomal maturation and the production of antimicrobial molecules such as reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI).

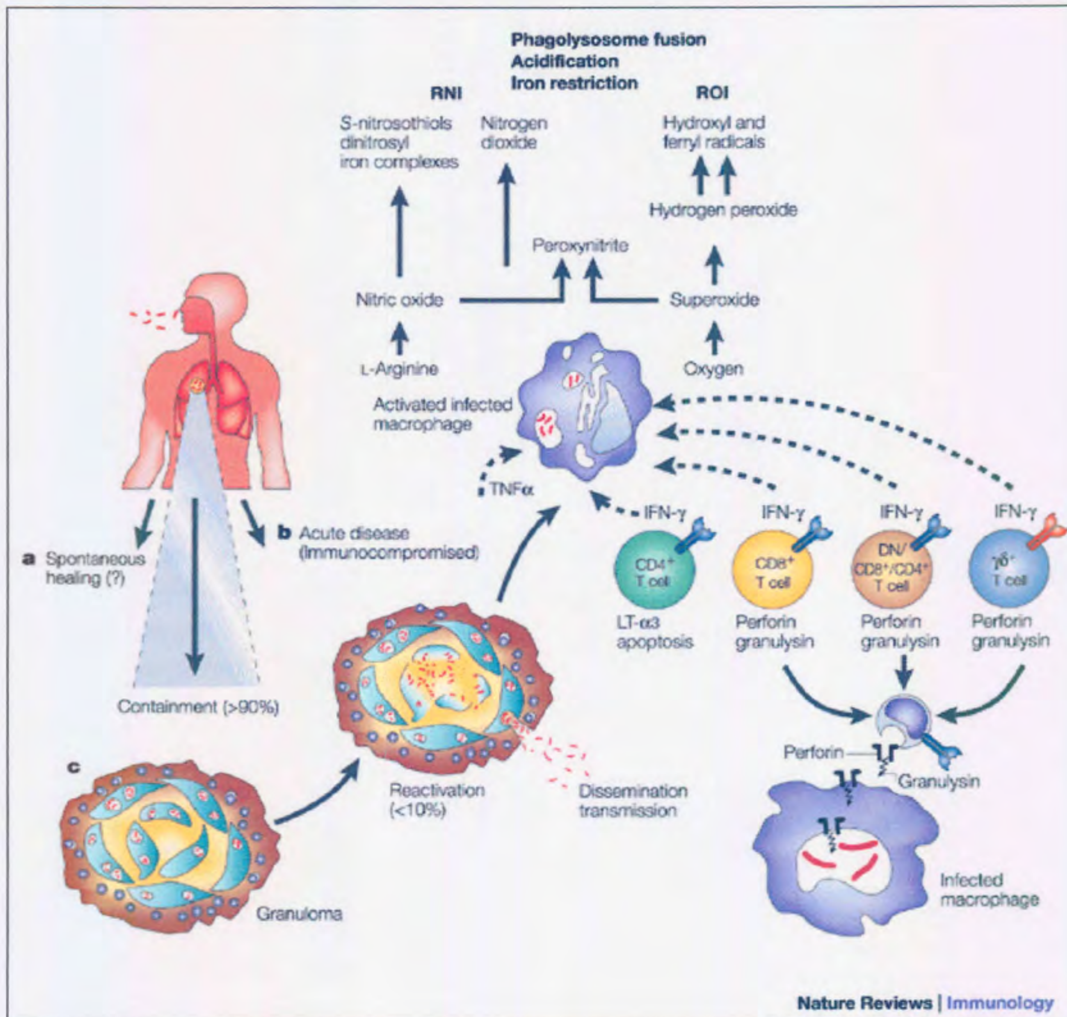

Figure 1.4 The host immune response to tuberculosis. Three potential outcomes of infection of the human host with *Mycobacterium tuberculosis.* a) Abortive infection resulting in spontaneous healing. b) Infections directly after infection in the immunocompromised host. c) Disease developing later as a result of reactivation after mycobacteria were initially contained. LT-α3, lymphotoxin- α3 [12].

Much attention has been focused on the role of the cytokine interferon gamma IFN-γ, in terms of its capacity to activate the macrophage's ability to inhibit mycobacterial growth. For example, activation of macrophages with IFN-γ before their infection with pathogenic *Mycobacterium* overcame the hold that the bacteria exerted on phagosome maturation. Unlike the phagosomes in resting macrophages, the vacuoles of activated macrophages acidify to pH~ 5.2 [36,37]. Acidification of the phagosomes is independent of bacterial viability because bacteria isolated

from macrophages at early time points were still fully viable. However, the bacterial load in these pre-activated cells drops with time, indicating that the cells are microbicidal [37]. Efficient killing of *Mycobacterium spp.* requires production of nitric oxide through the expression of the inducible nitric oxide synthase NOS (NOS2 in humans) [23,38,39]. The efficacy of nitric-oxide-mediated killing of mycobacteria is enhanced by the acidification of the mycobacterium-containing vacuole in the activated macrophage. Evidence supporting the key role of IFN-γ in the control of *M. tuberculosis* infection was demonstrated experimentally by studies showing that IFN-γ knockout mice are susceptible to virulent tuberculosis [40,41]. *M. tuberculosis* bacilli grew essentially unchecked in the organs of IFN-γ knockout mice and although granulomas formed, they quickly became necrotic. Macrophage activation is defective in these mice, and NOS2 expression is low [42,43]. Clinically, individuals defective in genes for IFN-γ or the IFN-γ receptor are prone to serious mycobacterial infections, including *M. tuberculosis* [44].

IL-12 is another cytokine that is critical in the development of a Th1 response [45] and the production of IFN-γ [46]. TNF can synergize with IFN-γ to activate macrophages [47]. Mice deficient in TNF and challenged with *M. tuberculosis* exhibited poorly formed granulomas in the lungs with areas of extensive necrosis. The failure to form organized granulomas resulted in widespread dissemination of *M. tuberculosis* and the rapid death of the animals. A comparison of macrophages within the lungs of TNF-mutant vs. wild-type animals revealed equivalent levels of major histocompatibility complex (MHC) class II and iNOS, suggesting that the critical role of this cytokine is in granuloma formation, rather than in the activation of T cells and macrophages [48]. As with many other infections, the production of TNF must be finely balanced, as its overproduction leads to increased cellular accumulation, compromising lung function and exacerbating tissue damage.

In contrast to the macrophage activating properties of IFN-γ, anti-inflammatory cytokines such as IL-10 play a number of important roles in down-regulating an active immune response in tuberculosis, including deactivation of macrophages [49], inhibition of T cell proliferation [50] and suppression of cytokine production by T lymphocytes [51], thereby controlling the harmful side-effects induced by the inflammatory cytokines.

1.1.4.2 Role of T-lymphocytes in host defense against mycobacteria

1.1.4.2.1 The Th1/Th2 paradigm applied to conventional CD4⁺ and CD8⁺ T cells

Although there is a role for many types of T-lymphocytes in host defense against *M. tuberculosis* (Fig. 1.4), undoubtedly the major effector cell in cell-mediated immunity in tuberculosis is the $CD4^+$ T-lymphocyte. In recent years, a paradigm for thinking about the functions of $CD4^+$ T cells and their relationship to the manifestations of disease has been developed based on experimental evidence gained from the murine model, but which has now also accumulated a substantial amount of support in a variety of human diseases. It holds that $CD4^+$ helper T-cells can be separated into at least two phenotypic classes, Th1 and Th2 [52-54]. These cells derive from a so-called Th0 cell under the control of cytokines such as interleukin-12 (IL-12) and IL-4. Type1 $CD4^+$ T-lymphocytes (Th1) and natural killer T-lymphocytes (NK cells) secrete interferon gamma, which activates alveolar macrophages to produce a variety of substances, including reactive oxygen and nitrogen species, which are involved in growth inhibition and killing of mycobacteria. Macrophages can also secrete interleukin-12 (IL-12) in a positive feedback loop to amplify this pathway. Phenotypically, Th1 cells are characterized mainly by their ability to produce the cytokines IFN-γ and IL-2, whereas Th2 cells produce cytokines such as IL-4, IL-5, and IL-10 (Fig. 1.5). Th1-type cytokines are those that activate other inflammatory and phagocytic cells capable of inhibiting the growth of pathogenic bacteria, while Th2 cells are involved in the inhibition of pro-inflammatory macrophage functions and the production of IgE and recruitment of eosinophils [55]. Cytokines such as IL-3, lymphotoxin, and granulocyte-macrophage-colony stimulating factor (GM-CSF) are secreted by both phenotypic classes of CD4+ cells.

The relationship of Th phenotype to disease manifestations in humans was demonstrated in studies published by Modlin and colleagues [56,57] in which skin biopsy lesions from patients with leprosy were examined to determine the state of expression of a variety of cytokine genes.

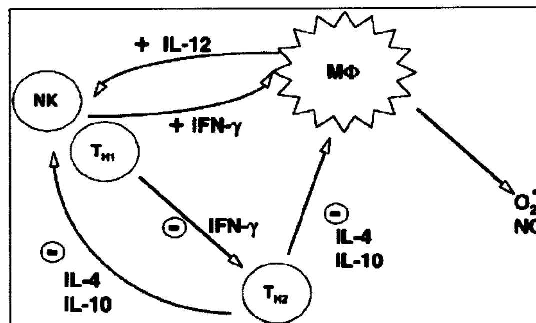

Figure 1.5 The Th1/Th2 paradigm applied to macrophage-T lymphocyte interactions in tuberculosis [55].

The clinical poles of leprosy are represented by lepromatous (susceptible) and tuberculoid (resistant) lesions. Lepromatous leprosy lesions are characterized clinically by extensive cutaneous damage with poorly defined lesions that infiltrate the dermis diffusely. Bacilli (*Mycobacterium leprea*) are easily demonstrated in normal skin by staining. In contrast, tuberculoid lesions are sharply demarcated and are singly or few in number on the skin. Histologically, noncaseating granulomas are present in tuberculoid lesions, and bacilli are rare or absent. By using the reverse transcriptase polymerase chain reaction (RT-PCR) to evaluate cytokine gene expression, Modlin and colleagues determined that lepromatous leprosy lesions contained cells expressing the Th2 cytokines IL-4, IL-5, and IL-10; whereas resistant, tuberculoid lesions contained cells expressing the Th1 cytokines IFN-γ and IL-2. Thus, the pattern of cytokine gene expression by helper T-cells seems to be associated with different manifestations of disease and the Th1/Th2 paradigm appears tenable in tuberculosis as well. Asthma, Crohn's colitis and organ transplantation are further examples of disease states with clinical manifestation that seem at least in part to be related to the Th1/Th2 phenotype present at the site of disease [58,59].

A popular notion of the Th1/Th2 paradigm is that there is a switch from a predominant Th1 response to a Th2 response leading to active disease (Fig. 1.6). Whether this applies to pulmonary tuberculosis remains unclear, since attempts at isolating Th2 cells and Th2-type cytokines from the site of infections have not been consistently successful [42,43,60]. In fact, IL-4 expression in samples from infected individuals were often shown to be lower than in those from uninfected

controls [61,62]. This warns against a simple interpretation of the role played by Th1/Th2 immunity in tuberculosis. It may be more accurate to argue that a true switch from a Th1 to a Th2 response does not necessarily occur in tuberculosis, but that the relative strength of the Th1 response determines latency or active disease.

Recently, however, evidence supporting the fact that antigen presenting cells such as macrophages and dendritic cells themselves may determine or direct immunologic outcomes is suggested by results such as those showing that *Leishmania* infection of macrophages can increase their ability to stimulate a Th2 response instead of a Th1 response [63]. In a related vein, it has been reported recently that dendritic cells have the potential to shepherd T lymphocytes into Th1- or Th2-dominant phenotypes [64]. Macrophages induced in Th1-dominated immune responses secrete multiple inflammatory mediators (e.g. IL-1, IL-6, and TNF-α) and are therefore termed inflammatory macrophages. Inflammatory macrophages possess cytotoxic and antimicrobial effector functions based on their ability to produce nitric oxide (NO) [23]. The production of NO is catalyzed by the enzyme inducible NO synthase (iNOS), which oxidizes the substrate L-arginine to form NO and L-citrulline. The fundamental importance of this metabolic pathway in murine macrophages as a key defense element in various infectious diseases as well as its role in diverse settings of immunopathology is today firmly established [65,66]. In contrast, the alternative pathway of macrophages to metabolize L-arginine and the functions associated with macrophages using this pathway are less well understood. This metabolic route is catalyzed by the enzyme arginase and leads to the products L-ornithine and urea. Th1 and Th2 cytokines [67,68] as well as the corresponding T cells [69] are competitively regulated by the balance of L-arginine metabolism in murine macrophages. While Th1 cells and cytokines induce specifically iNOS, Th2 cells and cytokines up-regulate arginase. Furthermore, a mutual negative feedback between both pathways suggested a competitive regulation; IFN-γ was found to suppress arginase activity in macrophages stimulated with LPS or IL-4 [67], whereas the iNOS-suppressing activities of IL-4 and IL-10 are well established [70,71].

Attempts to classify CD8+ T cells in type 1 and type 2 categories, similar to the Th1/Th2 paradigm of CD4+ T cells are not very convincing. CD8+ T cells, similar to CD4+ T cells, can produce IFN-γ, but their main function is target cell killing. By lysing infected host cells, CD8+

T cells could facilitate the translocation of *M. tuberculosis* from incapacitated cells to more proficient effector cells [72]. More recently human CD8+ T cells that express granulysin and perforin have been shown to kill *M. tuberculosis* directly [73-75]. Granulysin is responsible for bacterial killing and presumably gains access to *M. tuberculosis* that reside within macrophages through pores formed by perforin (Fig. 1.4). Antigen–specific T cells that achieve killing of infected macrophages mostly through apoptotic mechanisms, can also inhibit growth of *M. tuberculosis* [76]. Finally, ATP released by T cells can induce killing of mycobacteria [77].

