Mycobacterial mycolic acids as immunoregulatory lipid antigens in the resistance to tuberculosis

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"Morena ke modisa wa ka ga nkitla ke tlhoka sepe, o ntsamaisa mo mafulong a matalana"

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List of Abreviations

β2M	Beta-2-microglobulin
γδ	Gamma-delta
A	
AIDS	Acquired immune deficiency syndrome
AM	Alveolar macrophage
APC	Antigen presenting cell
ATCC	American Type Culture Collection
B	
BCG	Bacillus Calmette-Guérin
C	
CAM	Cytokine-activated monocytes
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming units
Chol	Cholesterol
CMI	Cell-mediated immunity
D	
DEPC	Diethyl pyrocarbonate
DN	Double negative
DNA	Deoxyribonueleic acid
DOTS	Directly observed treatment short course

DTH	Delayed-type hypersensitivity
E	
E. coli	*Eschirichia coli*
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EMB	Ethambutol
G	
GAP-DH	Glyceraldehyde-3-phosphate dehydrogenase
GLC	Gas-liquid chromatography
GLP	Good laboratory practise
GM-CSF	Granulocyte macrophage colony stimulating factor
H	
HCl	Hydrochloric acid
HEPA	High Efficiency Particulate Air
HIV	human immunodeficiency virus
HPLC	High-performance liquid chromatography
I	
i.n.	Intranasal
i.p.	Intraperetoneal
i.v.	Intravenous
IFN-γ	Interferon gamma
IgG	immunoglobulin G
IL-	Interleukin

INH	Isiniazid
iNOS	Inducible nitric oxide synthase
IRMA	Immunoradiometric assay
K	
KOH	Potassium hydrooxide
L	
LAM	lipoarabinomannan
LDL	Low-density lipoproteins
LPS	lipopolysaccharides
M	
M. avium	*Mycobacterium avium*
M. bovis	*Mycobacterium bovis*
M. leprae	*Mycobacterium. leprae*
M. tuberculosis	*Mycobacterium tuberculosis*
M.tb	*Mycobacterium tuberculosis*
MA	Mycolic acids
MAIDS	Murine acquired immunodeficiency syndrome
MCF	Mink cell focus forming
MDR TB	Multi-drug resistant tuberculosis
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
MOPS	3-(*N*-morpholino) propanesulfonic acid
MOTTS	Mycobacteria Other Than Tuberculosis

MRC	Medical Research Council
mRNA	Messenger RNA
MuLV	Murine leukemia virus
MΦ	Macrophage
N	
NAC	No amplification control
NaCl	Sodium chloride
NALC-NaOH	N-acetyl-L-cysteine-sodium hydroxide
NaOH	Sodium hydroxide
NK	Natural killer cells
NKSF	Natural killer cell stimulatory factor
NO	Nitric oxide
NTC	No template control
O	
OD	Optical density
P	
PBS/AE	Phosphate buffered saline azide EDTA
PC	Phospahatidyl choline
PCR	Polymerase chain reaction
PMBC	Peripheral blood mononuclear cells
PPD	Purified protein derivative
PZA	Pyrazinamide

R

RFP	Rifampetine
RIA	Radioimmunoassay
RIF	Rifampicin
RNA	Ribonucleic acids
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
RQ-PCR	Real time quantitative PCR
RQ-RT-PCR	Real-time quantitative reverse transcriptase PCR
rRNA	Ribosomal ribonucleic acids
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase PCR
RZL	Rifalazil

S

Sal	Saline
SC-1	Stromal cell line
Ser	Serum
SIV	Simian immunodeficiency virus
SM	Streptomycin
SOD	Superoxide dismutase
SQ-RT-PCR	Semi-Quantitative RT-PCR

SR	Scavenger receptor
T	
TB	Tuberculosis
TCR	T cell receptor
Th	T helper
TNF-α	Tumor necrosis factor alpha
W	
WHO	World Health Oraganisation

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Summary: Mycobacterial mycolic acids as immunoregulatory lipid antigens in the resistance to tuberculosis

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Tuberculosis has returned with vengeance mainly due to the resurgence of multi drug resistant strains incurred by non-compliance to the 6-9 months chemotherapy programme. Co-infection with HIV, which disorientates the immune response, has aggravated the situation. This study was built on previous observations that indicated that the major lipid cell wall component of *M. tuberculosis*, i.e. mycolic acids, a wax that envelopes and protects the bacillus from the hostile host immune system, can be purified and administered to animals for protection against subsequent tuberculosis induction. It was established in this study that mycolic acids pre-treatment can significantly protect mice upon subsequent intranasal infection with *M. tuberculosis* and that this protection is not attributed so much to the T helper cell immunity, but rather through induction of innate immunity. In the murine AIDS model, innate immunity induced by mycolic acids pre-treatment was not enough to protect the virally immunocompromised mice against subsequent *M. tuberculosis* infection. Mycolic acids administration in mice did not support tuberculosis chemotherapy to enable shortening of the duration of chemotherapy. In human tuberculosis patients, antibodies to mycolic acids could be measured in a specially adapted configuration of a resonant mirror biosensor. The preliminary investigation opened up the possibility that the prevalence of anti-mycolic acids

antibodies in tuberculosis patients may be measured as a surrogate marker for tuberculosis infection. An apparent cross-reactivity between mycolic acids and cholesterol in binding to tuberculosis patient antibodies may provide far reaching insight in the role of the mycolic acids in the cell wall to facilitate infection. This research contributed significantly to the understanding of the host-pathogen interaction in tuberculosis, to open up fresh approaches to improved diagnosis and chemotherapy.

Opsomming: Mikobakteiële mikolsure as immunregulatariese lipied-antigene in die weerstandigheid teen tuberkulose.

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Tuberkulose is terug met mening, hoofsaaklik vanweë die herlewing van multi-drogery-weerstandige patogeenstamme deur die onvoldoening aan die 6-9 maande lange chemoterapie-termyn. Ko-infeksie met HIV, wat die immuunrespons versteur, het die toestand vererger. Hierdie studie is gebaseer op vroeëre waarnemings wat daarop gedui het dat die dominante lipied-selwandkomponent van *M. tuberculosis*, d.i. mikolsure - 'n was wat die basillus omhul en beskerm teen die vernietigende immunstelsel van die gasheer - gesuiwer en toegedien kan word aan proefdiere om beskerming te verleen teen daaropvolgende tuberkulose-induksie. Daar is met hierdie studie vasgestel dat voor-infeksie behandeling met mikolsure muise aansienlik kan beskerm teen daaropvolgende intranasale infeksie met *M. tuberculosis* en dat hierdie beskerming nie soseer te wyte is aan die T-helpersel-immuniteit nie, maar eerder deur die induksie van ingeskape immuniteit. In die muis-VIGS-model, was die ingeskape immuniteit, geïnduseer deur voorbehandeling met mikolsuur, onvoldoende om teen *M. tuberculosis* infeksie te beskerm in die virale immuunverydelde muis. Toediening van mikolsure aan muise het nie die effektiwiteit van chemoterapie ondersteun om 'n verkorte duur daarvan moontlik te maak nie. In mens tuberkulose-pasiënte kon teenliggame teen mikolsure aangetoon word in 'n spesiaal aangepaste konfigurasie van die resonante spieëlbiosensor. Die voorlopige ondersoek het die moontlikheid onthul dat die bestaan van anti-mikolsuur-teenliggame in tuberkulose pasiënte gemeet kan word as 'n surrogaatmerker van tuberkulose infeksie. 'n Oënskynlike kruis-reaktiwiteit tussen mikolsure- en cholesterol-binding aan tuberkulose-pasiënt-teenliggame kan verreikende insig verleen aan die rol

van mikolsure in die selwand om infeksie te bevorder. Hierdie navorsing het bygedra tot 'n beter begrip van die gasheer-patogeen interaksie in tuberkulose, ten einde vars benaderings te skep vir verbeterde diagnose en chemoterapie.

CHAPTER 1

General Introduction

1.1 Tuberculosis history

Tuberculosis (TB) is actually not a new disease but is an ancient one. This disease has its antiquity displayed in prehistoric human bones, Egyptian mummies and Egyptian writings (Fätkenheuer *et al.*, 1999). The ancient Greeks coined a term *phthisis*, meaning wasting, with its etiology then unknown. The precise description of *phthisis* characterises the clinical appearance of active tuberculosis. The term tuberculosis was only seen in print for the first time in 1839, used by Schönlein to describe a spectrum of tuberculous diseases.

Tuberculosis became a disease associated with crowding and poverty, which became a public health crisis due to the rise of modern cities in the 17th century. In 1679 Franciscus Sylvius described lung nodules that developed during tuberculosis as "tubercula". This probably marked the first stage from calling the disease consumption to tuberculosis. Before the etiologic agent of TB was elucidated, it was believed to be a constitutional disease whereas its infectious nature was dismissed. Benjamin Martin was in 1722 the first to make credible speculation that TB was an infectious disease when he proposed that the disease could be transmitted from the lungs of an infected person through inhalation by a healthy person (Bloom and Murray, 1992). This notion was still dismissed even after Jean-Antoine Villemin had in 1865 demonstrated that transferring fluid from a person with TB to rabbits caused these animals to develop TB. Villemin's studies received a very hostile reception even though they were published 17 years before the causative agent of tuberculosis was isolated (Grange, 1980; Bloom and Murray, 1992).