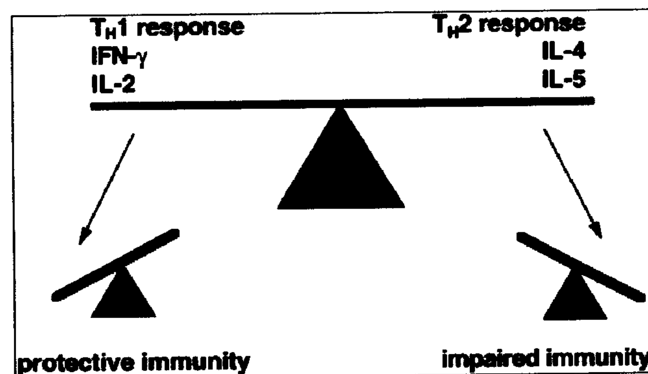

Figure 1.6 Popular view of the Th1/Th2 paradigm in infectious diseases [55].

1.1.4.2.2 The role of unconventional T cells

In addition to the two major CD4+/CD8+ T-cell populations, unconventional T cells, namely γδ T cells and CD1-restricted αβ T cells, apparently participate in protection against tuberculosis. A comparison of these different conventional and unconventional T cell populations in respect of their recognition of antigen presenting molecules are summarized in table 1. T cells expressing the γδ TCR partially protect against high, but not low inocula of *M. tuberculosis* [78] and regulate granuloma formation in mice [79,80]. Unlike mouse γδ T cells, human γδ T cells are stimulated by a group of non-proteinaceous phosphate containing antigens, apparently independent of any MHC restriction element. These 'phospholigands' include different prenopyrophosphates and nucleotide conjugates, all of which are abundant in mycobacteria. The phospholigands stimulate γδ T cells that express the Vγ2δ2 chain combination independently of their fine antigen specificity. Vγ2δ2 T cells comprise an important population of γδ T, constituting about 5% of all T cells in the peripheral blood in adults. This large γδ T-cell population readily produces IFN-γ

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after stimulation with phopholigands and expresses granule-dependent mycobacteriocidal activity [81].

Table 1

Comparison of expression, function and T cell recognition of CD1 and classical MHC molecules

	MHC Class I	MHC Class II	CD1a, b and c (group 1)	CD1d (group 2)
Species	Human, mouse	Human, mouse	Human	Human, mouse
Genomic location	MHC	MHC	Non-MHC	Non-MHC
Major cellular expression	Nucleated cells, *hematopoietic cells	B cells, *DCs (immature)	B cells (CD1c), *DCs (immature)	B cells, monocytes, macrophages, DCs
Antigens presented	Endogenous peptides	Exogenous peptides	Endogenous/exogenous glycolipids and lipids	Endogenous/exogenous glycolipids and phospholipids
Responding T cell subsets	CD8+ TCRαβ	CD4+ TCRαβ	DN, CD8+, CD4+, TCRαβ, TCRγδ	DN, CD4+, TCRα (NKT), diverse TCRαβ
Responding T cell functions	CTL	Th1/Th2-immune regulation	Th1/CTL activation	Th0/CTL activation?

CD 1 molecules share features of class I and class II MHC mediated antigen presentation, but uniquely present lipid antigens. *Indicates elevated cell surface expression levels. CTL, cytotoxic T lymphocyte [82].

T cells with specificity for mycobacterial glycolipids presented by CD1 molecules seem to have a unique role in human tuberculosis [83-86]. Group 1 CD1 molecules comprising CD1a, CD1b, and CD1c, are found in primates and guinea-pigs, but not in mice [84-86]. Generally CD1-glycolipid-specific T cells produce IFN-γ and express cytolytic activity. Group 1 CD1 molecules typically present glycolipids that are abundant in the mycobacterial cell wall, such as phosphatidylinositol, mannosides, lipoarabinomannan, mycolic acids and hexosyl-1-phosphoisoprenoids [85,86]. The different CD1 molecules prefer distinct intracellular locations,

with CD1a being almost exclusively expressed on the cell surface and in the early recycling endosome, CD1b residing primarily in late endosomes/lysosomes and CD1c being localized on the cell surface and in endosomes at different stages of maturation [87,88]. CD1a and CD1c apparently have ready access to mycobacterial glycolipids as *M. tuberculosis* arrests phagosomal maturation at early stages. Mycobacteria shed glycolipids inside the phagosome, and these enter the late endosome/lysosome, where they can also interact with CD1b [87]. Vesicles containing shed glycolipids can be released, and therefore be delivered to bystander cells. Group 1 CD1 molecules are abundantly expressed on DCs, but are virtually absent on macrophages, and CD1b surface expression is down-regulated in cells infected with *M. tuberculosis* [89]. The transfer of glycolipids from infected macrophages to bystander DCs therefore constitutes an important mechanism for promoting CD 1 presentation [87]. Group 2 CD 1 molecules (CD1d) appear not to play an important role in tuberculosis [90].

The main feature that distinguishes CD1-restricted T cells from conventional MHC-restricted T cells is the nature of the antigens they recognize. Foreign lipids make up the major component of bacterial cell walls and are also abundant in eukaryotic parasites. Because lipids are not directly derived from the genome, they fall into the category of 'innate' antigens that are not easily mutated, making them excellent targets for immune attack. As such, they may play an important role in host defense and correspondingly may be able to serve as recall antigens suitable for microbial vaccine development. A better understanding of the dialogue between the tubercle bacillus and especially its cell wall components and the host will provide important guidelines for the rational combat against this disease, which has become of major importance today.

1.1.5 Lipids of the mycobacterial cell envelope

The long-term habitation of *M. tuberculosis* within macrophages has resulted in the development of numerous pathogenic strategies to evade macrophage defenses, many of which involve lipid mediators within its cell envelope. The *M. tuberculosis* cell envelope differs substantially from the cell wall structures of both gram-negative and gram-positive bacteria. In addition to the cell membrane and peptidoglycan layers found in other bacteria, the mycobacterial cell envelope contains a large hydrophobic layer of mycolic acids. These are long chain branched

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β hydroxyl fatty acids that exist either covalently attached to the cell wall or non-covalently attached in the form of cord factor, i.e. trehalose dimycolate (TDM). An outer lipid leaflet, which is composed of a mixture of complex waxes, triacylglycerols and bioactive glycolipids is hydrophobically associated with the inner mycolyl leaflet (Fig. 1.7). Mycobacterial lipoglycans, LAM and related lipomannan (LM) and phosphomannosides (PIM) are also associated with the cell wall. A lipid moiety anchors these lipoglycans to the cytoplasmic membrane or the outer lipid leaflet. It is thought that most mycobacterial cell walls conform to the model whereby mycolic acid and LAM are the principal constituents.

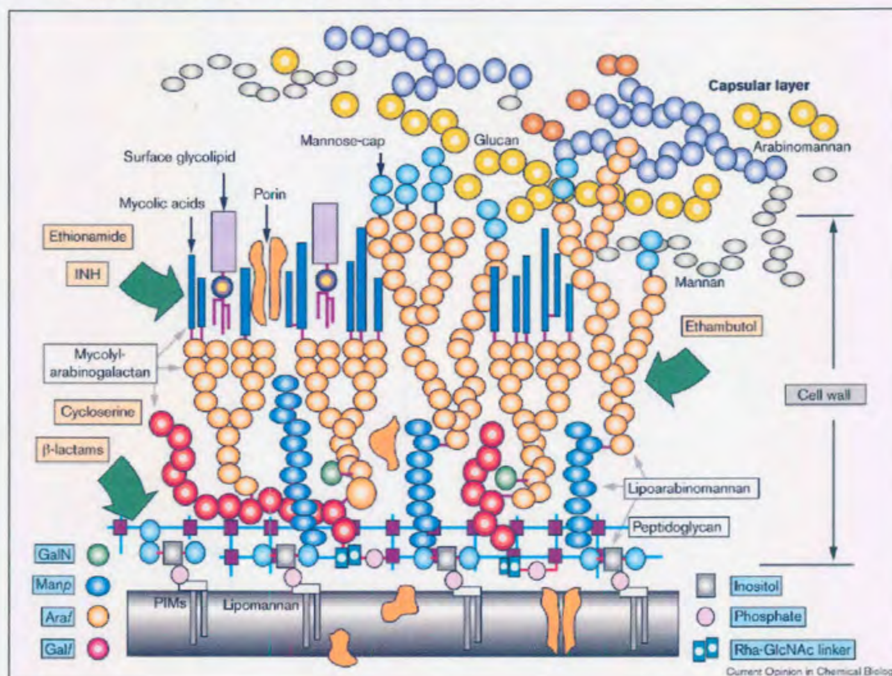

Figure 1.7 A model of the mycobacterial cell wall. The sites of action of some known antimycobacterial drugs are also depicted (green arrow) in the model [91].

The three-dimensional and spatial arrangement of the key molecules are largely unknown. The nature of the outermost surface of the cell wall remains unresolved. Most agree that the glycosylated groups of the complex lipids are exposed on the outermost surface of the cell wall [92], while others assert that a dense capsule-like matrix of free carbohydrates (glucans, mannans and arabinomannans) and secreted proteins conceals these lipids [93]. The surface glycolipids include a variety of species- and strain specific glycopeptidolipids, lipo-oligosaccharides, and phenolic glycolipid, the chemical identity and amount of which varies from one species to

another [94], which may explain the controversy. This vast array of lipids and glycolipids confers extreme hydrophobicity to the outer surface of the organism. The lipid-rich low permeability matrix contributes to the difficulty in combating mycobacterial infections by endowing the microorganism with innate resistance to therapeutic agents and host defences [95].

1.1.5.1 Biological effects of mycobacterial lipids on host cell function

A number of the mycobacterial cell wall lipids have been demonstrated to have potent biological activity when tested on eukaryotic cells in *in vitro* systems, raising the possibility that they may be important for pathogenesis. For example lipoarabinomannan, a major cell wall-associated glycolipid, inhibits IFN-γ activation of macrophages [96,97], induces TNF-α release from macrophages [98] and scavenges free oxygen radicals [99]. Trehalose dimycolate, a mycolic acid–containing glycolipid, produces granulomatous inflammation and thymic atrophy when injected into mice [100]. The phthiocerol dimycocerosate family of complex lipids in the cell envelope of pathogenic mycobacteria suppress lymphocyte responses [101,102]. This novel pathogenic strategy is reflected by the distinct *in vivo* phenotypes of the mutants in genes required for lipid pthiocerol dimycocerasate (PDIM) synthesis, mma4, and pcaA [93,94]. If these lipids were simply redundant members of an inert waxy coat, then the phenotypes of these mutants would be similar, yet they are distinct.

1.1.5.2 Export and intracellular trafficking of mycobacterial lipids

Considering the numerous biological properties of mycobacterial lipid moieties, one can view the cell envelope as an array of distinct lipid effector molecules, each with its own role in modulating the interaction of *M. tuberculosis* with its host. The finding that PDIM is transported into the media of the *in vitro* grown organisms by a protein encoded by the gene, mmpL7, suggests that the large family of mmpL proteins in the genome of *M. tuberculosis* may also be involved in lipid export [103]. Supporting this concept of exported lipid effectors, recent data demonstrates that mycobacterial lipids can be found within the cytoplasm of host macrophages, free of the intact bacterium. These lipids intercalate into the host membranes, are transported inside the cell and accumulate in a multivesicular lysosomal structure reminiscent of the MIIC compartment that is involved in antigen loading of MHC class II molecules. A similar fate was observed for bacterial proteins released from the surface of intracellular bacilli [104]. The

vesicular structures into which the bacterial lipids and proteins coalesce are shed into the external medium through a constitutive exocytic process [105]. Immunoelectron microscopy of the cell surface of infected cells showed that the plasma membrane is devoid of bacterial lipids except at sites of exocytosis where 100-200 nm vesicles seem to be released from the cell [105] and are subsequently internalized by neighbouring macrophages. These exosomes induce a pro-inflammatory response comparable to that of intact bacilli, and might function *in vivo* by expanding the influence of the bacterium beyond its host cell, facilitating modulation of surrounding cells. Therefore, further characterization of lipid cell envelope antigens and their biological- and immunomodulatory properties may contribute to better understanding of the physiopathology of tuberculosis.

1.1.5.3 Mycolic acid as major mycobacterial cell envelope component

In contrast to the well-studied properties of LAM, PDIM and cordfactor, the biological effects of mycobacterial mycolic acids, which form the most abundant and characteristic lipid component of the mycobacterial cell envelope, are less well characterized. Mycolic acids are high molecular weight (C_{60}-C_{90}), α-alkyl, β-hydroxy fatty acids which are produced by all species of mycobacteria and constitute 40-60% by weight of the complex cell envelope [106]. Mycolic acids are arranged in a bilayer with the cell wall lipids forming a permeability barrier of extremely low fluidity, distinct from the plasma membrane [107]. In addition to α-mycolic acid, which may contain double bonds and cyclopropane rings and which are synthesized by all mycobacterial species, most species also produce oxygenated mycolic acids. Alterations in the proportion and structures of mycolic acids have been shown to produce significant changes in the fluidity of this inner layer resulting in changes in permeability [108,109]. All natural members of the *Mycobacterium tuberculosis* complex (*Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis* and *Mycobacterium microti*) are pathogenic and synthesize the same combination of mycolic acids, i.e. α-mycolic acid, ketomycolic, and methoxymycolic acids different from that observed in non-pathogenic, fast-growing mycobacteria (Fig. 1.8). The attenuated vaccine strain, *M. bovis* BCG Pasteur, only produces α- and keto-mycolic acids.