This remained so until 1882, when Robert Koch isolated and identified *Mycobacterium tuberculosis*, an intracellular parasite, as the causative agent for tuberculosis (Chaparas, 1982; Fenton and Vermeulen, 1996; Mendez-Samperio *et al.,* 1995; Elhers, 1994).

Robert Koch followed up his discovery with an attempt to produce a vaccine from the culture medium. This was however very disastrous as it produced a typical cell-mediated delayed hypersensitivity reaction. This was later called a tuberculin test, as it became a useful tool for detecting active *M. tuberculosis* infection as described by Von Pirquet in 1907. The challenge was still on for the development of a successful vaccine against *M. tuberculosis* infection. In 1906 Albert Calmette and Camille Guérin found that the less virulent strain of *Mycobacterium bovis* rendered protection in calves against a lethal dose of infection. This then led to tests in humans that achieved success in 1921. The introduction of Bacillus Calmette-Guérin (BCG) vaccine helped to keep the spreading of the disease at bay at least for some time. The application of BCG has been controversial, given that it has been in use since 1921. BCG can protect or ameliorate severe forms of systemic tuberculosis in children, particularly tuberculosis meningitis, but it is of low or no protective value to adults (Huebner, 1996).

At the moment the only available vaccine is *M. bovis* bacillus Calmette-Guérin (BCG), of which its efficacy today still remains controversial. BCG induces high levels of resistance in tuberculosis animal models but human trials have demonstrated varying efficacy, ranging from 80% in some trials (in United Kingdom) to a lack of any significant protection in several trials in developing countries. BCG vaccination is currently not in use in the USA as they rely on the tuberculin test to guide therapy. All patients in the USA with human immunodeficiency virus (HIV) suspected to be co-infected with *Mycobacterium*, are recommended by the Centre for Disease Control (CDC USA) to receive chemotherapy (CDC, 1994).

1.2 Mycobacterium tuberculosis

The genus *Mycobacterium* incorporates over 70 species of both pathogenic and non-pathogenic types, but of these only a fractional percentage can cause disease (Grange, 1996; Shinners and Yeager, 1999). The pathogenic species include the causative agents of mainly leprosy (*Mycobacterium leprae*, discovered by Hansen in 1874) and tuberculosis (*M. tuberculosis*). The remaining *Mycobacterium* species are environmental organisms, collectively known as MOTTS (Mycobacteria Other Than Tuberculosis). *Mycobacterium tuberculosis* is a rod shaped bacterium with a lipid rich cell wall (Fig. 1.1). Most of the other mycobacteria can only cause disease in immune-compromised individuals (Gomes *et al.*, 1999). *Mycobacterium tuberculosis*, is responsible for more than three million deaths per year (WHO, 2001; Bloom and Murray, 1992; Kaufmann and Van Embden, 1993; and Draper, 2000).

These bacteria are resistant to most common antibiotics available, and are only susceptible to aminoglycosides (streptomycin), rifamycins and fluoroquinoles. They are also resistant to drying, alkali and most chemical disinfectants. It is believed that resistance is related to the unusual cell-wall structure of the bacteria that makes them different from other bacteria as shown in Fig. 1.1 (Barksdale and Kim, 1977; Steyn, 1996; Brennan and Nikaido, 1995).

All mycobacteria are acid fast, aerobic, contain mycolic acids and have 59-65% GC content in their genomes. The chromosome is not bounded by a nuclear membrane but wrapped into a nuclear body, which constitutes the classification of mycobacteria as prokaryotes.

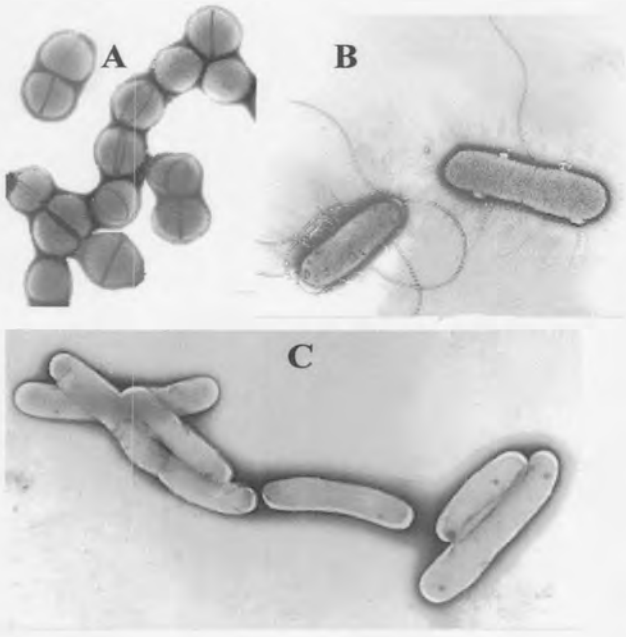


Figure 1.1: Electron micrographs of bacteria indicating the difference in appearances. Gram-positive bacteria (*S. aureus*) is depicted on A, Gram-negative bacteria (*E. coli*) is depicted on B and mycobacteria (*M. tuberculosis*) is depicted on C. (Adapted from http://www.uct.ac.za/depts/mmi/lstyn/cellwall)

Mycobacterial cell walls are thick; making them efficient barriers, while Gram-positive bacteria are covered by a porous peptidoglycan layer and Gram-negative bacteria are covered by two membranes which contain lipopolysaccharides (LPS) and porins as depicted in Fig. 1.2. The cell envelope of *M. tuberculosis* is made out of a plasma membrane with a cell wall consisting of peptidoglycan linked to arabinogalactan and mycolic acids (Brennan and Nikaido, 1995; Yuan *et al.,* 1997).

The cell wall is also made out of lipoarabinomannan (LAM) and trehalose-2'-sulfates, which are associated with the virulence of the mycobacteria (Fenton and Vermeulen, 1996). Mycobacterial mycolic acids are linked to carbohydrates forming trehalose

dimycolate (also named cord factor) that can be isolated from free lipid extracts of mycobacteria (Orbach-Arbouys *et al.,* 1983; Gotoh *et al.,* 1991). Mycolic acids may also be linked to glucose to form mono-mycolate (Goren, 1979; Gotoh *et al.,* 1991). Studies have suggested that cord factor might be the substance responsible for the inhibition of phagosome-lysosome fusion in macrophages (Spargo *et al.,* 1991).

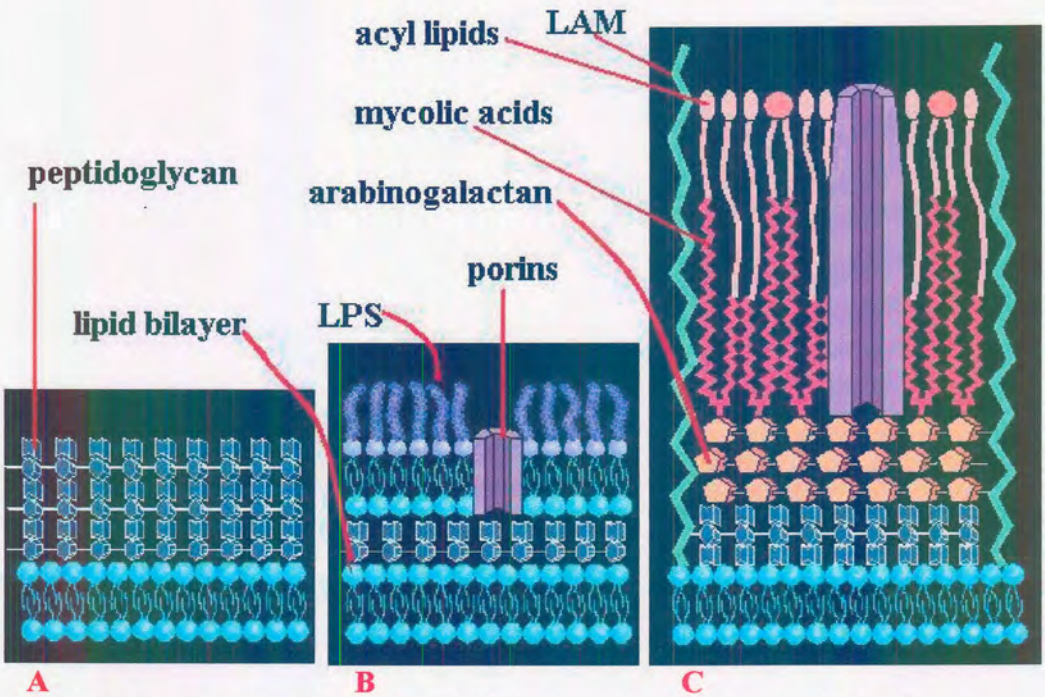


Figure 1.2: Bacterial cell walls of Gram-positive organisms (A), Gram-negative organisms with a double lipid layer (B) and mycobacteria rich in mycolic acids (C). (Adapted from http://www.uct.ac.za/depts/mmi/lstyn/cellwall)

1.3 Tuberculosis: an emergency that does not cease

It is estimated that approximately 10% of people infected with *M. tuberculosis* are expected to develop tuberculosis during their lifetime. Depending on the blood supply

and oxygen tension in the organs, disease may or may not occur. Adult tuberculosis normally occurs through reactivation. The world incidence of tuberculosis in developing countries has always been high, but in industrialized countries the disease has been on the decline for a few decades. However, the acquired immune deficiency syndrome (AIDS) epidemic and the appearance of multi-drug resistant *M. tuberculosis* strains have changed the situation. Today one third of the world population is infected with *M. tuberculosis*.