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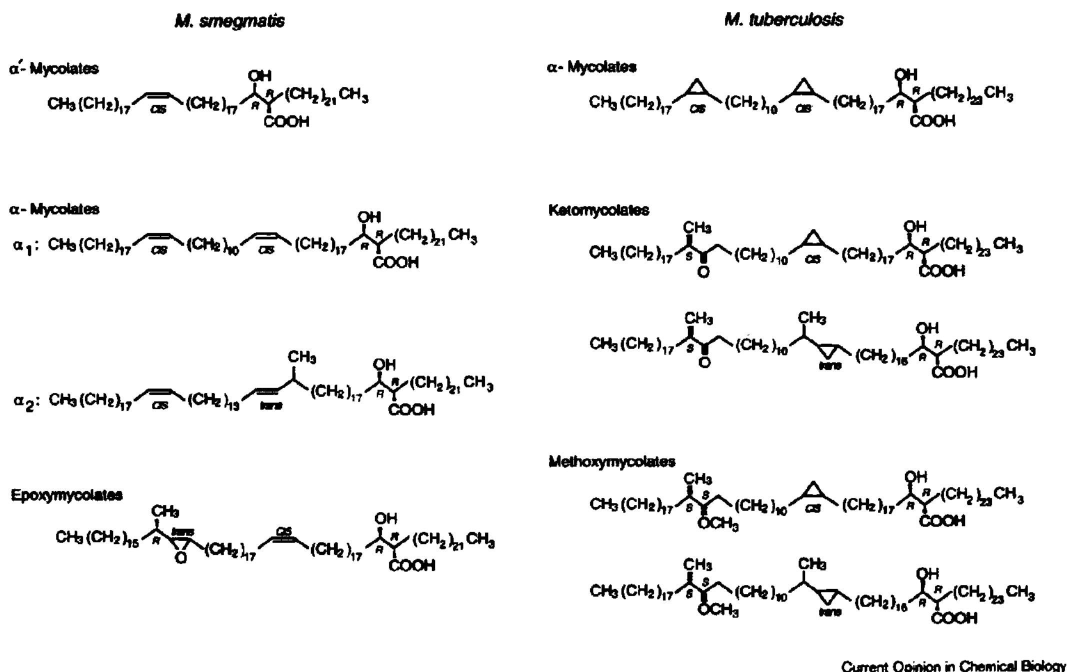

Figure 1.8 Structures of mycobacterial mycolic acids in fast growing non-pathogenic (*M. smegmatis*) and slow growing pathogenic (*M. tuberculosis*) mycobacteria [91].

Evidence for the role of mycolic acids in *M. tuberculosis* replication *in vivo* came with the disruption of the mmaA4 gene [110]. MmaA4 is one member of a large family of S-adenosylmethionine-dependent methyl transferases that modify the mycolic acids of the *M. tuberculosis* cell wall by introducing cyclopropane rings and methyl branches [111]. This mutant did not synthesize oxygenated mycolic acids, therefore lacked two of the three major classes of mycolic acids present in the cell envelope, was impaired for replication in macrophages *in vivo*, but could persist normally *in vitro* at a lower titer growth rate than wild-types [110]. This work established a role for a specific subclass of mycolic acids in mycobacterial replication *in vivo*.

1.2 OBJECTIVES

Previous results by Verschoor *et al.* [112] demonstrated that pre-treatment with mycolic acid prolonged the survival of mice infected with *M. tuberculosis*. This supports the notion that mycobacterial lipids such as mycolic acid can act as immunomodulatory molecules. This study investigated the fundamental interaction of mycolic acids with macrophages, in order to contribute to the understanding of how this protective immunity is established.

The hypothesis was that a distinct morphotype, phenotype and activation status in macrophages may result from interaction with mycolic acids, leading to increased microbicidal capacity of these cells.

CHAPTER 2

EFFECT OF MYCOLIC ACID ON MACROPHAGE FUNCTIONS

2.1 Introduction

Understanding the pathology of mycobacterial diseases such as tuberculosis requires knowledge of the interaction of the mycobacterium with its host cell, the macrophage. Although mycobacteria can infect other cell types, the macrophage is its primary target in all stages of the disease and *M. tuberculosis* employs novel pathogenic strategies for both replication and persistence inside these macrophages. Many of these involve lipid mediators in the mycobacterial cell envelope. Further characterization of cell envelope antigens and their effects on macrophage function and immunomodulatory properties may contribute to understanding the physiopathology of tuberculosis.

Mycobacterial mycolic acids have been shown to have immunomodulatory properties, as demonstrated previously by Verschoor *et al.* [112]. They showed that pre-treatment with MA prolonged the survival of mice infected with *M. tuberculosis*. Whether this protective effect could be due to the direct effects of MA on macrophage functions leading to increased microbicidal activities and inhibition of intracellular survival of *M. tuberculosis* is the subject of this investigation.

In order to study the *in vivo* modulating properties of mycobacterial mycolic acid (MA) on macrophage functions, a suitable carrier had to be found to allow the administration of this hydrophobic molecule into animals. Numerous carriers were investigated for their effectiveness of incorporation of MA and possible toxic effects, work that has been performed mostly by Stoltz [113]. The incorporation into liposomes was found to be the most effective vehicle for MA. Unlike other carriers tested (e.g. polystyrene microbeads), liposomes had the advantage of being completely biodegradable, thereby diminishing the effect of accumulated inert particles and focusing the effect on the mycobacterial biolipid.

The present study focuses on the physical changes as a result of the macrophage interaction with MA and on the modulation of macrophage functions involved in innate and adaptive immunity. The functional significance of these modulated functions were evaluated in terms their ability to inhibit the intracellular survival of *M. tuberculosis*. Since macrophages are able to direct subsequent immune responses, the immuno-modulation functions brought about by MA may have important implications for diseases caused by mycobacteria.

2.2 Materials and Methods

2.2.1 Animals

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

2.2.2 Isolation of Mycolic acid from *M. tuberculosis* (H37Rv)

Mycolic acids were isolated from the cell wall of the virulent Erdman strain of *M. tuberculosis* as described by Goodrum *et al.* [114]. Purified mycolic acids were dissolved at a concentration of 1mg/ml in chloroform before the start of each experiment.

2.2.3 Heat-killed *M.tuberculosis* (H37Ra)

Heat-killed, lyophilized *M. tuberculosis* (H37Ra) (Difco) was suspended in PBS and an amount of $2.5x10^7$/100µl/mouse was administered intraperitoneally.

2.2.4 Preparation of liposomes as carrier for mycolic acid

The original methods of Bangham *et al.*, [115] 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. Briefly, mycolic acid (250 µg, unless otherwise indicated) were mixed with 45 µl phosphatidylcholine (Sigma) at 100mg/ml in chloroform. The organic solvent was evaporated, and the lipids recovered in sterile PBS (1ml). The suspension was processed in an ultrasound bath (Branson 1200) at 60°C for 20 min followed by a final vortex step. Control liposome suspensions were prepared similarly but without the addition of MA. A sample of 100 µl of these liposome suspensions was used for intraperitoneal injection. Mycolic acids were also adsorbed onto 1 µm fluorescent polystyrene microspheres (Molecular Probes, Leiden, The Netherlands). Saponified MA (125 µg) in chloroform was put into a silanated Eppendorf tube and dried with 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 molten MA at 100°C. The hot suspension was then sonicated, cooled down to room temperature and sonicated again. The amount of mycolic acid injected per mouse corresponded to the extract obtained from 2.5 x 10^7 bacilli.

2.2.5 Isolation and culture of macrophages

Mice were sacrificed 48 hours after the intraperitoneal administration of MA-liposomes, liposomes or PBS unless otherwise indicated. The peritoneal macrophages were collected by peritoneal lavage with 10% sucrose water (w/v). After centrifugation at 200 x g for 10 min, the cell pellet was resuspended in complete RPMI-1640 medium containing 10% FCS. In all other experiments, macrophages were enriched by a 2 hour adherence step. Non adherent cells were removed by washings with RPMI medium, unless otherwise indicated.

2.2.6 Uptake studies

In order to determine the uptake of mycolic acid by peritoneal cells, mycolic acids were fluorescently labeled by the derivatization with 5-bromofluorescein. 5-Bromofluorescein (1mg/200 µl DMF) was added to dried, freshly saponified MA (2 mg). This was followed by the addition of 18-crown-6 ether (2.4 mg/500 µl chloroform) and heating to 90°C for one hour. After the vial was cooled down, chloroform (1ml) was added and the sample was incubated at room temperature for 1 hour. Non-covalently bound reagents were removed from the mycolic acid preparation by 5 washes with chloroform saturated hydrochloric acid water (1:1), followed by 5 washes with chloroform-saturated water (1:1). 5-BMF-MA was incorporated into liposomes as described earlier and 100 µl of these suspensions were introduced into mice intraperitoneally. After two hours the peritoneal cells were isolated and analyzed for the internalization of 5-BMF-MA-liposomes by flow cytometry and confocal microscopy.

2.2.7 Laser scanning confocal microscopy

To evaluate phagocytosis of the 5-BMF-MA-liposomes, isolated peritoneal cells were seeded into 8-well cell chambers, (chambered cover glass systems, Lab-Tek, Nunc International, U.S). Enriched macrophage cultures were analyzed by laser scanning confocal microscopy (LSM 410 invert; Zeiss; Germany). For co-localization experiments, the acidotropic dye LysoTracker Red DND-99 (Molecular Probes, Eugene, OR) (1:20 000 in RPMI medium) was used. Macrophages were preloaded with LysoTracker for 2 hours, after which cells were washed, fixed and mounted on glass slides.

2.2.8 Light microscopy

Peritoneal cells were seeded onto #1 thickness, 12 mm diameter glass coverslips in 24-well tissue culture plates at density of $5x10^5$ or $2x10^5$ cells per coverslip. Macrophages were enriched as described previously and fixed with 1% paraformaldehyde. For experiments determining the neutral lipid content, the cells were stained with 0.5% (w/v) Oil Red O in propane-1,2-diol.

2.2.9 Electron microscopy

Peritoneal cells were fixed with 2.5% glutaraldehyde for 20 minutes followed by osmium fixation for another 10 minutes. 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 before analysis on a Philips EM301 transmission electron microscope.

2.2.10 Cholesterol determination

Macrophages were enriched by adherence and harvested by gentle scraping. The cells were washed three times with PBS and suspended by addition of NaOH (0.2 M, 0.6 ml) for 15 min at 4°C. Then 0.5 ml of the cell lysate was made up to 1.0 ml with ice cold PBS containing butylated hydroxytoluene (Sigma; final concentration 20 μM) and EDTA (2mM final concentration), methanol (2.5 ml) added and the total lipids extracted into hexane (10 ml) as previously described [116]. The sterols, which partitioned into the hexane phase, were dried by evaporation and redissolved in HPLC mobile phase. The mobile phase consisted of acetonitrile-isopropanol 30:70 (v/v). Cholesterol and cholesterol esters (linoleate, arachidonate, oleate) were separated on a Vidac C18 reverse phase high performance liquid chromatography HPLC column at room temperature. Cholesterol heptadecanoate was added to the samples before extraction as an internal standard for detection was at 210 nm.

2.2.11 DNA synthesis

Peritoneal cells were directly seeded into 96-well tissue culture plates at a density of $2x10^5$ cells per well. After washing away the non-adherent cells, DNA synthesis was measured by the incorporation of [methyl-^{3}H] thymidine (TdR) (2 μCi/ml) over 24 hours. Uptake was stopped by freezing the plates at –20°C. After thawing, the trichloroacetic acid-precipitable material was recovered using an Inotech cell harvester and the incorporated radioactivity was counted on a

digital autoradiographic counter (Microplate Scintillation Counter, TopCount, Packard, The Netherlands).

The DNA content of enriched macrophages was measured by staining with propidium iodide and flow cytometric analysis. Macrophages were enriched as described previously, harvested by gentle scraping, centrifuged and resuspended in PBS. Aliquots of 50 µl of a stock staining solution comprising 250 µg/ml propidium iodide (PI; Sigma Chemical Co), 5 mg/ml RNAse (EC 3.1.27.5; Sigma), and 1% Triton-X in distilled water were added to $2x10^5$cells/200 µl. After being stained with PI the cells were incubated in the dark at 4°C for a minimum of 3 hours, after which PI fluorescence was measured using a fluorescence-activated cell sorter (FACS Calibur flow cytometer, Becton Dickinson). Cell cycle analysis was performed on a gated, single population by using Modfit LT cell cycle analysis software (Verity Software House, Inc). Acquisition was restricted to 10 000 events for each sample.

2.2.12 FACS analysis

Total peritoneal cells were suspended at $2x10^5$cells/100µl in FACS buffer (PBS supplemented with 0.5% BSA, 0.01 % NaN_3, and 100 mM EDTA), followed by incubation with anti-FcγRII (CD32) Pharmingen (San Diego, USA) for 15 min at 4°C. Immunostaining for cell surface molecules was performed for 1 hour at 4°C using the antibody against F4/80 (anti-F4/80 PE, Serotec, Oxford, U.K.) in combination with either Mac-1 (anti-CD11b-FITC), $I\text{-}A^b$ (anti-$I\text{-}A^b$ - biotin), CD-40 (anti-CD-40-biotin), or B7-1 (anti-CD80-biotin). The cells were then washed and samples receiving biotin antibodies were then incubated with streptavidine-FITC (2 µg/ml). All antibodies were of rat origin and were used in concentrations recommended by the manufacturer (Pharmingen, San Diego, CA and Serotec, Oxford, U.K.). To correct for a specific staining, an appropriate isotype control antibody was used. Cells were fixed with 2 % paraformaldehyde before analysis on FACS.

2.2.13 Proliferation assays

For experiments testing the ability of MA-treated macrophages to generally trigger T cell proliferation, purified $CD4^+$ T cells (4 x 10^4 cells/well) were cultured in complete RPMI medium on adherent peritoneal macrophages (3 x 10^3 cells/well) with increasing doses of anti-CD3 mouse

antibody. $CD4^+$ T cells were purified according to a negative selection method described by StemSep™ cell separation systems [48] from spleens of C57/BL/6 mice. Reagents, columns and magnet for purification were obtained from the same company. For experiments testing the ability of MA-treated macrophages to trigger antigen specific T cell responses, adherent macrophages (3 x 10^3 cells/well) were loaded overnight with different concentrations of the influenza virus surface haemagglutinin. The excess antigen was removed by washing the antigen loaded macrophage cultures with RPMI medium before the addition of T-HA cells (4 x 10^4 cells/well), a haemagglutinin specific Th1 T cell line restricted to the I-A^b haplotype. The haemagglutinin was purified and the T-HA cells created and maintained as described by Desmedt *et al.* [117]. The macrophage/T-cell co-cultures were further incubated for 3 days at 37°C in a 5% CO_2 environment and pulsed with [^{3}H]thymidine (2 µCi/ml) for the last 12 hours. The cells were then harvested using a cell harvester (Microplate Scintillation Counter, TopCount, Packard, The Netherlands) and the incorporated radioactivity was counted using a digital autoradiographic counter (Microplate Scintillation Counter, TopCount, Packard, The Netherlands).