In April 1993 the World Health Organisation (WHO) declared tuberculosis a global emergency after noticing that an alarming number of people still die from this disease. It was predicted that at least thirty million people would have died between 1990 and 1999. With these projections already noted earlier on, deaths due to tuberculosis are still very high. This led the WHO to develop a new initiative early in 1999 to accelerate a global action against tuberculosis. This initiative, called Stop TB, noted that 80% of the world tuberculosis fall within a few countries that could be addressed individually from the rest, as tuberculosis management is primarily the major cause of the escalation (Fig. 1.3). A workshop held in South Africa (Pretoria, August 1999) engaged all countries represented in the region. South Africa represented the fourth highest tuberculosis incidence with an estimated of 495 per 100 000 (Maher *et al.*, 1997; Coghlan and Concar, 2001; WHO, 2002). Co-infection with HIV plays a major part in the increasing rate of infection (Verschoor and Onyebujoh, 1999; Corbett and De Cock, 2001).

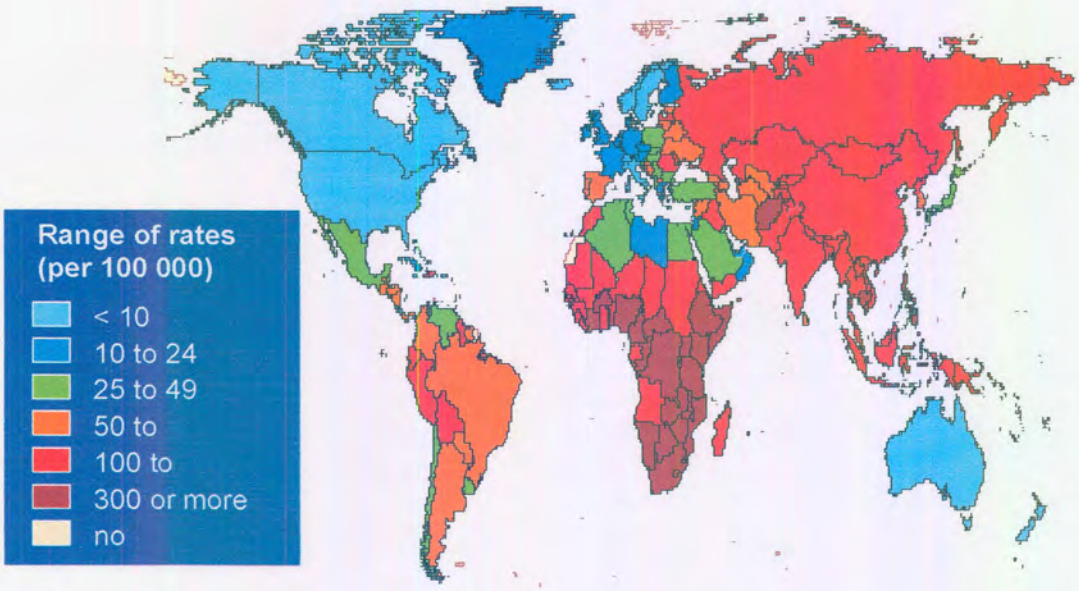


Figure 1.3: Countries with the highest tuberculosis cases in the world. (http://www.stoptb.org/tuberculosis/).

1.4 Tuberculosis and HIV

In 1981, there were reports of a disease that comprised unusual impairment of the immune system. This disease came to be known as acquired immune deficiency syndrome (AIDS). The etiology of the disease was in 1983 discovered by French scientists to be a retrovirus later named human immunodeficiency virus (HIV) (Chermann *et al.*, 1983; Levy *et al.*, 1984). HIV is part of a family of retroviruses in which genetic material is stored in ribonucleic acids (RNA) rather than deoxyribonucleic acids. Once inside the cell the retroviral RNA is transcribed into deoxyribonucleic acid (DNA) by its own reverse transcriptase enzyme. This will allow the proviral DNA to be integrated into the host DNA. The infected cell will harbour the virus in the latent state as long as it lives. When the virus was discovered it was only found in 1 out of 1000 $CD4^+$ cells infected because the virus could slip into the host DNA without any signals to show. The presence of HIV infection over time leads to AIDS. The association between HIV and tuberculosis was discovered in 1983. Persons with HIV infection are more likely to

progress to tuberculosis after an initial infection with *M. tuberculosis* (Selwyn *et al.*, 1989; Shafer and Edlin, 1996). As HIV increases susceptibility for *M. tuberculosis* infection and disease progression, tuberculosis similarly influences the course of HIV infection by production of stimulatory cytokines and by decreasing $CD4^+$ T-cells (Schauf *et al.*, 1993; Wallis *et al.*, 1993).

The hallmark of AIDS is the loss of T lymphocytes with the CD4 surface marker. T-cells are effective primary players in the development of cell-mediated immunity (CMI), which controls *M. tuberculosis* infection. The loss of T-cell function reduces cytokine productions, which in turn compromises activation of macrophages. As described before, the activation of macrophages stimulated by T-cell cytokines, is required to control *M. tuberculosis* infection. Tuberculosis cannot be diagnosed with the tuberculin test in AIDS patients, as anergy can occur in about 30% of the patients (WHO, 1999). Countries with greatest numbers of HIV/AIDS also have the greatest numbers of tuberculosis cases because AIDS is associated with the loss of CMI. Nowhere in the world has the combined tuberculosis-AIDS epidemic become more evident than in Africa. Since 1984, when HIV infection became prominent in Africa there has been a dramatic concomitant increase in the incidence of tuberculosis (Fätkenheuer *et al.*, 1999).

1.1 Infection and transmission of *M. tuberculosis*

M. tuberculosis infection (human and animal) can occur in any organ, but the lung is virtually always the port of entry (Dutt and Stead, 1999; Toossi, 1996; Fenton and Vermeulen, 1996; Bermudez and Goodman, 1996; Flynn and Chan, 2001[b]). *Mycobacterium tuberculosis* has the ability to survive and to withstand the hostile environment within the phago-lysosome in the lung (Fig. 1.4). The mechanism of *M. tuberculosis* survival is primarily based on its interaction with the macrophage (MΦ). The MΦ is the crucial cell in the host defence against pathogenic bacteria.

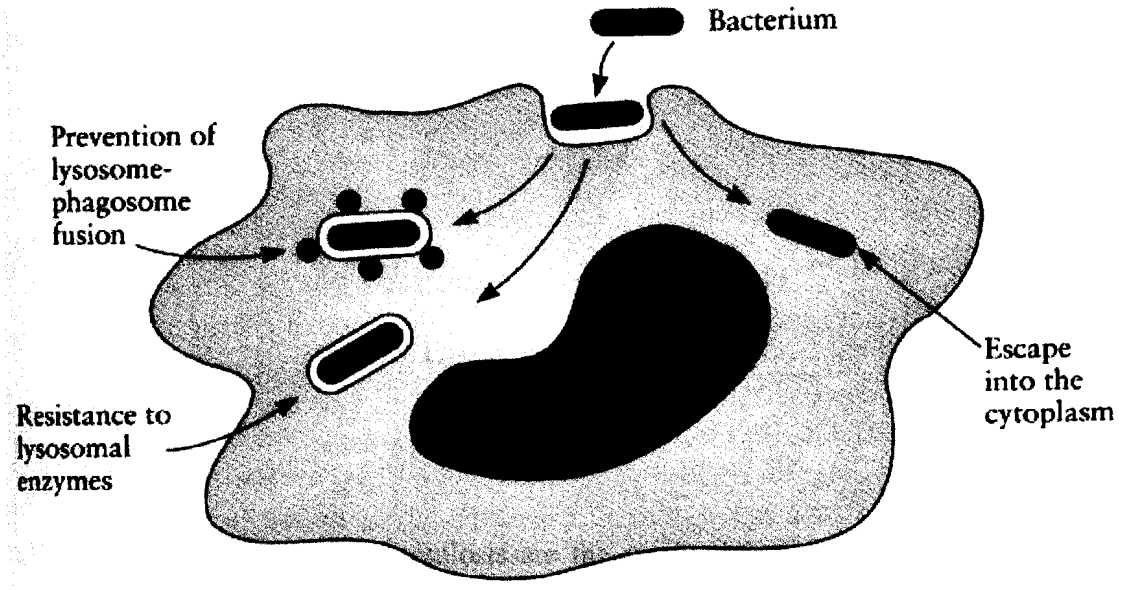


Figure 1.4: Mechanism of *M. tuberculosis* survival and escape into the cytoplasm.

The bacilli are commonly discharged into the atmosphere through aerosolisation by sneezing and coughing by an infected individual. The aerosol droplet may then dry out to tiny droplet nuclei, some of which may contain a few bacilli. Droplets of the size range 1-10 μm are trapped in the upper nasal passages or expelled into the pharynx by the mucociliary mechanism of the lower respiratory tract and digested. Tuberculosis patients produce droplet nuclei carrying tubercle bacilli in proportion to the liquidity and the number of bacilli excreted in the secretions (Dutt and Stead, 1999). Factors that determine transmission are indicated in Fig.1.5. The figure also indicates that the probability of disease contraction caused by airborne infection is not high. The ability of the bacilli to cause infection upon exposure depends on the adequacy of the individual's innate immunity (Comstock, 1982). Should there be an infection, cell-mediated immunity is evoked and depending on the adequacy of the immune system, the organisms become sequestered in dormant foci without causing disease (Dutt and Stead, 1999).

Figure 1.5: Factors that determine transmission of infection from a source case to contacts and the normal progression to tuberculosis in infected contacts (Adapted from Dutt and Stead, 1999).

1.6 Tuberculosis

In humans, *Mycobacterium tuberculosis* infection occurs upon the inhalation of bacteria into the deep lung. Only one to three bacteria are sufficient for an effective inoculum. After the inhalation of the bacteria, only 10% will reach the respiratory bronchioles and alveoli of the lung. Most bacilli will however settle in the upper respiratory epithelium where they are likely to be expelled by the mucociliary escalator (Fenton and Vermeulen, 1996; Andersen, 1997). The tubercle bacillus is itself relatively non-toxic. Most of the

clinical manifestations of tuberculosis are the result of interactions between the host's immune system and the infecting *Mycobacterium*. The pathogenicity of tuberculosis is determined by the battle between the host and *M. tuberculosis*. In this battle each is armed with different weapons and the ability to utilize each weapon and vulnerability determines who gets the upper hand.

In this war the host is armed with activated MΦs (powerful phagocytes able to kill ingested bacilli) and the ability to stop intracellular bacterial growth by killing non-activated infected MΦ. The non-activated infected MΦs are a source of vulnerability, as they provide a favourable environment for mycobacterial growth that brings about the liquefied caseous material that supports extracellular growth of *M. tuberculosis*. The bacillus' strategy in this war is the logarithmic multiplication within the non-activated infected MΦs and extracellular multiplication in the walled off liquefied caseous material, including those next to the lumen cavities, to reach tremendous numbers. The mycobacteria, on the other hand are unable to survive in activated MΦs and cannot multiply in solid caseous tissues (Dannenberg, 1999).

1.2.1 Stages of tuberculosis

There are five stages of pulmonary tuberculosis. Once the bacillus is inhaled, the first stage begins with the alveolar macrophage (AM) ingesting it for destruction. Destruction of the bacillus is dependent on the inherent microbicidal power of the AM and the genetic and phenotypic virulence of the ingested *Mycobacterium*. Most AMs are activated cells that have been activated non-specifically by a variety of inhaled and ingested stimulants. In the second stage, if the original AM failed to destroy the *Mycobacterium*, the tubercle bacilli multiply until the MΦ bursts. The bacillary load is then ingested by other AMs and by blood-borne MΦs (Dannenberg, 1991; Dannenberg, 1999). These MΦs and a variety of chemotactic factors of host origin are attracted to the site of bacillus release. During this stage the bacilli grow logarithmically in non-activated MΦs without being destroyed,

as delayed-type hypersensitivity (DTH) is not activated. Only MΦs activated via CMI are able to destroy ingested bacilli. The third stage is characterised mainly by caseous necrosis, occurring due to DTH resulting in killing of infected non-activated MΦs. The fourth stage determines whether the disease becomes clinically apparent. Cell-mediated immunity plays a major role in this stage on infection. Should poor CMI develop, the bacilli could escape and grow in partly or non-activated Macrophages. The cytotoxic DTH immune response continues to kill these MΦs and enlarge the caseous centers and increasing disease progression. The final stage involves liquefaction of the caseous centres. At this stage even the well-developed CMI is ineffective in controlling infection (Dannenberg, 1999; Fenton and Vermeulen, 1996). This demonstrates the importance of CMI and DTH in the pathogenesis of tuberculosis.

1.3 Tuberculosis immunity

The interactions of immune-competent-cells, such as T-cells and MΦs, characterise the immunologic response to tuberculosis as either acquired or cell-mediated. CMI can be considered to be a beneficial host response characterized by the sensitisation of T-cells (Th1 cells) that respond by releasing pro-inflammatory cytokines such as interferon-γ (IFN-γ), Interleukin-2 (IL-2) and tumour necrosis factor-α (TNF-α) (Flynn *et al.*, 1993; Saunders and Cheers, 1994; Hernandez-Pando and Rook, 1994; Flynn *et al.*, 1995; Flynn and Chan, 2001[a]). These cytokines would in turn activate MΦs that would then be able to destroy phagocytosed bacilli. Pro-inflammatory cytokines also play a role in the recruitment of monocytes, natural killer (NK) cells and other T-cells to the infection site (Bermudez *et al.*, 1995; Fenton and Vermeulen, 1996; Toossi, 1996; Rhoades *et al.*, 1995; Ehlers *et al.*, 1994). As originally described by Koch, DTH is immunologically similar to CMI, in that it is a process that involves Th1 cells and their cytokine secretion. DTH develops concomitantly with CMI in naturally infected individuals suggesting expression of immunologic events where the same immune-competenT-cells play critical roles.

The activation of MΦs by cytokines and the recruitment of T-cells to the site of infection, are no doubt crucial for growth inhibition and killing of mycobacteria. Macrophages and T-cells secrete immuno-suppressive cytokines whose balance between activation and deactivation determines the outcome of the host defence system (Fenton and Vermeulen, 1996; Toossi, 1996; Bermudez *et al.*, 1995).

1.4 Cell-mediated immune response

Because pathogenic mycobacteria have an intracellular habitat, they are shielded from humoral defence mechanisms. During intracellular replication, some bacterial antigens become accessible to the host-cell processing and are thereby expressed on the surface of the infected cell. This would trigger recognition by T-cells and trigger T-cell proliferation into effector T-cells (Kaufmann and De Libero, 1988). The T-cell-mediated immune response can be divided in two subclasses: T helper 1 (Th1) and T helper 2 (Th2) characterised by their cytokine profiles. Cytokine secretion, that plays a role in the induction and mediation of protection against *M. tuberculosis* infection, is not, however, limited to T-cells only.

Cytokine secretion comes from all cell types including $CD4^+$ T-cells, $CD8^+$ T-cells, MΦs and NK cells. There are type I (Th1) and type II (Th2) immune responses, depending on the type cytokine secreted (Rook and Hernandez-Pando, 1996). Th1 is characterised by secretion of TNF-α, IFN-γ and IL-2 whereas Th2 is depicted by secretion of IL-4, -5, -6, -10 and -13 (Aliprantis *et al.*, 1996; Dipiro, 1997). In murine models of tuberculosis, the initial inflammatory type I immune response against *M. tuberculosis*, needed for effective DTH, is followed by an anti-inflammatory type II immune response. This may be required to limit inflammation and minimize tissue damage on the site of infection (Fenton and Vermeulen, 1996).

1.4.1 CD4+ T-cells in tuberculosis

In *M. tuberculosis* infection, Th1 cytokines are secreted to render CMI (DiPiro, 1997). The $CD4^+$ T-cells are essential for the protection against *M. tuberculosis* infection. This is illustrated in $CD4^+$ T-cell depleted HIV patients. In animal models, it was shown that transfer of $CD4^+$ T-cells from immunised mice to non-immunised *M. tuberculosis* infected mice provided protection (Orme *et al.*, 1987; Barnes *et al.*, 1989). The $CD4^+$ T-cells depletion in mice infected with *M. tuberculosis* resulted in increased mycobacterial multiplication in the lungs and spleens (Orme, 1996; Barnes and Modlin, 1996; Toossi, 1996). The $CD4^+$ T-cells transfer provided protection by transferring DTH (Pedrazzini and Louis, 1986; Boom *et al.*, 1987). The $CD4^+$ T-cells protection against *M. tuberculosis* is mediated by cytokine secretion. The $CD4^+$ T-cells function by recognising the antigen on the major histocompatibility complex (MHC) class II molecules of antigen presenting cell (APC) through their T-cell receptors (TCR).

1.4.2 CD8+ T-cells in tuberculosis

Evidence from animal models suggest that $CD8^+$ T-cells also play a role in controlling tuberculosis infection (Kaufmann and Andersen, 1998; Barnes and Modlin, 1996). Studies with antibody blocking and gene knockout mice indicated that a $CD8^+$ T-cell deficiency caused an increased sensitivity to both *M. tuberculosis* and *M. bovis* (Boom, 1996).

The $CD8^+$ T-cells recognise antigen presented on MHC class I molecules (Barnes and Modlin, 1996). It has been shown that mice lacking MHC class I (β2m KO) are more susceptibile towards *M. tuberculosis* infection. The $CD8^+$ T-cells are also implicated in the destruction of infected cells in granulomatous lesions in the lungs (Andersen, 1997; Rook and Hernandez-Pando, 1996; Barnes and Modlin, 1996; Tascon *et al.*, 1998). Mice immunised with *Mycobacterium vaccae* were shown to elicit $CD8^+$ T-cells that killed *M.*

tuberculosis infected MΦ. These $CD8^+$ T-cells were shown to secrete IFN-γ and enhance IL-12 production (Skinner *et al.*, 1997). Cytotoxicity and IFN-γ production are typical functions of $CD8^+$ T-cells in antimicrobial immunity.