2.2.14 NO production by macrophages

Peritoneal cells were seeded out in 96-well tissue culture plates at 2x 10^5cells/well. The macrophages were enriched by a 2 hour adhesion step followed by washing with RPMI medium. This was followed by the addition of either LPS (1µg/ml) and IFN-γ (100U/ml), or IL-10 (100U/ml) and IL-4 (100U/ml) in order to steer the macrophages in either classical (Th1-inducing) or alternative (Th2-inducing) activation, respectively. These macrophage cultures were cultured for a further 72 hours before the supernatant was removed for NO determination and the cells lysed for subsequent arginase activity measurements (see below). NO synthesis by macrophages was measured indirectly as the nitrite accumulation in the culture medium by reaction with Griess reagent, as described by Ding *et al.* [118]. Briefly, 100 µl supernatant was mixed with with 100 µl Griess reagent consisting of 1% sulphanilamide, 0.1% naphthylethylene-diamine-dihydrochloride and 2.5% phosphoric acid. After incubation for 15 min at room temperature, nitrite concentration was determined by absorbance at 550 nm with reference to $NaNO_2$ as standard.

2.2.15 Determination of arginase activity

Arginase activity was measured in cell lysates from the same macrophage cultures in which NO was determined in the supernatants, with slight modifications, as previously described [119]. Briefly, cells were lysed after 72 hours of culture with 50 μl of 0.1% Triton-X-100. After 30 min on shaker, 17 μl of 25 mM Tris-HCl were added. To this lysate, 6 μl of 10 mM $MnCl_2$ were added and the enzyme activated by heating for 10 min at 56°C. Arginine hydrolysis was conducted by incubating the lysate with 40 μl of 0.5 M L-arginine (pH 9.7) at 37°C for 60 min. The reaction was stopped with 320 μl of H_2SO_4 (96%)/H_3PO_4 (85%)/ H_2O (1/3/7, v/v/v). The urea concentration was measured at 540 nm after addition of 8 μl α-isonitrosopropiophenone (2%, in 100% ethanol) followed by heating at 95°C for 30 min. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of urea per min.

2.2.16 Total and differential cell analysis

Total peritoneal cells were quantitatively isolated 48 hours after the administration of either PBS, liposomes, MA-liposomes or heat-killed *M. tuberculosis*. Total cells were counted in a Neubauer chamber and expressed as total number of cells /ml fluid recovered during the peritoneal washings. Differential analysis was performed by optic microscopy in smears prepared in a cytocentrifuge and stained with May-Grünwald-Giemsa.

2.2.17 Cytokine assays

Total peritoneal cells from MA-treated or non-MA-treated mice were seeded out in 24-well tissue culture plates at $1x\ 10^6$cells/well and macrophages enriched as described above. Cultures were stimulated overnight with LPS (0.1 μg/ml) or heat-killed *M. tuberculosis* ($1x10^6$/ml). Supernatants were collected after 72 hours and cytokines were measured by specific ELISAs for IFN-γ, IL-6, IL-10, GM-CSF (Pharmingen, San Diego, CA), TNF-α (R&D Systems, Minneapolis, MN), and IL-12 (p70 and p40) (Biosource International, Inc, California, USA) using matched antibody pairs according to the manufacturer's instructions.

2.2.18 MPO assay

Myeloperoxidase activity was measured using the technique described by Bradly *et al.* [120]. Peritoneal cells were seeded out in 96-well tissue culture plates at $1x\ 10^5$ cells/well and the

macrophages were enriched as described previously. Overnight culture of the cells with or without LPS (0.1 µg/ml) or *M. tuberculosis* ($1x10^6$/ml) was followed by MPO extraction. Briefly, cells were suspended in 100 µl ice-cold 0.5% hexadecyltrimethylammonium bromide (HTAB) (Sigma) in 50 mM potassium phosphate buffer, pH 6.0 and freeze-thawed three times. Aliquots (50 µl) of the lysate were transferred to a new 96-well plate and 150 µl of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml o-dianisidine dihydrochloride (Sigma) and 0.0005% hydrogen peroxide (Sigma) were added. After 20 min incubation at 25°C, the absorbance was measured at 460 nm using a Biometra spectrophotometer. MPO activity was calculated from a MPO (Sigma) standard curve and expressed in units. One unit of MPO activity was defined as that amount that degrades 1 µmol of hydrogen peroxide per minute at 25°C. Each sample was measured in duplicate.

The cytochemical localization of myeloperoxidase was done with a Myeloperoxidase Detection Kit (Sigma Diagnostics) according to the manufacturer's instructions. The method is based on the ability of myeloperoxidase to oxidize the substrates p-phenylenediamine and catechol in the presence of hydrogen peroxide to form a black insoluble reaction product. Light microscopic analysis of cells treated with either PBS, liposomes or MA-liposomes for 48 hours *in vivo* with or without the *in vitro* re-stimulation with *M.tb* was performed.

2.2.19 Intracellular survival assay

Macrophage monolayers were exposed to mycobacteria to obtain a multiplicity of infection (MOI) of 1:5 (1 macrophage for every 5 bacteria) and the plates incubated at 37°C for 5 hours before the extra-cellular mycobacteria were removed by extensive washing. The plates were maintained at 37°C for a further 96 hours, followed by lysis of the macrophages in distilled water and transfer of the lysate into BACTEC medium. The radioactive CO_2 produced by the bacteria as a result of the consumption of ^{14}C-palmitate in the medium was measured daily in an automated BACTEC 460 machine as described by Middlebrook *et al.* [121].

2.3 Results

2.3.1 MA-liposomes are phagocytosed by peritoneal macrophages

A specific problem when studying the biological properties of lipids, such as mycolic acid (MA), is their poor solubility in water. To overcome this, the mycobacterial MA was incorporated into liposomes as vehicles for subsequent intraperitoneal administration into mice. Since the liposome size influences the way of internalization, the sizes of the liposomes were determined by flow cytometric analysis of their light scattering properties in comparison with microbeads of known sizes. The results indicated a size range of the liposomes from 0.1 – 10 μm (Fig. 2.1 A-B), compatible with phagocytosis as route of internalization.

In order to determine the type of phagocyte responsible for the *in vivo* phagocytosis of the intraperitoneally injected liposomes, MA was fluorescently labeled by derivatization to 5-bromofluorescein (5-BMF) and incorporated into liposomes before intra-peritoneal administration. Peritoneal cells were isolated two hours after administration of 5-BMF-MA-liposomes and stained for the macrophage-specific marker F4/80. Flow cytometric analysis of the 5-BMF-MA-liposomes-treated cells indicated that only the F4/80-positive population internalized the fluorescent liposomes (Fig. 2.1 C-D), identifying macrophages as the responsible cell type for internalization of the MA-liposomes. Internalization of MA-liposomes was confirmed with confocal microscopic analysis of the 5-BMF-MA treated cells, as shown in the cross-sectional images of the adherent macrophage monolayers (Fig. 2.1 E).

To determine the degree of maturation of the phagosomes containing the labeled MA, their co-localization with LysoTracker Red DND-99, a weak base conjugated to a red fluorophore, was investigated. Due to its acidotropic nature, LysoTracker Red serves as a marker of acidified, mature phagosomes. Confocal microscope images of peritoneal cells loaded *in vivo* with 5-BMF-MA-liposomes, followed by *ex vivo* loading with Lyso Tracker, demonstrated that the majority of the internalized 5-BMF-MA co-localized with Lyso Tracker Red-containing vesicles (Fig. 2.1 F), thus indicating the maturation of MA containing phagosomes into late stage phagocytic compartments.

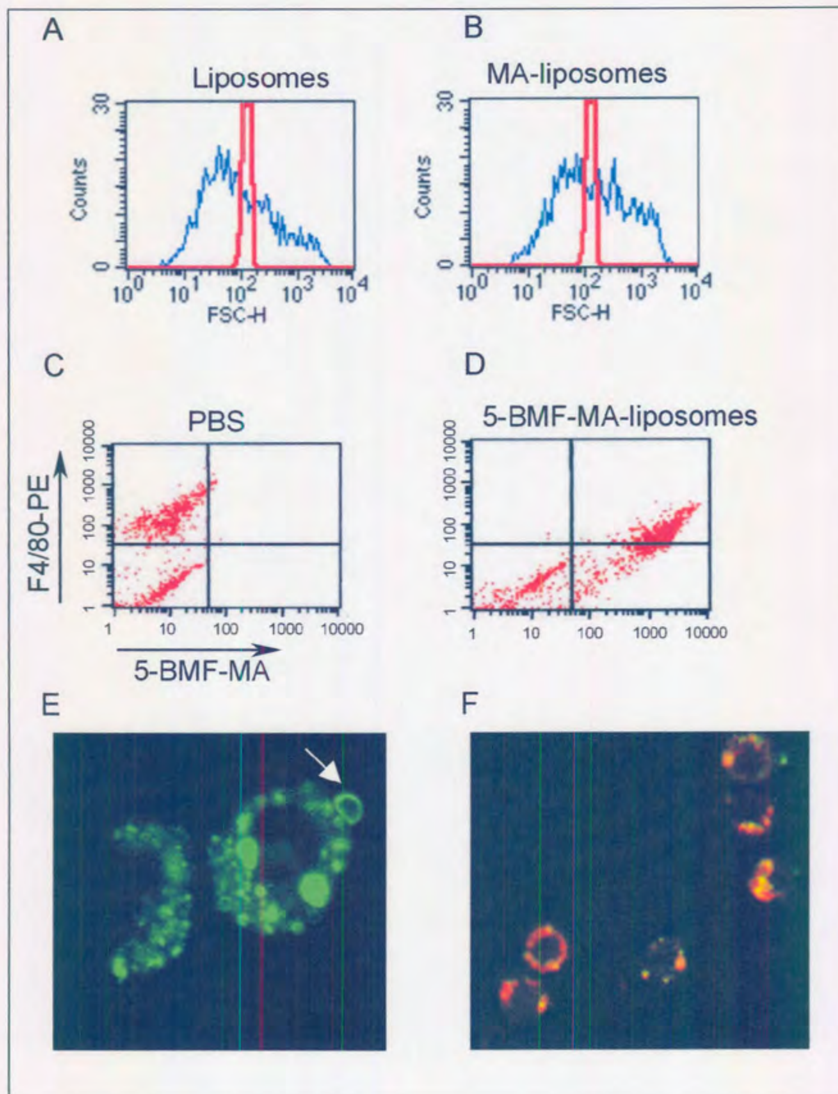

Figure 2.1 MA-liposomes are internalized by murine peritoneal macrophages. Flow cytometric analysis of the sizes of liposomes (A) and MA-liposomes (B) as compared to microbeads (1 μm in size). Red line represents the microbeads and the blue line the liposomes or MA-liposomes. Flow cytometric analysis of peritoneal cells labeled with the anti-macrophage Ab, F4/80-PE, two hours after the i.p. administration of PBS (C), or 5-BMF-MA-liposomes (D). Cells originating from mice injected with PBS, and stained with a control Ab were contained in the LL quadrant. Confocal microscope images of adherent peritoneal cells loaded *in vivo* for two hours with 5-BMF-MA-liposomes (E), and followed by the loading in *vitro* with Lyso Tracker Red DND-99 (F). The cross-sectional images indicate that the majority of the 5-BMF-liposomes are internalized by the adherent peritoneal cells, while only few are located on the surface of the cell (indicated by white arrow) (E). In addition, co-localization of 5-BMF-lipsomes (green) and Lyso Tracker Red DND-99 (red) are indicated by the yellow areas (F).

2.3.2 Phagocytosis of MA-liposomes induces the formation of foam cells

To verify how phagocytosis of MA-liposomes affected the macrophages, the morphology of the isolated macrophages was determined. Interestingly, macrophages isolated from the peritoneum 48 h after administration of MA-liposomes (Fig. 2.2 A+B) or MA-coated microbeads (Fig. 2.2 C), showed an increase in size and multiple vacuoles, features indicative of foam-like morphology.