1.4.3 Double-negative αβ T-cells

The double negative (DN) $CD4^-$ $CD8^-$ T-cells with the αβ antigen receptor were observed to become activated upon recognition of lipid antigen presented on CD1 molecules (Porcelli *et al.*, 1992; Beckman *et al.*, 1994). The CD1 molecules are non-polymorphic gene products encoded outside the MHC. They are similar to the MHC class I molecules in that their surface-expression is beta-2-microglobulin (β2M)-dependent. The DN T-cells can promote CMI at the site of infection as they have the ability to produce IFN-γ and lyse infected MΦs (Daugelat and Kaufmann, 1995; DiPiro, 1997; Barnes and Modlin, 1996; Rook and Hernandez-Pando, 1996).

1.4.4 γδ T-cells in tuberculosis

Of all peripheral T-cells in human and mouse, the $CD4^+$ and $CD8^+$ types constitute 90% of the T lymphocytes. The γδ T-cells are generally DN as they lack expression of CD4 and CD8 markers. The γδ T-cells and $CD4^+$ T-cells functionally produce the same amount of IFN-γ and are equally cytotoxic for MΦ (Fathman and Frelinger, 1988; Tsukaguchi *et al.*, 1995). The population of γδ T-cells has been shown to increase with *M. tuberculosis* infection. These cells can play a role in the first line of defence against the infection, because they recognize low molecular weight non-peptide ligands directly on the surface of infected macrophages (Andersen, 1997; Rook and Hernandez-Pando, 1997; Barnes and Modlin, 1996; Orme, 1996).

In humans γδ T-cells have an innate capacity to recognize *M. tuberculosis* antigens without prior exposure to these antigens and secrete pro-inflammatory cytokines such as

IL-2 and IFN-γ (Barnes *et al.*, 1990; Barnes and Modlin, 1996). Recently it has been indicated that γδ T-cells also respond to glycolipid antigens and nucleotide moieties (Tanaka *et al.*, 1995; Boom, 1996; Daugelat *et al.*, 1995).

1.4.5 Other cells in tuberculosis

Natural killer T-cells ($NK1.1^+$ $CD4^+$ T-cells) are a subgroup of T-cells expressing surface markers naturally associated with natural killer (NK) cells and can recognise glycolipid presentation on murine CD1d molecules (Bendelac *et al.*, 1995; Kawano *et al.*, 1997; Kawano *et al.*, 1998). NK cells are naturally occurring, cytolytic effector cells not restricted by the MHC complex. Other cells that play a role in the protective immune response against *Mycobacterium* infection include NK cells, neutrophils, and MΦs. These cells are able to secrete IFN-γ after TNF-α and MΦ-derived IL-12 stimulation. NK cells are known to exhibit non-MHC restricted cytotoxic activity towards the infected alveolar macrophages (Andersen, 1997, Dougelat and Kaufmann, 1996). The MΦs are involved in the primary immune response towards *M. tuberculosis* infection. The major cell types involved in the immune reaction against *M. tuberculosis* are listed in table 1.1.

Table 1.1: Cells involved in specific and non-specific defence against *M. tuberculosis*

Cell Type	Function
Macrophages (MΦ)	o Non-activated monocytes/macrophages allow tubercle bacilli to multiply within them. o Highly activated MΦs destroy or inhibit tubercle bacilli
Lymphocytes	o T-cells (from the thymus) and B cells (from the bone marrow): these cells provide immunologic specificity to the host defence against tubercle bacilli. o Antigen-activated T-cells activate MΦs by producing cytokines. o Antigen-activated B cells produce antibodies. T-cells have been subdivided in a variety of ways based on (1) their surface markers (CD4 and CD8 T-cells), (2) the cytokines they produce (Th1 and Th2 T-cells), and (3) their functions(helper, regulatory, and cytotoxic T-cells)
Antigen-Presenting Cells (APCs)	o Dendritic cells (both local and circulating)are the most efficient APCs. o Macrophages and B-cells are also efficient APCs. Antigens of the tubercle bacilli are presented to the lymphocytes both locally in the tuberculous lesion and more distally in the draining lymph nodes.
Natural Killer (NK) Cells	o NK cells (both local and circulating) are important early defence cells against intracellular microorganisms (viruses, bacteria, fungi, and protozoa). o In tuberculosis, NK cells kill bacilli-laden MΦs and produce IFN-γ, which activates MΦs and stimulate a Th1 cytokine immune response.

(Adapted from Dannenberg, 1999)

1.9 Cholesterol and mycobacterial survival

The ability of *M. tuberculosis* to survive within the MΦ has its key features in arresting intracellular trafficking, which allows bacteria to replicate and survive in the host. A 50-kDa protein has been detected in phagosomes containing live mycobacteria but not in phagosomes containing heat-killed mycobacteria (Ferrari *et al.*, 1999; Gatfield, Ferrari and Pieters, 2000). This molecule was termed TACO. The association of TACO with the phagosome prevents maturation into or fusion with lysosomes, and thereby allowing mycobacteria to survive within the phagosome (Fig. 1.6).

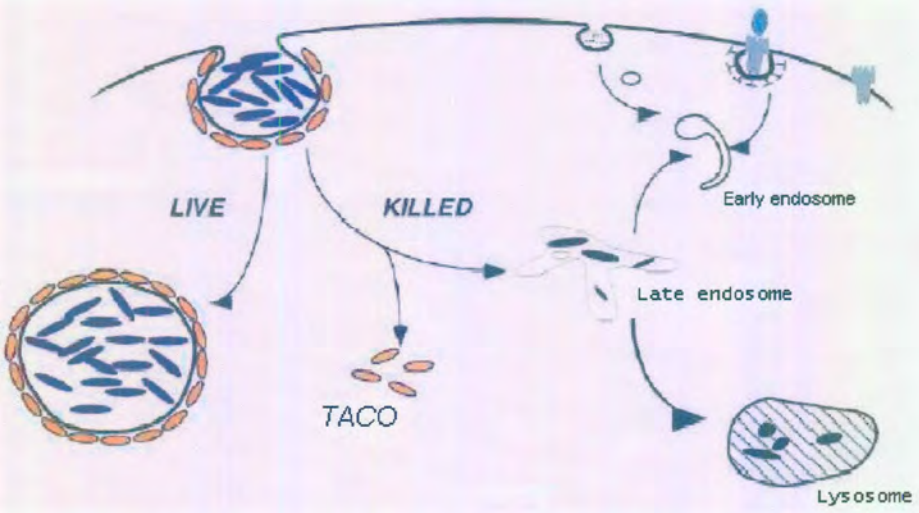


Figure 1.6: Mycobacterial evasion of the host macrophage. (Adapted from Gatfield, Ferrari and Pieters, 2000).

Molecular mechanisms behind mycobacterial entry into the MΦs are still poorly understood. Studies have indicated cholesterol accumulation at the site of mycobacterial entry. Depletion of plasma membrane cholesterol specifically inhibited mycobacterial uptake. Gatfield and Pieters (2000) have also indicated that the association of TACO to

the membranes is cholesterol dependent. The observations were made that when a cholesterol sequestering digitonin is administered TACO is separated from the membrane. The specific relevance of these observations to tuberculosis was demonstrated by showing that depletion of cholesterol affects the uptake of *M. tuberculosis*, but not the uptake of other bacterial pathogens.

1.6 The macrophage (MΦ)

In primary tuberculosis, the neutrophils are the first line of defence followed by the more important MΦs. These cells have a predominant role in the immune system by their removal and processing of particulate antigens and presenting them to the T-cells. The T-cells then produce cytokines that activate MΦs. The activated MΦ is a large cell with large nucleus and abundant cytoplasm often containing vacuoles. Alveolar MΦ are strategically located to play an important role in the defence against *M. tuberculosis* infection. (Roitt *et al.*, 1993; Janeway and Travers, 1996). The migration of monocytes (through blood-vessel walls) into infected organs induces MΦ differentiation (Toossi *et al.*, 1996; Fenton and Vermeulen, 1996). These cells mature into phagocytes with the ability to remove invading pathogens by engulfing them and exposing them to lysosomal enzymes such as non-specific esterase, peroxidase and lysozyme which assist in bacterial degradation. Several specific surface receptors for *M. tuberculosis* intake are visible on the cell membrane including specialized microorganism adhesion receptors, immunoglobulin G (IgG) Fc-, mannosyl-, fucosyl- and cytokine receptors (Van Oss and Regelmortel, 1994).

1.6.1 Macrophage (MΦ) activation

The T-cells control activation of MΦ by IFN-γ secretion. However after infection, MΦs must also sensitise T-cells through presentation of mycobacterial antigens. Once the MΦ is in contact with the *Mycobacterium*, IL-1 is secreted to activate T-cells to release a

number of cytokines. Activated MΦs change their morphology and have increased enzymic activity. These cells are able to fuse their lysosomes more effectively to phagosomes, which contain the ingested bacteria. The ingested bacterium is exposed to a variety of highly potent lysosomal enzymes that are bactericidal. Activated MΦs also produce oxygen radicals and nitric oxide, both of which have potent antibacterial activity (Schebesch *et al.,* 1997; Desmedt *et al.,* 1998; Bonecini-Allmeida, 1998).

Enhanced expression of MHC class II molecules and TNF-α receptors on the cell surface of the activated MΦ amplifies the immune response. This enables the MΦ to present more antigens and sensitise more T-cells. Binding of TNF-α to its receptor on the MΦ cell surface enhances expression of microbicidal reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) (Leenen *et al.,* 1994; Bilyk and Holt, 1995; Murch, 1995; Allendoerfer and Deepe, 1998; Sato *et al.,* 1998). The production of nitric oxide (NO) is one of the effector pathways necessary for the containment of *M. tuberculosis* infection. In the MΦs, NO and other RNI are derived from L-arginine through inducible nitric oxide synthase (iNOS) controlled pathway (Ding *et al.*, 1988). Inhibition of NO was observed to aggravate the *M. tuberculosis* infection (Rook and Hernandez-Pando, 1996; Fenton and Vermeulen, 1996).

1.6.2 Fate of intracellular *M. tuberculosis* in the MΦ

In the lung, the air stream carries small infections particles to all parts of the lung where the *M. tuberculosis* bacteria are taken up by AMs by means that include complement activation or mannose-mannose receptor interaction. Mannose is expressed by most of the virulent bacteria and the mannose receptor is better known as MΦ entry protein (Andersen, 1997). Once phagocytosed the growth of the intracellular bacteria depends on their ability to avoid destruction via lysosomal enzymes, RNI, and ROI. At this stage the pathogen must find a suitable site for replication while evading or circumventing destruction. Mycobacteria have an ability to survive and withstand the hostile

environment through impairment of the phagosome. The capacity to block fusion of mycobacterial containing phagosomes with lysosomes becomes critical and involves excluding the proton ATPase responsible for acidification, thus creating an altered environment for growth, to achieve this (Ferrari *et al.*, 1999). In addition, the interplay of T-cell and MΦ-derived cytokines is important for the control of *M. tuberculosis* infection. The killing of ingested *M. tuberculosis* is likely to occur in the MΦ phagosome fused with lysosomes that produce toxic ROI such as H_2O_2 and O_2^-, and RNI such as NO and NO_2^-. Cytokines were found to be good modulators of MΦ RNI in mouse models. Containment of bacterial growth is dependent on the production of cytokines, especially monokines, that include IL-12, TNF-α and TNF-β (Toossi, 1996; Anderson, 1997; Pece *et al.*, 1997). Activated monocytes secrete IL-12, IFN-γ and TNF-α, mediating the immune response towards the Th1 mode, known to enhance the action of $CD4^+$ T-cells against the MΦ infected with *M. tuberculosis.* (De Libero *et al.*, 1988; Flynn *et al.*, 1995; Cooper *et al.*, 1997). IL-12 would then recruit NK cells to the site of infection. The NK cells subsequently secrete another cytokine, TNF-α. TNF-α induces the formation of granulomas and the subsequent confinement of the bacteria within the MΦs. This cytokine also induces iNOS, required for the formation of nitric oxide and other RNI (Malaviya *et al.*, 1996; Averil *et al.*, 1995; Aung *et al.*, 1996).

Macrophages are able to take up and break down some of the organisms at the primary focus of infection in the lung where mycobacteria grow slowly. The antigens obtained are then presented on MHC class II molecules for TCR recognition on T-cells (Anderson, 1997; Fenton and Vermeulen, 1996; Rhoades *et al.*, 1995; Bermudez and Goodman, 1995). This interaction results in T-cell secreting IFN-γ, which in turn stimulates the MΦs to secrete IL-1, IL-6 and chemokines. IL-1 induces proliferation of neighbouring T-cells, which in turn secrete IFN-γ (Ellner, 1994; Porter *et al.*, 1993; Andersen, 1997).

1.7 Inflammation

Inflammation is a response caused by tissue damage after infection. Inflammation of the lungs after infection with *M. tuberculosis* proceeds with the bacteria and the MΦ first appearing to have a symbiotic relationship. This is followed by the first stage of the innate immune response, characterised by the activation of AMs to secrete IL-12 (Andersen, 1997; Kaufmann and Anderson, 1998; Vanham *et al.*, 1997). In tuberculosis experimental animals, cytokine release is then triggered in T-cells ($CD8^+$-, DN-, NKT- and $CD4^+$), and MΦs, two weeks after the infection. Macrophages subsequently show bacteriostatic activity, recruit lymphocytes to the site of infection and stimulate T-cells to secrete IL-2 and IFN-γ (Anderson, 1997; Ellner, 1997; Rhoades *et al.*, 1995). Monocytes and lymphocytes are attracted and committed to the site of infection by IFN-γ, which acts as a chemotaxin. The activated monocytes then secrete IL-1 and TNF-α. The accumulation of MΦs, T-cells and NK cells results in granuloma formation. Some of the MΦs become epithelial cells as they turn weakly phagocytic due to cytokines, while some may fuse with each other and become multiple nucleiated giant cells (Fenton and Vermeulen, 1996; Janeway and Travers, 1996). Giant cells usually form the centre of these granulomas and are surrounded by T-cells to regulate MΦs and prevent the spread of tissue damage (Janeway and Travers, 1996).

1.8 Cytokines

In the 60s reports appeared suggesting that soluble factors are secreted following antigen activation. Cytokines are small peptide mediators of inflammation, produced by many different cell types. The name interleukin (IL) was initially intended for cyokine produced by leukocytes and acting on leukocytes. Monokines, lymphokines, and cytokines are products from MΦs, lymphocytes and all cell types respectively. As different cell types can secrete the same cytokine, the term cytokine is therefore often used. Cytokines generally exhibit common features such as:

- Molecular weight of less than 25 kDa
- Being produced during effector phases of natural and acquired immunity
- Regulation of development, activation, differentiation, and effector functions of immune cells
- Production by a variety of cells and action on more than one
- Action mediated through ligand receptor interactions
- Small quantities produced that are effective at ultra-low concentrations

Despite the ability of many cells to produce cytokines, monocytes and MΦs tend to generate these mediators in the greatest quantities (Allendoerfer and Deepe, 1998; Sato *et al.*, 1998; Bilyk and Holt, 1995; Leenen *et al.*, 1994).

As indicated in Fig. 1.7, T-cells and MΦs communicate through cytokines to activate one another and thus control *M. tuberculosis* infection. The MΦ serves as both target and activator of the T-cell cytokine response. Cytokines are not only mediators of protection, but can also mediate pathology and susceptibility towards *M. tuberculosis* infection. Protection against *M. tuberculosis* is of a Th1 type, with IFN-γ being the major mediator of MΦ activation and IL-2 the major inducer of T-cell activation (Cooper *et al.*, 1993; Flynn *et al.*, 1993; Murray, 1994; Trinchieri, 1994; Zhang *et al.*, 1994; Trinchieri, 1995; Abbas *et al.*, 1996). In experimental animals, gene depletion of IFN-γ or its receptor rendered the animals more susceptible to *M. tuberculosis* and *M. bovis* infection (Flynn *et al.*, 1993; Cooper *et al.*, 1995; Huang *et al.*, 1993).

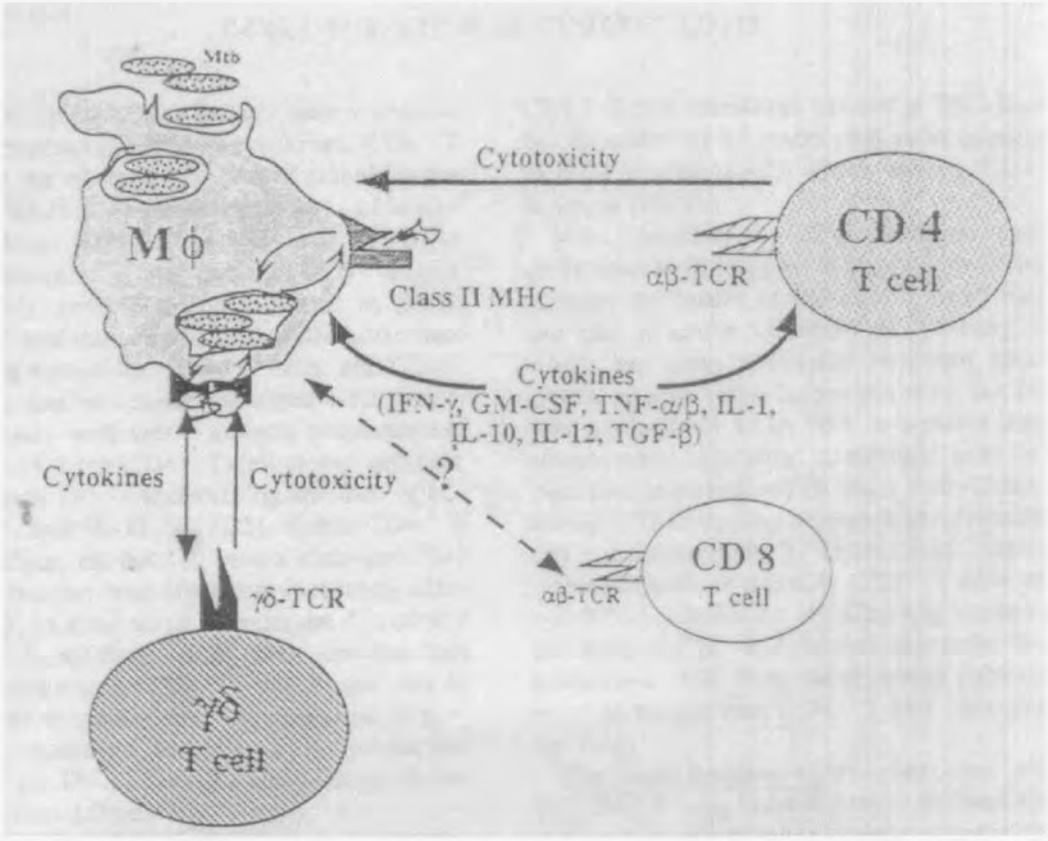


Figure 1.7: Model of the bi-directional cellular and cytokine interactions between T-cell subsets and macrophages (MΦ) infected with *M. tuberculosis* (Mtb) (Boom, 1996).