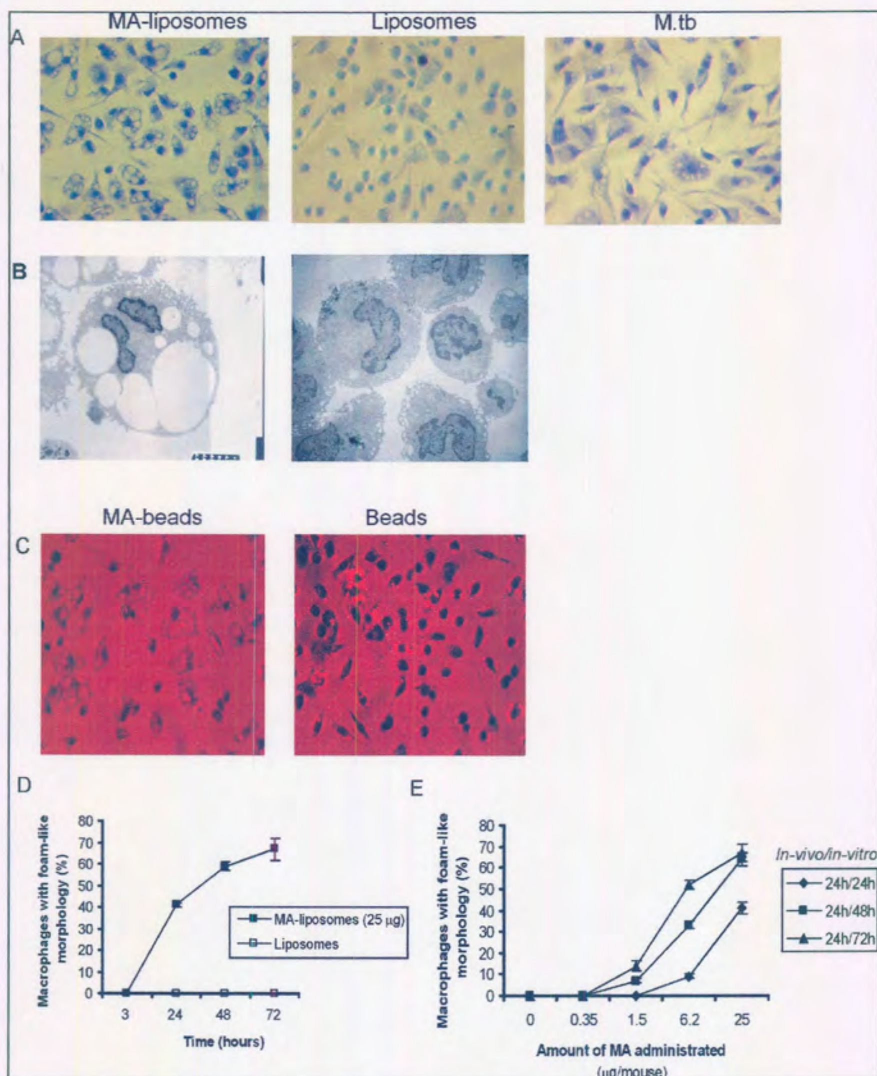

Figure 2.2 Macrophages undergo altered morphology following internalization of MA-liposomes. Light microscope- (A) or electron microscope images (B) of peritoneal macrophages pre-loaded *in vivo* for

48 h with MA-liposomes, liposomes or heat-killed *M. tuberculosis*. Fluorescence microscopic analysis of macrophages loaded *in vivo* for 48 hours with beads-MA or beads only (C). The formation of large vacuoles typical of foam cells can be visualized in all cases where MA was administered but not in cases where the vehicle only is administered. The time dependent induction of foamy macrophages following intra-peritoneal administration of either liposomes or MA-liposomes (D). Percentage of foamy macrophages, obtained 24 hours after the administration of increasing doses of MA, at different time points after introducing the cells in culture (E).

Both features were absent in macrophages treated with the empty liposome (Fig 2.2 A+B), uncoated beads (Fig. 2.2 C) or PBS alone. Some foam cells derived from animals injected with MA-beads did not contain any beads (Fig. 2.2 C). A similar foam-like morphology was induced by administration of heat-killed *M. tuberculosis* (Fig. 2.2 A) although vacuole formation in the cells was less pronounced. The induction of this specific type of activation in macrophages by MA may therefore be representative for the whole bacterium.

Analysis of the time dependence of this response to MA revealed that cells with foam-like morphology appeared between 3 h and 24 h after injection of the MA-liposomes (Fig. 2.2 D), further increasing up to 70% after 72 hours. *Ex vivo* cultures of peritoneal macrophages, collected 24 hours after the intraperitoneal administration of MA-liposomes, showed a further increase of cells exhibiting the characteristic foam-like morphology (Fig. 2.2 E). Doses of MA as low as 1.5 to 6.2 μg were effective in inducing this peculiar type of macrophage activation, although at a slower rate and with a longer lag period (Fig. 2.2 E). Foam cell formation is therefore a gradual process that, once triggered by MA *in vivo*, can also proceed *ex vivo*. This suggests that MA-induced foam cell formation may occur through soluble factors to explain the presence of foam cells without beads in the MA-beads treated mice.

2.3.3 Foam cells have increased cholesterol content and undergo cell division

Accumulation of large amounts of lipids, especially cholesterol, in the cell characteristically accompanies foam cell formation [122].

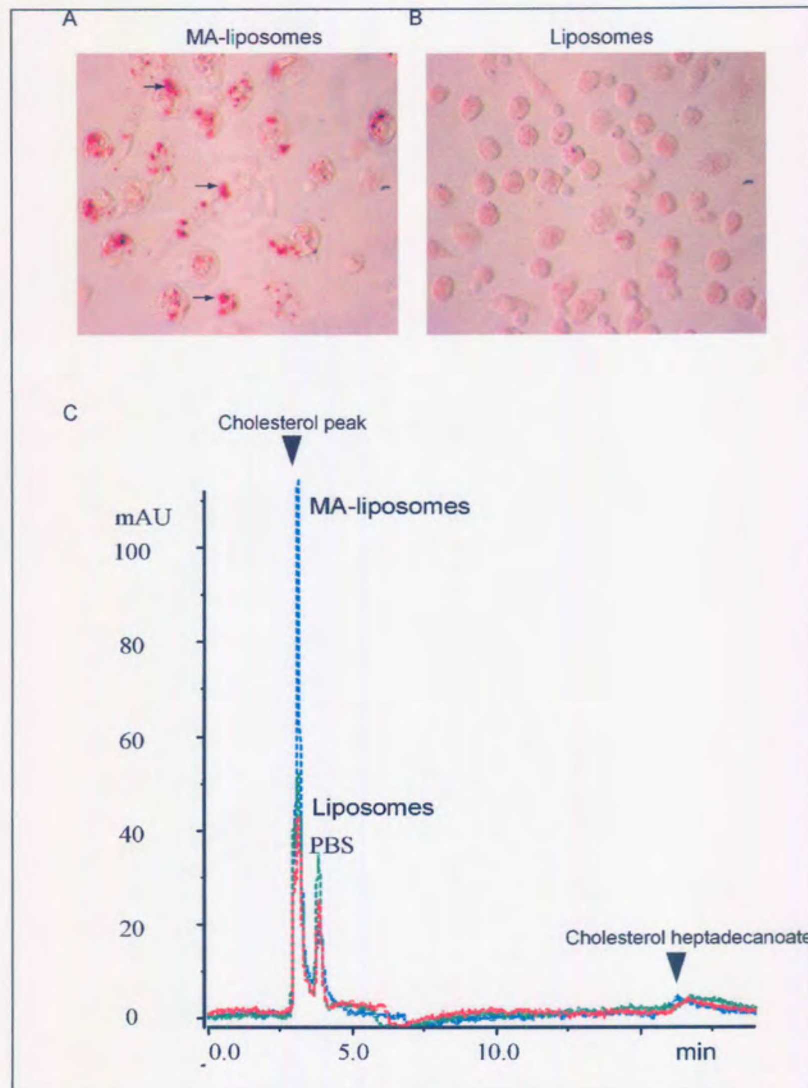

Figure 2.3 Macrophages show increased lipid content following uptake of MA-liposomes. Light microscopic images of peritoneal macrophages treated in-vivo for 48 hours with either MA-liposomes (A) or liposomes (B) followed by neutral lipid staining with Oil Red O. Dark spots visible on macrophages pre-loaded *in vivo* with MA-liposomes are indicative of accumulated neutral lipids in (Black arrows). HPLC elution profile for cholesterol obtained from lipid extraction of peritoneal macrophages isolated from mice, pre-treated *in vivo* for 48 hours with either PBS (red line), liposomes (green line) or MA liposomes (blue line) (C).

To determine if the MA-induced foam-like morphology is accompanied by increased lipid accumulation, the intracellular lipid content of MA-treated cells was evaluated. Staining with the neutral lipid stain, Oil Red O, produced a cytoplasmic staining pattern in macrophages pre-treated

in vivo with MA-liposomes (Fig. 2.3 A), but not in macrophages treated with liposomes (Fig. 2.3 B) or PBS only (not shown). To determine whether the increase in intracellular lipid detected by staining with Oil Red O is due to accumulation of cholesterol, MA-treated cells were extracted with non-polar organic chemicals, and the lipids separated on HPLC. Results showed an increase in cholesterol peak height for cells isolated from MA-liposomes treated mice compared to cells from liposomes or PBS treated mice (Fig. 2.3 C).

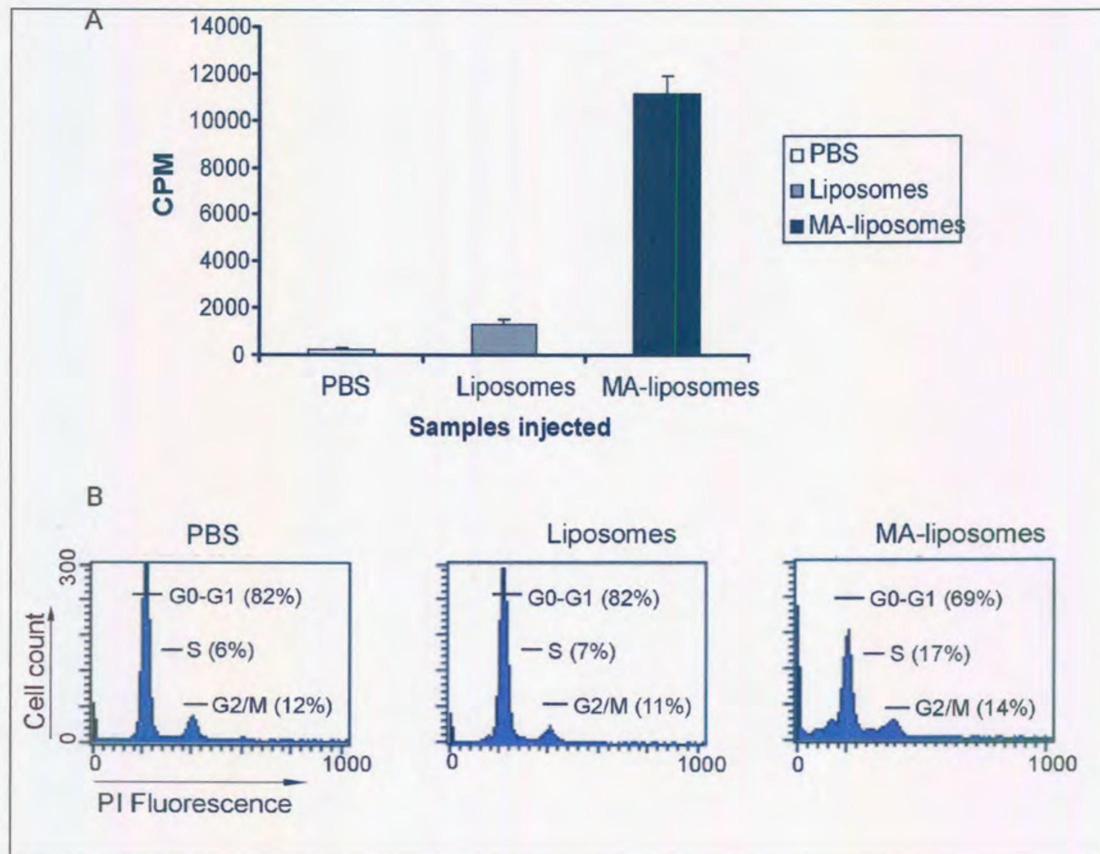

Figure 2.4 Increase in DNA synthesis as a result macrophages-MA interaction. Peritoneal cells were collected 48 hours after the intra-peritoneal administration of either PBS, liposomes, or MA-liposomes and cell proliferation estimated by [^{3}H]TdR incorporation over 24 hours. Flow cytometric analysis of the DNA content of PI stained-macrophages pre-loaded in-vivo for 48 hours with either PBS (B), liposomes (C) or MA-liposomes (D).

Macrophage monolayers from peritoneal cells, isolated 48 h after administration of MA-liposomes, showed pronounced [^{3}H]TdR incorporation over a 24 h culture period accompanied by

an increased proportion of cells in the S phase (Fig. 2.4). Both features were absent in cultures of untreated cells or cells treated with vehicle only. This result demonstrates entry of the MA-induced macrophage cells into the mitotic cell cycle.

2.3.4 Immune properties of MA-induced foamy macrophages

To determine the effect of MA on macrophage functions in both the innate and adaptive immune responses, cell surface expression of markers involved in antigen presentation, their functional antigen presenting capacity to T cells, along with production levels of (anti-) inflammatory cytokines and of the bactericidal mediator, myeloperoxidase (MPO) were analyzed.

2.3.4.1 Foamy macrophages express phenotypic markers involved in antigen presentation

Peritoneal macrophages from MA-treated mice showed surface expression of the Mac-1 (CD11b) and F4/80 differentiation markers, characteristic of mature macrophages [123,124]. Surface expression of MHC class II molecules and of the T cell co-stimulatory molecules CD40 and B7-1 on F4/80-positive cells were verified by two-colour FACS. As shown in table 2, MA induced a slight upregulation of all three markers analyzed, indicating that the macrophage-derived foam cells are likely to function as efficient APCs in adaptive immune responses.

Table 2 Phenotypic characterization of macrophage derived foam cells*

Samples injected	*Cell surface marker*			
	CD11b	*CD40*	*B7-1*	*I-A^b*
PBS	209.3±23.4	44.5±15.8	23.3±5.8	12.2±1.02
Liposomes	278.5±32.4	39.7±11.7	17.4±4.6	13.0±1.3
MA-liposomes	260.6±56.4	75.5±14.2	41.7±7.4	30.0±9.7

*Peritoneal macrophages were harvested 48 hours after the intra-peritoneal administration of either PBS, liposomes or MA-liposomes and the cells were stained as described in *materials and methods*. Values represent mean peak fluorescence ± SD after subtraction of the background obtained with isotype control Abs or streptavidine-FITC only. Results are from one representative experiment repeated at least three times.

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2.3.4.2 MA-induced foamy macrophages provide T cell costimulation and induce antigen specific T cell proliferation

The antigen presenting capacity of MA-induced foamy macrophages was investigated to test the functional implications of the APC^+ phenotype. Firstly, in order to determine the MA-induced foamy macrophages' ability to provide T cells with costimulatory signals, purified $CD4^+$ T cells were incubated with anti-CD3 mAb as the first signal on adherent MA-treated peritoneal macrophage cultures and the proliferative responses of the $CD4^+$ T cells assessed. In addition, the ability of the MA-induced foamy macrophages to trigger antigen specific proliferative T-cell responses was determinied. MA-induced foamy macrophages were pre-loaded with the human influenza virus haemagglutinin (HA) and subsequently co-cultured with HA specific T cells (T-HA cells). Proliferative responses of both $CD4^+$ cells as well as T-HA cells could be triggered by the MA-induced foamy macrophages (Fig. 2.5). These proliferative responses were similar to that triggered by control macrophages, indicating that MA-induced foamy macrophages fully retain their capacity to activate T cells towards an immune response.