The emphasis on the Th1 mode of protection was observed by the lowered IL-4 production (Orme, 1993). Further studies have suggested that the Th1/Th2 polarity is determined by the innate immune system production of IL-12 and IL-4. In the presence of an excess of both cytokines, IL-4 dominates over IL-12 and thereby shifting the immune system towards a Th2 system (Hsieh, 1993). The Th1/Th2 balance is later maintained by competing IFN-γ and IL-4. Studies in animal models have indicated that Th2 cytokines are disease exacerbating (Sher and Coffman, 1992). Major mycobacterial infections can be defined in terms of Th1/Th2 concept. *Mycobacterium leprae* infection form the

leprotous pole, characterised by Th2, whereas *M. tuberculosis* infection forms the benign tuberculoid pole is characterised by Th1 cytokine dominance.

1.8.1 Interleukin-1

Interleukin-1 (IL-1) is the term for two polypeptides (IL-1α and IL-1β) with diverse roles in immunity and inflammation having both growth stimulatory and pro-inflammatory effects (Dinarello, 1984). This cytokine is synthesised by many cell types particularly activated MΦs. Mycobacterial cell wall antigens such as lipoarabinomannan (LAM) stimulate human monocytes to produce IL-1 (Wallis *et al.*, 1990; Zhang and Rom, 1993). IL-1 is produced as large precursor molecules cleaved by specific serine protease enzymes to yield a mature protein. IL-1 acts on T and B-lymphocytes. It particularly stimulated T-cell proliferation in the classical co-stimulatory assay (Dinarello, 1991). Secretion of IL-1 by MΦs directs expression of IL-2 by $CD4^+$ T-cells (Barnes and Modlin, 1996).

1.8.2 Interleukin-4

Interleukin-4 (IL-4) causes differentiation of B lymphocytes, acts as a growth factor for T lymphocytes and mast-cells, and exerts other effects on granulocytes, megakaryocytes and MΦs. Interleukin-4 and IL-10 are cross-modulatory in that they reduce Th1 responses and increase antibody production (Bogdan and Nathan, 1993). Even though *M. tuberculosis* is strongly counteracting the development of Th2 cell types, IL-4 secretion is observed at later stages of the disease (Orme *et al.*, 1993). Mononuclear phagocytes favour Th1 responses, whereas B cells promote a shift towards the Th2 pole. Mycobacteria potently induce IL-12 and down-regulate IL-4, and this may occur in adjunct. Mycobacterial infection may also promote the Th1 response without induction of IL-12 but by curtailing IL-4 production (Emoto *et al.*, 1997).

1.8.3 Interleukin-10

Interleukin-10 (IL-10) plays an important role in mycobacterial diseases as it was detected in pleural fluids of tuberculosis patients. The major cells producing IL-10 are monocytes which can be activated by mycobacterial cell-wall components (Barnes *et al.*, 1992; Sieling *et al.*, 1993). Naturalization of IL-10 was observed in vitro to enhance mycobacterium-specific proliferation of PBMC and increase IL-12 and IFN-γ expression (Sieling *et al.*, 1993). IL-10 also inhibits proliferation of DN-T cells and expression of CD1 molecules (Barnes *et al.*, 1992; Sieling *et al.*, 1993).

1.8.4 Interleukin-12

Interleukin-12 (IL-12), first described in 1989 by two independent laboratories, is an important regulatory cytokine mainly produced by infected MΦs. Mononuclear phagocytic cells, dendrtitic cells, neutrophils and B cells also produce IL-12 in response to bacterial infection. This cytokine has its major function in the activation of NK cells and the promotion of Th1 cell development; hence it is also known as natural killer cell stimulatory factor (NKSF) or cytotoxic lymphocyte maturation factor (CLMF). Interleukin-12 has been shown to promote the development of Th1 both *in vivo* and *in vitro* (Kobayashi *et al.*, 1989; Stern *et al.*, 1990; Scott, 1993; Hienzel *et al.*, 1993; Hsieh *et al.*, 1993; Gazzinelli *et al.*, 1993; Trinchieri and Scott, 1994; Snijders *et al.*, 1996).

The structure of IL-12 is heterodimeric consisting of two subunits. A p35 light chain (showing homology to other cytokines) and a p40 heavy chain (showing homology to the extra-cellular portion of several cytokine receptors) are covalently linked to form a biologically active heterodimer. The p40 heavy chain is secreted in large excess over the biologically active heterodimer. In murine models, the p40 heavy chain homodimers have been observed to have antagonistic activity towards the biologically active IL-12. The biological significance of the homodimer formation is still obscure, and has not been indicated in humans. Many cell types that are unable to produce IL-12 express the p35

light chain mRNA (Trinchieri and Scott, 1994; Bost and Clemments, 1995; Trinchiery and Gerosa. 1996).

Several studies have demonstrated that IL-12 expression mediates the initiation of a Th1 cell-mediated immune response *via* the induction of IFN-γ by T-cells (Koyabashi *et al.,* 1989; Sypec *et al.,* 1993; Gazinelli *et al.,* 1993; Flynn *et al.,* 1995; Bost and Clemments, 1995; Cooper *et al.,* 1995; Ladel *et al.,* 1997; Cooper *et al.,* 1997). IFN-γ is crucial for MΦ activation at the onset of the disease as it plays a protective role against *M. tuberculosis* infection in murine models tested (Flynn *et al.,* 1993; Chensue *et al.*, 1995).

Other studies have also suggested that IL-12 regulates functions of NK cells. Interleukin-12 was shown to stimulate NK cells to secrete TNF-α, GM-CSF and IFN-γ, important in host defence against bacterial infections (Chantry *et al.*, 1990; Bermudez *et al.,* 1995). These IL-12 activated NK cells trigger MΦs to inhibit intracellular growth of the mycobacteria (Bermudez and Goodman, 1995). In mice infected with *M. tuberculosis*, IL-12 administration reduced the bacterial load, reduced the pathology and prolonged survival, thus confirming the protective role of IL-12 (Zang *et al.,* 1994; Cooper *et al.,* 1995; Flynn *et al.,* 1995). Investigations have also indicated that mice resistant to *M. tuberculosis* have high base level expression of IL-12 (Flynn *et al.,* 1995; Pretorius, 1999).

In SCID mice infected with *Toxoplasma gondii*, the therapeutic effects of IL-12 suggested that this cytokine might have utility in treating opportunistic infections in HIV infected patients (Scharton-Kersten *et al.*, 1996; Walker *et al.*, 1997; Neyer *et al.*, 1997; Scharton-Kersten *et al.*, 1998). This was again supported by observations that (1) peripheral blood mononuclear cells (PBMC) from HIV patients were found to be deficient in IL-12 but showed elevated levels of IL-1 and TNF-α and (2) PBMC can respond to IL-12 stimulation by IFN-γ secretion and enhancement of NK cell lytic activity (Cantry et al., 1989; D'Addario *et al.*, 1992; Kohno *et al.*, 1992). *In vitro* studies

also demonstrated that IL-12 augmented the cytolytic activity of NK cells in HIV-positive patients toward the *M. tuberculosis* infected monocytes (Koyabashi *et al.*, 1989; Denis, 1991[a]; Denis, 1991[b]; Saunders and Cheers, 1995). Furthermore human patients infected with *M. tuberculosis* have shown an increase in the amount of IL-12 secreted (Murch, 1995).

1.8.5 Interferon-gamma (IFN-γ)

Gresser and Naficy had in 1964 described the presence of viral inhibitory activity in cerebrospinal fluids derived from patients with infectious and non-infectious diseases; they described the substance as "interferon-like" as it differed from other interferons because it was acid labile (Gresser and Naficy, 1964). Hoskins and other scientists that followed had already described an interferon since 1960. For many years IFN-γ was referred to as "acid-labile-interferon' and subsequently as "type II interferon", as opposed to acid stable IFN-α and IFN-β. IFN-γ is a cytokine that is secreted by activated T-cells and natural killer (NK) cells (Flynn *et al.*, 1993; Ogasawara *et al.*, 1998). Significant levels of IFN-γ are found in plasma, lymph nodes, and cerebrospinal fluid of HIV-infected individuals.

Secretion of IFN-γ activates MΦs as a mechanism for controlling pathogens such as *Leishmania* and *M. tuberculosis* (Flynn *et al.*, 1995; Kemp *et al.*, 1997). This cytokine stimulates killing of intracellular parasites through induction of ROI production in the MΦs. Release of hydrogen peroxide, induced by IFN-γ, was found in both human and murine MΦs (Nathan and Yoshida, 1988; Nathan, 1987; Bogdan and Nathan, 1993; Sato *et al.*, 1998). Exogenous administration of recombinant IFN-γ induced migration of Th1 cells to the site of infection and initiated granuloma formation (Bonecini-Almeida *et al.*, 1998). In experimental animals, the removal of IFN-γ in IFN-γ-gene knockout mice, exacerbated *M. tuberculosis* infection (Flynn *et al.*, 1993). Although immune-modulation

is the primary role of IFN-γ, it also has antiviral activity by working in synergy with other cytokines such as IFN-α, IFN-β, and TNF-α (Kaplan *et al.*, 1986; Hamblin, 1993).