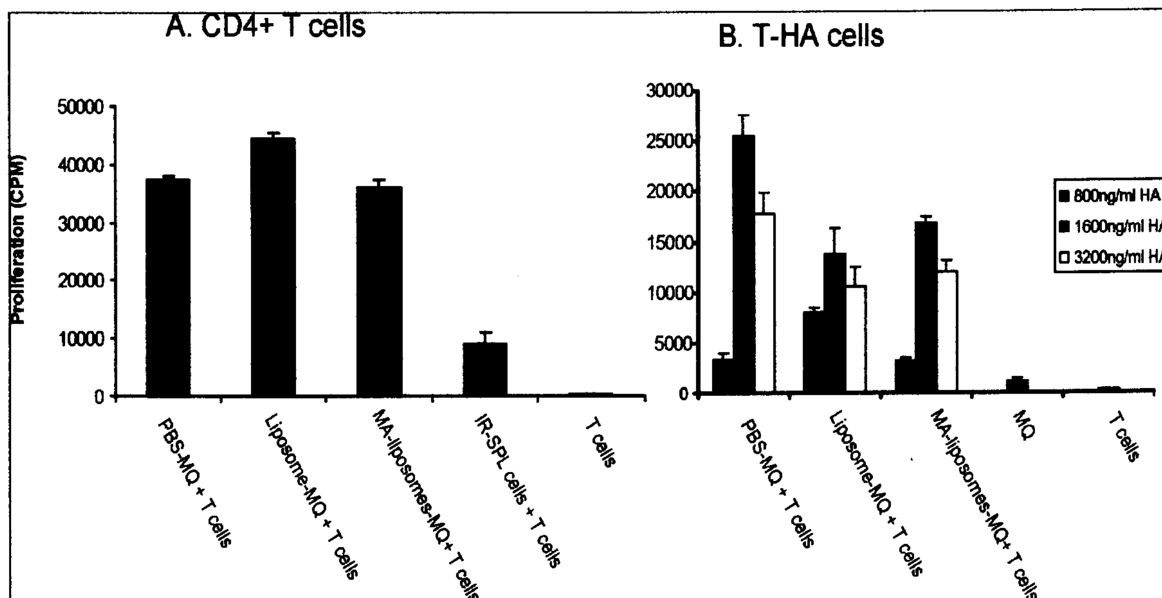

Figure 2.5 Antigen presenting capacity of foamy macrophages as induced by mycolic acid. T cell proliferation by MA-induced foamy macrophage co-stimulation (A). Purified CD4+ T cells from spleens of C57/BL/6 mice were cultured in complete medium with anti-CD3 mAb (1μg/ml) alone or with macrophages (3 x 10^3/well) from PBS-, liposomes- or MA-liposomes-treated mice. Proliferation of $CD4^+$ T cells in the presence of anti-CD3 mAb (1μg/ml) and co-cultured with irradiated spleen cells (IR SPL cells)

as APCs (3 x 10^3/well), was used as positive control. Similar results were obtained with lower concentrations of anti-CD3 mAb. Proliferation of HA-specific T cells (T-HA cells) induced by HA loaded foamy macrophages (B). Macrophages (indicated as MQ) from PBS-, liposomes-, or MA-liposomes-treated mice were loaded overnight with increasing doses of HA before removal of excess antigen and co-culturing with T-HA cells. In all experiments macrophages at a concentration of 3 x 10^3/well from PBS-, liposome- or MA-liposomes-treated mice alone or loaded with antigen and pulsed for 12 hours produced counts in the range of 1000-2000 CPM.

2.3.4.3 *The distinct activation status induced by MA counteracts induction of arginase activity in Th2-inducing macrophages*

In order to characterize the activation status of MA-induced foamy macrophages as either Th1- or Th2-inducing, their ability to metabolize arginine by two alternative pathways involving the enzymes *inducible NO synthase* (iNOS) or *arginase* was investigated. These two enzymes are competitively regulated by Th1 and Th2 helper cells via their secreted cytokines: Th1 cells induce iNOS, and Th2 cells induce arginase activity in the macrophages. Results demonstrated that MA-treatment as such did not result in significantly increased NO secretion or arginase activity (Fig. 2.6).

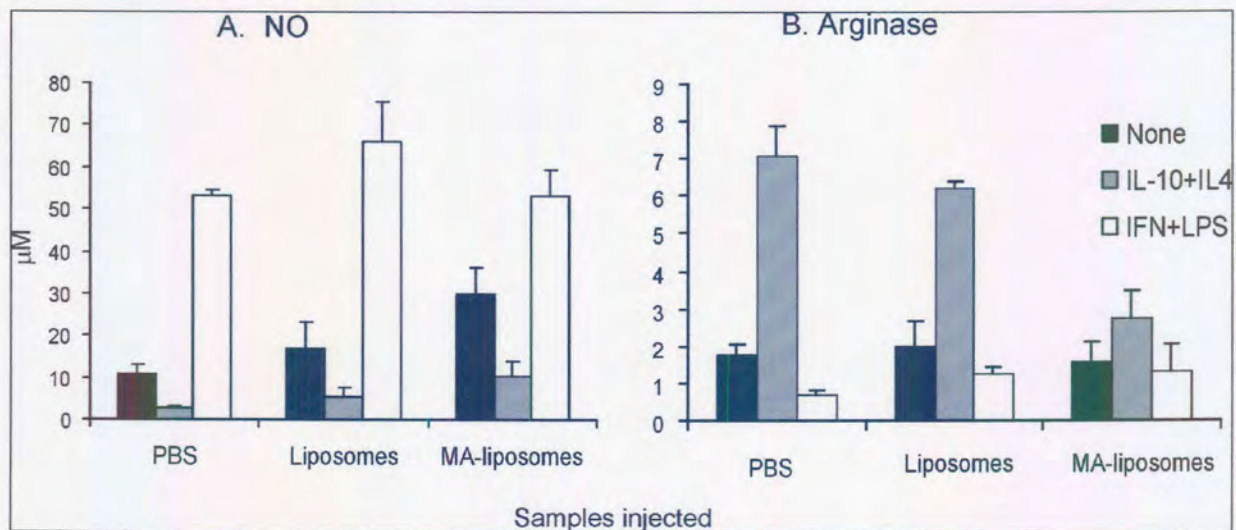

Figure 2.6 NO and arginase production by MA-induced foamy macrophages. NO and arginase production from macrophages isolated from PBS-, Liposome- or MA-treated mice with or without steering them into Th1-inducing macrophages by the addition of LPS and IFN-γ, or Th2–inducing macrophages by the addition of IL-10 and IL-4. NO production was measured as the amount of nitrite accumulated in

medium while arginase activity was measured in the cell lysates as the amount of urea produced. Results represents the mean +/- SD of 3 mice tested individually from one representative experiment repeated at least two times.

Cytokine treatment confirmed that the peritoneal macrophages can be steered into either a classical or alternatively activated status based on the increased production of NO and arginase, respectively. Prior *in vivo* treatment with MA, however, resulted in reduced arginase activity in macrophages steered with IL-4 and IL-10, while NO levels were not significantly affected in macrophages steered with IFN-γ and LPS (Fig. 2.6). This suggests that MA-induced foamy macrophages may strongly counteract Th2 biased immune responses.

2.3.4.4 Effect of MA versus heat-killed M. tuberculosis on acute cell influx into the peritoneal cavity

To determine the types of cells recruited by macrophages following the phagocytosis of MA, in comparison to whole heat-killed *M. tuberculosis,* cytospin preparations of quantitatively harvested peritoneal cells, were assessed after staining with May-Grünwald/Giemsa stain. Results obtained indicated increased numbers of total cellular infiltration following administration of either MA, or heat-killed *M. tuberculosis.* Both treatments resulted in increased neutrophil intrusion into the peritoneum, with an additional increase in the number of mononuclear cell with the administration of heat-killed *M. tuberculosis.* The results demonstrate therefore that MA induces an acute phase innate immune response similar to that induced by heat-killed *M. tuberculosis.*

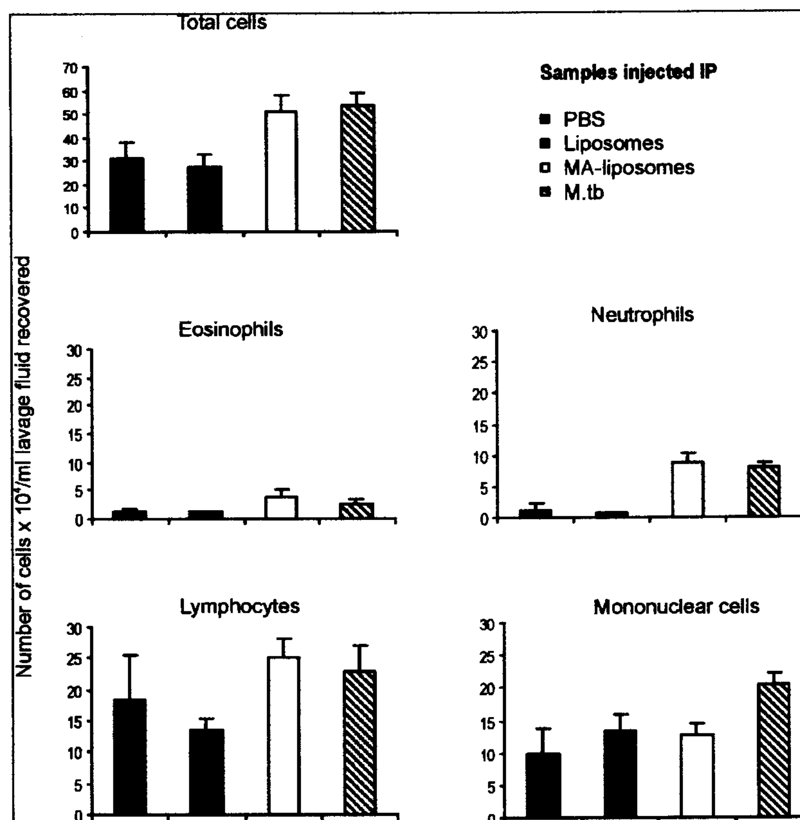

Figure 2.7 Recruitment of neutrophils into the peritoneal cavity following the administration of MA-liposomes. The cellular composition infiltrating the peritoneal cavity was determined in mice pre-treated *in vivo* for 48 hours with PBS, liposomes, MA-liposomes or heat-killed *M. tuberculosis* (*M. tb*). Cytospin preparation of peritoneal cells isolated from each individual mouse was prepared and the slides were fixed and stained with May-Grünwald/Giemsa before microscopic analysis and determination of the differential cells counts. Results represent the mean +/- SD of 5 mice tested individually from one representative experiment repeated at least two times.

2.3.4.5 MA primes macrophages for the production of the bactericidal mediator, MPO

To determine the inflammatory and bactericidal capabilities of MA-induced foam cells myeloperoxidase (MPO) activity was quantified with a spectrophotometric assay on adherent macrophages. MA-treatment did not result in a significant increase of MPO levels. However, exposure to a second inflammatory stimulus, either heat-killed *M. tuberculosis* or LPS, resulted in a 3-fold increase in the levels of MPO activity (Fig. 2.8 A). This was confirmed by cytochemical staining (Fig. 2.8 B), thus suggesting foamy macrophages as the source of MPO activity.

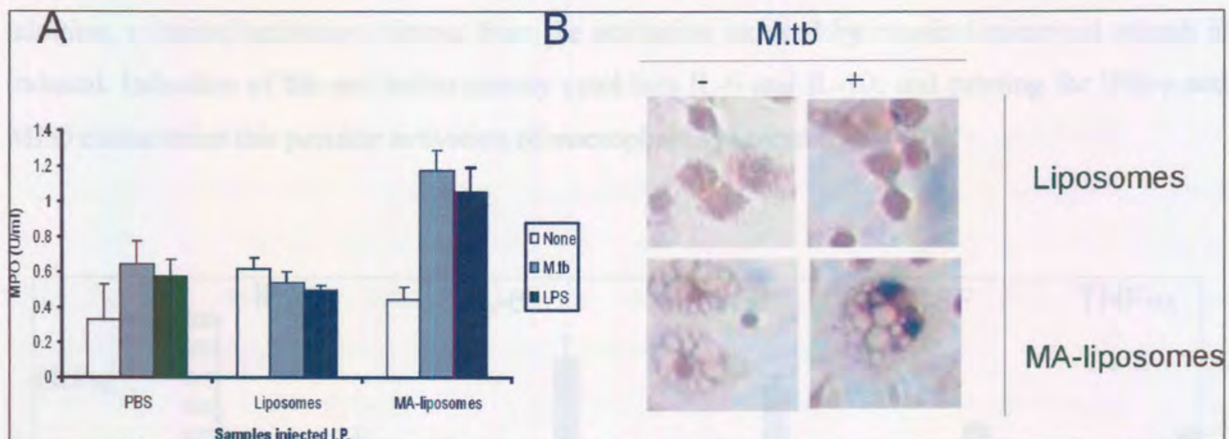

Figure 2.8 MA primes macrophages for the bactericidal mediator, MPO. Spectrophotometric assay for MPO activity in macrophage monolayer pre-treated *in vivo* with either PBS, liposomes, MA-liposomes or M.tb. The cells were re-stimulated *in vitro* with either none, LPS or M.tb (A). Cytochemical localization of MPO activity in macrophages pre-treated with liposomes, or MA-liposomes with or without the *in vitro* restimulation with M.tb (B). Results represent the mean +/- SD of five mice tested individually from one representative experiment repeated at least two times.

2.3.4.6 Cytokine profile of MA-induced foamy macrophages

Analysis of the supernatants from macrophage cultures revealed no significantly elevated level of the inflammatory cytokine IFN-γ as a result of MA-treatment (Fig. 2.9). IL-6 as well as the anti-inflammatory cytokine, IL-10, showed increased levels in cultures of MA-liposome-treated macrophages (Fig. 2.9). Exposure to an additional inflammatory stimulus resulted in a significantly increased production of IFN-γ, exclusively in MA-treated macrophages. IL-6 and IL-10 expression were upregulated to a similar degree in untreated- as well as MA-treated macrophage cultures upon exposure to the additional inflammatory stimulus. In contrast, MA-treatment did not significantly affect expression of IL-12 (data not shown), GM-CSF or TNF-α (Fig. 2.9) as such or in combination with LPS or heat-killed *M. tuberculosis* .

These results provide evidence that MA-induced foamy macrophages retain their capacity to respond to microbial danger signals by production of both pro- and anti- inflammatory cytokines, and their capacity to initiate adaptive immune responses by MHC class II antigen presentation. In

addition, a limited activation distinct from the activation induced by classical microbial stimuli is induced. Induction of the anti-inflammatory cytokines IL-6 and IL-10, and priming for IFN-γ and MPO characterize this peculiar activation of macrophages induced by MA.

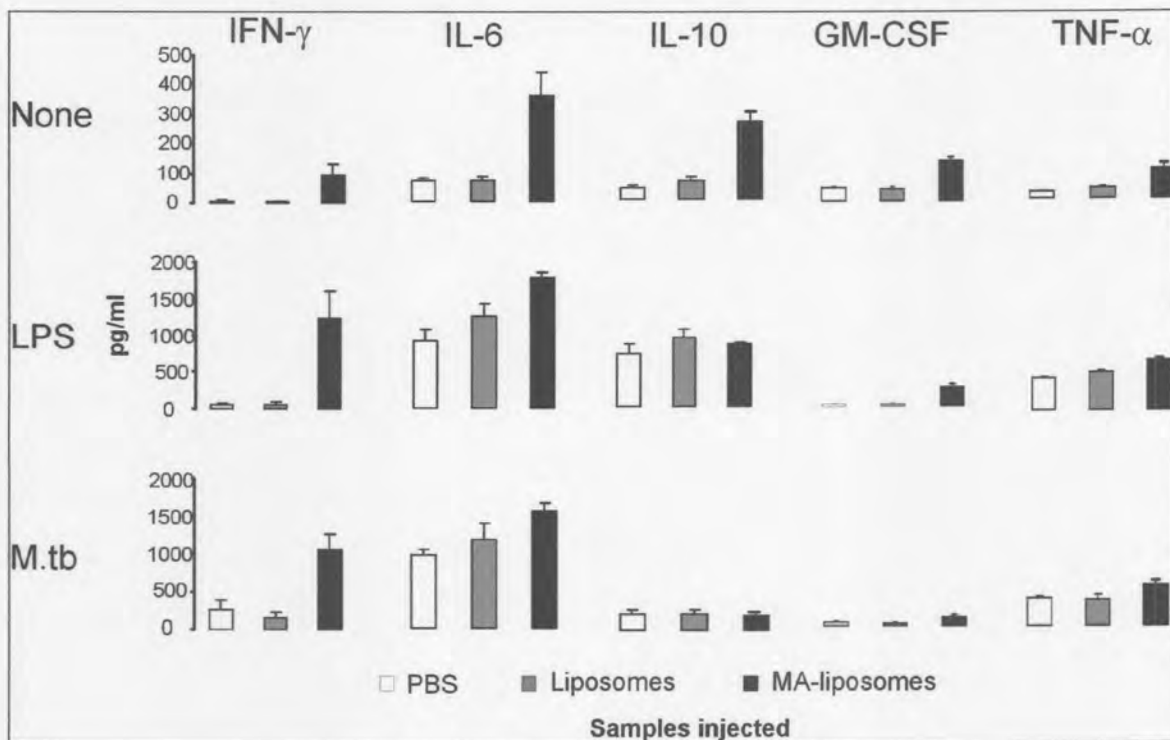

Figure 2.9 Effects of MA on macrophage-mediated immune and inflammatory functions. Cytokine production of murine peritoneal macrophages following *in vivo* treatment with either PBS, liposomes or MA-liposomes. Cells were re-stimulated *in vitro* with or without LPS or *M. tuberculosis*, cultured for 72 hours and the cell culture supernatants collected were analyzed with ELISA. Results represent the mean +/- SD from 5 mice tested individually from one representative experiment repeated at least three times.

2.3.5 MA-induced foamy macrophages inhibit the intracellular growth of *M. tuberculosis*

To test the functional implications of this distinct activation status and priming effect induced by MA, the foamy macrophages' ability to inhibit the intracellular growth of *M. tuberculosis* was evaluated.

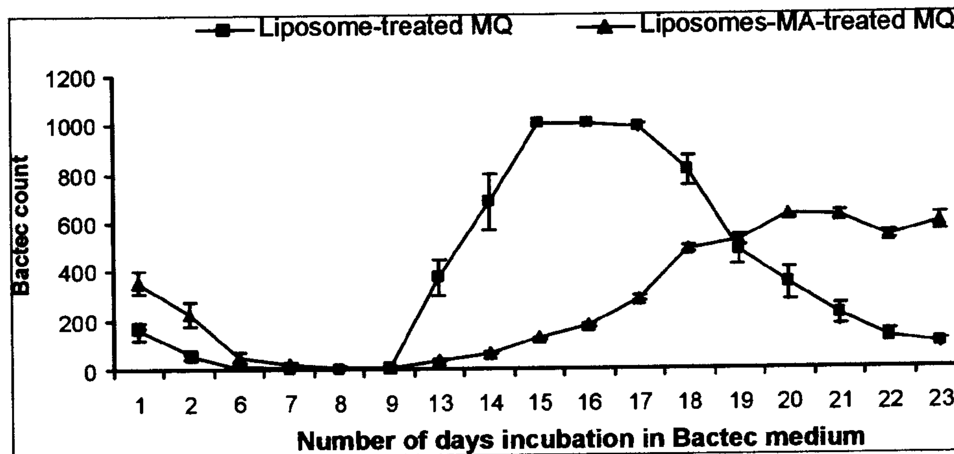

Figure 2.10 Foamy macrophages as induced by MA-liposomes inhibits intracellular growth of *M. tuberculosis*. Differences in growth potential of *M. tuberculosis* surviving intracellularly in macrophages loaded *in vivo* for 3 hours with liposomes (■) or MA-liposomes (▲). Each bar represents the mean of 3 samples +/- SD.

Preliminary results obtained indicated an approximately 85% reduction, after 14 days incubation, in the intracellular growth of mycobacteria from macrophages isolated from MA-liposome treated, compared to liposome-treated mice. Although the initial uptake of mycobacteria by these *ex vivo* macrophage cultures were not compared in this experiment, they showed similar phagocytic capabilities of fluorescently labeled mycobacteria (data not shown). This result demonstrates therefore the functional capability of MA to enhance microbicidal activities in murine peritoneal macrophages, thereby counteracting the intracellular survival of *M. tuberculosis*.

2.4 Discussion

The most remarkable consequence of the interaction and subsequent engulfment of MA with murine peritoneal macrophages was the formation of macrophage-derived foam cells. These cells exhibited the characteristic morphology of foam cells, namely increased cell size and multiple vacuole formation, entry into mitosis, as well as the characteristic intracellular accumulation of neutral lipids, including cholesterol.

Activated macrophages are a feature common to many inflammatory conditions. However, the presence of foam cell macrophages is a more restricted phenomenon. Although observed in some other diseases [125-130], foam cells are most commonly found in atherosclerotic lesions. This study demonstrates the formation of foam cells in murine peritoneal macrophages as a result of the interaction with the *M. tuberculosis* cell wall lipid, mycolic acid. As foamy macrophages could also be induced with heat-killed *M. tuberculosis*, albeit to a lesser extent, the question arises as to what role this peculiar cell type may have in mycobacterial diseases.

Recently the presence of foamy macrophages in murine tuberculosis granulomas has been documented in histopathological studies [131-133]. Parallels with the histopathological spectrum of leprosy can be drawn, murine tuberculous granulomas being closer to human 'lepromatous' than 'tuberculoid' lesions. Lepromatous granulomas are rich in macrophages with a high intracellular bacillary concentration and numerous foamy macrophages are seen in regressing lesions. In contrast, tuberculoid lesions are characterized by the presence of epithelioid cells and Langerhans-type giant cells.

The formation of foamy macrophages in primary granulomas is believed to be due to scavenging of necrotic debris and bacilli from the centre of granulomas, where cell destruction takes place [131]. *M. tuberculosis*-derived mycolic acids may contribute to this, based on the observation reported here that the biolipid induces the formation of foamy macrophages. Probably, a more detailed description of the mechanism involved in the formation of foam cells comes from the characterization of foam cells present in atherosclerotic lesions. The large amount of cholesterol accumulated in these foamy macrophages is believed to originate from extracellular cholesterol-rich lipoproteins such as LDL, which are endocytosed and degraded by

acidic lysosomal hydrolases (Fig. 2.11) [134,135]. Although cellular cholesterol levels tightly regulate expression of the normal LDL receptor, other receptors are expressed on macrophages, which can mediate uptake of LDL, modified either chemically or physically, and which are not subject to sterol regulation. These receptors include the collagenous AcLDL, scavenger receptors (SR-A1/A-2 and MARCO) and CD36 [136]. Some of the modifications to LDL, such as oxidation and association with extracellular matrix components, may occur *in vivo*. The lysosomal lipoprotein-derived unesterified cholesterol is then released into the cytosol and transported to cell membranes thereby explaining the subsequent lipid accumulation in the macrophages. Other modifications to LDL, such as acetylation, have been reported, but these are only relevant as *in vitro* experimental tools.

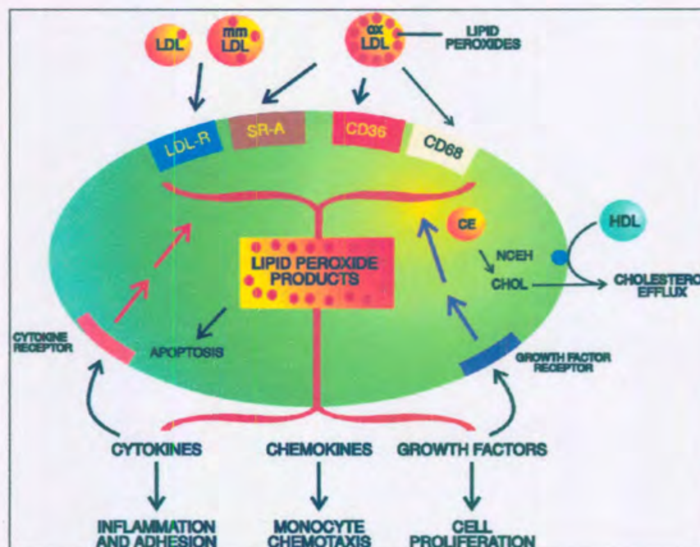

Figure 2.11 A diagrammatic representation of cholesterol homeostasis in macrophages. Factors regulating macrophage foam cell formation include native and modified lipoprotein ligands, expression and function of various scavenger receptor family members, intracellular accumulation of cholesterol esters, and cholesterol efflux. In addition, scavenger receptor-mediated internalization of lipid products can activate the cell to produce inflammatory cytokines, chemokines and growth factors, further enhancing foam cell formation [137].

Under normal conditions, the cell regulate the amount of free cholesterol available, since its concentration in membranes affects properties such as fluidity and permeability. In the presence of an appropriate extracellular acceptor, excess cholesterol can be exported from the cell via the

plasma membrane, a process known as reverse cholesterol transport. In conditions where export is restricted, for example when acceptor availability is limiting, excess cellular cholesterol is esterified by acyl coenzyme A: cholesterol acyltransferase (ACAT). The cholsteryl ester products are deposited in the cytosol as pools of insoluble lipid droplets. These esters are substrates for cytosolic neutral cholesteryl ester hydrolase(s) (nCEH) (Fig. 2.11). This results in excess cholesterol undergoing a futile cycle of esterification and ester hydrolysis. Because only unesterified cholesterol can be transported from the cells, the activity of nCEH is essential for clearance of cellular cholesteryl esters.

Therefore the nett accumulation of cholesterol is characteristic of the atherosclerotic foam cells, whose appearance clearly indicates a failure of those mechanisms that normally regulate cellular cholesterol content. In addition to the possibility of unregulated lipoprotein uptake, discussed above, cholesteryl ester export may also become compromised. The rate limiting step in mobilizing cholesterol export *in vivo* is not presently known. The most likely candidates are either the availability of extracellular acceptors for excess cholesterol or cellular factors. These may include transport from intracellular compartments to the plasma membrane, activity of neutral cholesteryl ester hydrolase(s) and ease of cholesterol removal from the plasma membrane. Whether foam cells as induced by MA and those observed in tuberculous granulomas also result from the failure of the these mechanisms of cellular cholesterol regulation remains to be investigated.

Remarkably little is known of the lipid content of foam cells and its influence on their function. To determine the effects of MA on macrophage-mediated immune and inflammatory functions, expression levels of surface ligands required for TCR activation of T cells along with MHC class II, APC activity and secretion levels of pro- and anti-inflammatory cytokines were analyzed. The unaltered APC capacity and increased expression of MHC class II and costimulatory ligands and receptors clearly indicate that MA-induced foamy macrophages fully retained their capacity to activate cellular immune reactivity. These results contradict beliefs that foam cells are biologically inactive cells.

The results demonstrate that the MA-induced foamy cells are not only biologically active, but

are able to respond to cytokine signals. Foamy macrophages produced NO when steered into a classical activation status with IFN-γ and LPS and similarly, produced arginase when steered into an alternative activation status with IL-10 and IL-4. However, foamy macrophages, steered into an alternatively activation status, showed significantly lower arginase activity than control macrophages, while the levels of NO remained unaltered after steering these macrophages into a classical activation status. MA as such did not induce significant amounts of either NO or arginase, indicating that MA, other than bacteria or bacterial compounds, did not induce a clear classical or alternative activation status in macrophages.

Previous reports have suggested a role of macrophage foam cells in progression of atherosclerosis since they are a source of inflammatory mediators such as cytokines. Demonstration of both mRNA and protein expression of a number of cytokines co-localized with macrophages in human atherosclerotic plaques support their potential involvement in disease progression. These include platelet-derived growth factor-β (PDGF-β) transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, IL-8, and macrophage colonystimulating factor [138-143]. In most cases of these studies cytokine mRNA or protein were not uniformly detected among the macrophages and were demonstrated in both foam and non-foam cells. Elevated levels of several of these cytokines have also been identified in macrophages (both foam and non-foam cell type) isolated from human lesions [138,143,144].

MA-induced foam cells by themselves did not induce significantly elevated levels of inflammatory cytokines GM-CSF, IFN-γ, IL-12 or TNF-α, although slightly elevated levels of IL-6 and the anti-inflammatory cytokine IL-10 were observed. Exposure to microbial danger signals such as LPS and heat-killed *M. tuberculosis*, however, strongly stimulated IFN-γ production. Therefore on the basis of these results and previous *in situ* studies it remains unclear whether foam cell macrophages have an inflammatory or anti-inflammatory profile. Rather, it seems likely that the activation status induced by MA is one distinct from other types of activation of macrophages.

The increased production of IFN-γ by MA-induced foam cells upon re-stimulation with LPS or heat killed *M. tuberculosis* could contribute to host defense during the chronic phase of

M. tuberculosis infection. IFN-γ has been shown to play a role in the control of *M. tuberculosis* infection, whether produced early in infection as a by-product of the activation of innate immune defense mechanisms, or by antigen-specific T cells following the induction of specific immunity [40,44]. IFN-γ is known to increase the oxygen burst in human monocytes to promote development of Th1 responses and to activate NK cytocidal activity, properties that may help to control infection.

Similarly to the results for IFN-γ, MA primed macrophages for production of MPO when exposed to heat-killed *M. tuberculosis*. MPO is an abundant heme enzyme from activated phagocytes that generates oxidants proposed to play critical roles in mycobacterial killing [145]. Neutrophils are the predominant source of MPO and by degranulating, they release the MPO into the microenvironment. Once in the microenvironment, MPO is rapidly inactivated. Both MPO and enzymatically inactive MPO (iMPO), because of their high mannose content, will bind to the macrophage mannose receptor (MMR) and be taken up by adjacent macrophages. The low levels of MPO observed in MA-induced foamy macrophages, but high levels after subsequent stimulation with LPS and heat-killed *M. tuberculosis*, may be explained by this mechanism of uptake of either iMPO or MPO released from infiltrating neutrophils. However this does not exclude the possibility that the foamy macrophages by themselves produce MPO. Although macrophages were considered to lack MPO upon maturation, recent studies demonstrated co-localization of MPO with CD68-positive macrophages in brain tissues and with lipid/cholesterol-enriched macrophages in artherosclerotic lesions [146-148]. The presence of MPO in MA-induced macrophage foam cells after stimulating the cells with LPS or heat-killed bacteria, supports the idea that MA is able to activate macrophages without triggering an actual inflammatory response. Rather, it endows them with the capacity to exert or promote microbicidal activities upon encountering bacteria or bacterial components.

Besides its bactericidal capabilities, MPO has also been implicated in the pathway for macrophage foam cell formation [148]. MPO catalyzes the formation of a number of reactive species that can modify low-density lipoprotein (LDL) to oxidized LDL (Ox-LDL) which is avidly taken up by macrophages, leading to massive cholesterol deposition and foam cell formation [149-152]. These foam cells form the hallmark of atherosclerotic lesions. Foam cells

have also been observed with infections caused by pathogens such as *Chlamydia pneumoniae* [153]. Once again, whether these oxidative species are produced by MPO originating from neutrophils or macrophages remains an unanswered question in this study. In addition, numerous enzymes along numerous pathways could contribute to oxidation of LDL and subsequent foam cell formation. Therefore, although MPO is a likely candidate, the actual involvement of MPO in foam cell formation induced by MA, constitutes another unanswered question.

Combined, the results indicate severe alterations in macrophage morphology, lipid accumulation, phenotype, activation status and, most importantly, priming for microbicidal activities upon exposure to MA. The functional significance of these modulated macrophage functions was demonstrated by the foamy macrophages' ability to inhibit the intracellular survival of *M. tuberculosis*. In the next chapter, the question of what the implications may be that these altered macrophage functions might have on the mycobacterial disease, is discussed.

CHAPTER 3

3.1 CONCLUDING DISCUSSION

The pathogenicity of mycobacteria is directly related to their ability to survive within macrophages, thereby circumventing host defense responses. This ability to resist degradation in macrophage phagosomes/lysosomes derives in large part from the complex structure of the cell wall of *Mycobacterium tuberculosis*. Surface exposure of lipid and glycolipid components of the mycobacterial cell wall is considered to be a major factor in the virulence of the pathogen by orchestrating the dialogue with host cells. Export of lipid effectors from *M. tuberculosis* to the host cells was recently described by Beatty and co-workers who demonstrated the presence of mycobacterial lipids in the cytoplasm of infected and non-infected host macrophages [104]. In infected cells, lipids intercalate into host cell membranes in multivesicular lysosomal structures, which are shed into the external medium and subsequently internalized by neighbouring macrophages [105]. Here the effect of the most abundant mycobacterial cell wall lipids, i.e. the mycolic acids, on infected and non-infected host macrophages were investigated. This was expected to contribute to a better understanding of the role of these molecules in the pathogenesis of tuberculosis.

One remarkable consequence resulting from the interaction of macrophages with MA, was the formation of macrophage derived foam cells. The accumulation of cholesterol in MA-induced foam-like cells is also a property of macrophage derived foam cells induced by oxidized-Low Density Lipoprotein (oxLDL) [154]. The presence of foamy macrophages was also observed in murine tuberculosis granulomas [131-133], which raises the question of the functional significance of this cell response. One important aspect may be the active lipid-import that is characteristic of foam cells. As the macrophage phagosomes are poor in nutrients, intracellular bacilli may have to rely on a mechanism of lipid accumulation to provide the substrates necessary for their survival and replication. In agreement with this concept, analysis of *Mycobacterium* spp. isolated from infected macrophages demonstrated an up-regulation of genes involved in lipid metabolism [31,155,156]. Moreover tissue mycobacteria preferentially metabolise fatty acids over glucose [30,157]. An unusually high proportion, approximately 30%, of the mycobacterial genome encodes genes

involved in lipid synthesis or metabolism [158]. This supports the notion of foam cell formation as a means for the mycobacterium bacilli to ensure access to metabolic substrates.

In contrast to MA-induced lipid accumulation in the macrophage, which might contribute to the pathogenesis of the disease, macrophage interaction with MA established a distinct but limited activation status and primed these macrophages for increased microbicidal and innate defense. This priming was demonstrated by the foamy macrophages' ability to inhibit the intracellular growth of *M. tuberculosis,* thereby giving a functional significance to the host cell response to MA. Whether this intracellular killing of mycobacteria is established via an IFN-γ- or MPO-mediated mechanism, remains to be investigated. Both of these mediators are likely candidates to explain the observed increased microbicidal acitivities in macrophages. Previous studies revealed that MPO released by neutrophils and phagocytosed by macrophages induced the secretion of reactive oxygen intermediates (ROI) [159-161] and the cytokines TNF-α, IL-1, IL-6, IL-8 and GM-CSF [162,163]. This consequently resulted in enhanced microbicidal activities of these macrophages [161], providing a mechanism by which the intracellular killing of mycobacteria is estabilished following treatment with MA. Whether MPO originates from the infiltrating neutrophils or are produced by these macrophages is not clear, but nevertheless the presence of MPO in foamy macrophages upon exposure to mycobacteria could contribute to the enhanced microbicidal activity observed.

IFN-γ constitutes another candidate for the observed enhanced innate defenses in MA-induced macrophages following exposure to mycobacteria. IFN-γ production by antigen presenting cells is still a matter of controversy, however. Recent findings of high-level production and intracellular expression of IFN-γ by IL-12-stimulated macrophages and dendritic cells contradict this initial paradigm in which IFN-γ production was restricted to lymphoid cells. Fenton *et al.* [164] showed that human alveolar macrophages produce IFN-γ mRNA *in vitro* upon infection with *M. tuberculosis* and that IFN-γ acts in an auto-regulatory manner. Prior to this observation, it had been accepted that alveolar macrophages were inefficient at controlling initial infection and that only after recruitment of lymphocytes producing IFN-γ did a protective response occur [164]. More recently, Wang *et al.* [165] have shown that macrophages from mice infected with *M. bovis* BCG produce IFN-γ in an IL-12-dependent fashion. Recent work demonstrated that CD68-positive foamy macrophages in human tuberculous granulomas also produce IFN-γ mRNA [166]. This corroborates

our results, suggesting that the macrophage may be able to autoregulate its activation state at the site of infection and pathology in tuberculosis. These findings have many implications for the immunopathogenesis of the disease, as well as for strategies for immunotherapy.

IFN-γ production by MA-primed macrophages, upon exposure to mycobacteria, might therefore play an important role in the initial stages of infection, prior to specific recognition by T cells. In addition, since it is well known that macrophages influence Th1 versus Th2-cell differentiation, it seems probable that the IFN-γ production by macrophages, at the time of antigen presentation, could provide a major mechanism for resistance to tuberculosis induced by MA. In this manner, MA-induced foamy macrophages, upon encountering of *M. tuberculosis* bacilli, would act not only to enhance innate immunity, but also to establish a link between innate immunity and the adaptive immune response against tuberculosis.

Foamy macrophages' functional ability to influence Th1/Th2 immune responses was demonstrated by their capacity to inhibit arginase production after steering towards an alternatively activated, Th2 supporting status. This could have important implications during the chronic stage of infection, characterized by increased Th2 responses and decreased Th1 responses. By down-regulating Th2 responses, Th1 effector functions are no longer hindered to control infection. It remains to be determined whether this mechanism underlies the MA-induced protection towards tuberculosis observed in mice.

The immunoregulatory influence of MA on the foam cell facilitated Th1/2 bias also opens up the possibility of using the MA as immunotherapy in other Th2 diseases, such as allergic asthma. Prior activation or priming of the APC towards Th-1 orientated antigen presentation, seems to be important in order to counteract the Th-2 biased immune response characteristic of allergic asthma. This has been demonstrated by recent studies showing that IFN-γ activated macrophages are capable of functioning as Th1-promoting APC's, thereby suppressing the development of Th2-type cells in both the primary and secondary immunity [117]. Moreover, the ability of IFN-γ activated lung macrophages to attenuate allergic inflammation in a murine model of allergic asthma has been demonstrated, by mounting Th1 responses in the bronchial mucosa that antagonized Th2 responses to inhaled allergen [167].

Combined, our results imply that MA exerts diverse activities during the course of tuberculosis infection. During primary infection, the induction by MA of foamy macrophages may contribute to the survival of the intracellular bacteria by generating a lipid rich phagosome environment. In contrast, engulfment of extracellular MA released by reactivation of *M. tuberculosis* later in infection, may prime neighbouring macrophages to produce IFN-γ and MPO to kill the pathogen.

This study formed part of an investigation that was supported by the pharmaceutical industry to determine whether the administration of MA may be utilized as chemo- or immunotherapy for tuberculosis patients. Rather than providing an unequivocal answer to this question, the research came up with a potential role for MA in both the pathogenesis of *M. tuberculosis*, as well as the induction of protective immunity in the murine host. The final answer may be a balance between these two effects, making MA an extremely interesting element in both pathogenesis and immune resistance, but far too complex to be safely considered as a pharmaceutical aid to combat tuberculosis. The study did, however, open up interesting new avenues for research towards a unique solution to this serious disease.

SUMMARY

The pathogenicity of mycobacteria is directly related to their ability to survive within macrophages, thereby circumventing host defense responses. This ability to resist degradation in macrophage phagosomes/lysosomes derives in large part from the complex structure of the cell wall of *Mycobacterium tuberculosis*. Surface exposure of lipid and glycolipid components of the mycobacterial cell wall is considered to be a major factor in the virulence of the pathogen by orchestrating the dialogue with host cells. Their interactions and modulating properties on host macrophage functions may contribute to our understanding of the pathogenesis of tuberculosis.

In this study the modulating properties on macrophage functions by the major mycobacterial cell wall lipids, mycolic acids, were investigated. The investigation focused not only on the physical changes induced in macrophages as a result of the interaction with mycolic acids but also on the modulation of macrophage functions involved in innate and adaptive immunity. It was concluded that MA was involved both in mechanisms of pathogenesis of *M. tuberculosis*, as in induction of protective immunity. By opening up some of the secrets of pathogenesis and immunity of tuberculosis, it provided new avenues for research to pursue a timeous and efficient solution to the disease.

OPSOMMING

Die patogenisiteit van mikobakterieë is direk verwant aan hulle vermoë om binne-in makrofae te oorleef. Hierdie vermoë om die afbreekproses binne-in makrofaag fagosome/lisosome te verhoed, kan grootliks toegeskyf word aan die komplekse struktuur van die selwand van *Mycobacterium tuberculosis*. Oppervlakuitdrukking van lipied- en glikolipiedkomponente op die mikobakteriële selwand word beskou as een van die hooffaktore vir die virulensie van die patogeen deurdat dit die interaksie met die gasheer dirigeer. Kennis van die interaksies en modulerende eienskappe op gasheer-makrofaag funksies kan bydra tot ons begrip van die patogenisiteit van tuberkulose.

In hierdie studie word die modulerende eienskappe op makrofaag funksies van die hoof selwand lipied, mikolsuur, ondersoek. Die fokus van die ondersoek word nie alleen geplaas op die fisiese veranderinge geïnduseer in makrofae as `n gevolg van die interaksie met mikolsuur nie, maar ook op die modulering van makrofaag funksies betrokke by ingeskape en aanpasbare immuniteit. Die gevolgtrekking was dat MA betrokke was by beide meganismes vir patogenisiteit van *M. tuberculosis*, sowel as by die induksie van beskermende immuniteit. Deur die onthulling van sommige van die geheime van patogenisiteit en immuniteit van tuberkulose, is nuwe navorsingsvelde oopgemaak vir die nastrewing van `n effektiewe oplossing vir hierdie ernstige siekte.

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