Other cytokines such as IL-12 and IL-18 are known to be inducers of IFN-γ, and it has been suggested that IL-12 and IL-18 could act in synergy in enhancing expression of IFN-γ (Micallef *et al.*, 1996; Kohno *et al.*, 1997). In knockout mice with the p40 heavy chain subunit of IL-12 deleted, infection with *M. tuberculosis* caused a decreased IFN-γ mRNA expression even though IL-18 levels were equivalent to those of the control mice (Cooper *et al.*, 1997).

1.12.6 Tumour necrosis factor-alpha (TNF-α)

The knowledge we have today of tumour necrosis factor (TNF) is the culmination of over two decades of research. Tumour necrosis factor-alpha (TNF-α) was first isolated in 1975 by Carswell and colleagues (Helson *et al.*, 1975; Carswell *et al.*, 1975). TNF-α is generally considered to be a product of activated macrophages, but is also secreted by lymphocytes, that include T-cells, thymocytes, B cells, mast-cells and NK cells. The production of TNF-α is partially stimulated by lipopolysacharides (LPS), other cytokines and the cell-wall components of mycobacteria (Carswell *et al.*, 1975). Although cytotoxicity may not be the major activity of TNF-α *in vivo*, it was one of the first activities attributed to it. TNF-α represents the most important member of the inflammatory cytokines (Tracey and Cerami, 1993[a]). TNF-α is considered both protective and pathologic because of its central role in granuloma formation and maintenance (Tracey and Cerami, 1993[b]). Macrophages infected with *M. tuberculosis* are major sources of TNF-α. TNF-α activates murine MΦ to produce RNI. TNF-α induces the activation of the L-arginine dependent cytotoxic pathway, which results in the generation of NO.

Transgenic mice unable to produce TNF-α have enhanced susceptibility to BCG and *M. tuberculosis* infection (Yoshida *et al.*, 1995; Garcia *et al.*, 1997). *In vitro* depending on the cell type or growth conditions, TNF-α has been suggested to induce necrosis or apoptosis with infection. Apoptosis is mainly induced by NO production stimulated by TNF-α. Addition of antibodies against TNF-α resulted in inhibition of NO production and apoptosis stopped (Aung *et al.*, 1996; Rojas *et al.*, 1997). Administration of TNF-α in *M. avium* infected mice inhibited intracellular and exogenous growth of mycobacteria (Denis, 1991[a]; Denis, 1991[b]).

Dendritic cells infected with *M. tuberculosis* were shown to produce elevated levels of TNF-α and IL-12 (Henderson *et al.*, 1997). The mechanism involved in the macrophage-mediated killing or growth restriction of the bacteria is still unclear. There is evidence for the involvement of ROI. Inactivated MΦ exert a base level of killing, which could be blocked by inhibiting the intrinsic L-arginine-dependent pathways by metabolic inhibition. Macrophages are fully activated by TNF-α and thus exerting full microbicidal activity. It has been demonstrated *in vitro* that TNF-α treatment of cells can efficiently kill non-virulent *Mycobacterium avium* (*M. avium*), whereas virulent *M. avium* has been prevented from growing. Superoxide dismutase (SOD) addition into cell cultures protected non-virulent *M. avium* from being killed by TNF-α-activated macrophages (Denis, 1991[a]).

1.13 Tuberculosis control

Mycobacterial diseases are today continuing to be associated with a high burden of morbidity and mortality in humans. Despite great advances in medical science and a range of effective drugs, which for some time created the impression that the disease has been conquered, tuberculosis remains a major global health problem. Pathogenic mycobacteria have a low rate of multiplication and reside intracellularly in macrophages of the host. As such they are hard to eradicate. The treatment for mycobacterial diseases

consists of a long course of combination chemotherapy. The long course of treatment easily leads to non-compliance and thus may contribute to the emergence of multi-drug resistant strains of mycobacteria (Heym *et al.*, 1994). The use of isoniazid (INH) as an antituberculous drug in 1952 has been one of the greatest achievements in the fight against tuberculosis. This drug functions in preventing biosynthesis of mycolic acids in the cell wall of mycobacteria.

1.9.1 Tuberculosis notification and treatment

The control of *M. tuberculosis* infection is primarily dependent on availability of effective structures for diagnosis and treatment management. At present there is available a treatment system known as directly observed treatment short course (DOTS). The World Health Organisation (WHO) has adopted this strategy to control *M. tuberculosis* infection after declaring tuberculosis a global emergency. Case notification of tuberculosis (Fig. 1.8.) closely approximates the true incidence in countries with effective tuberculosis control programmes. Case notification in countries like South Africa, where only a minority of the population have effective tuberculosis care, represents only a fraction of the true incidence (http://www.who.int/)

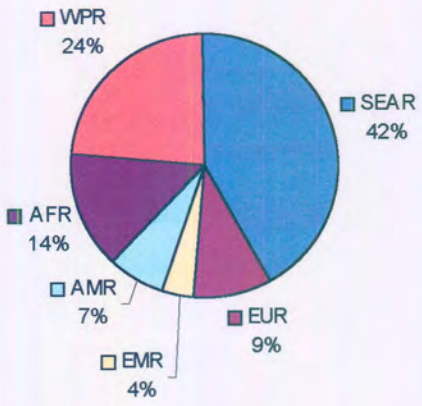


Figure 1.8: World distribution of notified tuberculosis cases by World Health Organisation (WHO) region. AFR-sub-Saharan Africa; AMR, Americas; EMR, Eastern Mediterranean; EUR, Europe; SEAR, South-East Asia; WPR, Western pacific (http://www.who.int/).

Even in the era of effective chemotherapy, scores of deaths occurs due to *M. tuberculosis* infection. This is not only due to co-infection with HIV, which is predominant in sub-Saharan Africa, but also to the lack of compliance with the DOTS programme. Non-compliance with the DOTS programme results in tuberculosis relapse. This could then lead to a drug resistant tuberculosis. Multi-drug resistant tuberculosis may result either from primary drug resistance, due to transmission of an already existing resistant strain, or secondary drug resistance, which refers to the emergence of the disease due to non-compliance to the six months DOTS programme. In developing countries with poor TB management programmes, this situation is aggravated by lack of fast and accurate diagnosis and testing methods (http://www.who.int/).

1.9.2 Tuberculosis diagnosis

Effective tuberculosis control requires fast and accurate methods for diagnosing infection. Various methods are today available for tuberculosis diagnosis based on DTH, staining, chromatography, DNA detection, and antibody responses. Techniques based on antibody detection are discussed in chapter 5.

1.9.2.1 Tuberculin test

The tuberculin skin test is the most widely used method of diagnosing *M. tuberculosis* infection. Despite a significant degree of variability, this test is considered to be reliable and available (Chaparas *et al.*, 1985). This is a skin test based on the modification of the Robert Koch experiment that used killed *M. tuberculosis*. This test measures the delayed-type hypersensitivity (DTH) to the tuberculosis protein (Lordi and Reichman, 1999).

1.9.2.2 Acid-fast staining

Because mycobateria have high lipid content and are thus difficult to Gram stain, acid-fast staining is usually used. The two techniques that are commonly used are the carbol-fuschin and the fluorescence methods. The carbol-fuschin method comprises of the Ziehl-Neelsen and the Kinysen procedures. These stain mycobacterial cells red against a methylene blue counter stain. The fluorescence stain method apply auramine O or auramine-rhodamine dyes. Auramine O stains bright yellow and auramine-rhodamine stains gold against a dark background. The fluorescence methods have an advantage of sensitivity over the carbol-fuchsin methods. The major disadvantages of both these methods are that they are relatively non-specific as they also stain non-viable bacteria (Zheng and Roberts, 1999).

1.9.2.3 Chromatographic identification

Gas-liquid chromatography (GLC) requires extraction of lipids, which are then analysed by a computer-aided gas liquid chromatograph (Etemadi, 1967). The advantage of this technique is that results can be obtained in a few hours and that it requires small amounts of mycobacteria. In developed countries, 77% of tuberculosis have been identified by GLC. The use of high-performance liquid chromatography (HPLC) is now more popular for diagnosing *M. tuberculosis*, as with this technique a particular species of mycobacteria can be identified (Glickman *et al.*, 1994). Mycolic acids, the abundant long-chain fatty acid in mycobacterial cell walls, produce distinguishable species-specific chromatographic patterns when separated by HPLC. The number, heights and positions of cluster of peaks can thus be used as a fingerprint of a particular species of mycobacteria (Roberts *et al.*, 1996; Zheng and Roberts, 1999).

1.9.2.4 DNA based techniques

Studies have indicated that nucleic acid amplification methods are promising in *M. tuberculosis* detection. This is mainly due to the advent of polymerase chain reaction (PCR) methods. These detection methods include target, probe, and signal amplification techniques. These techniques could either use ribosomal ribonucleic acids (rRNA) or parts of the genomic deoxyribonucleic acids (DNA) as selected targets. There are today commercially available kits that have been demonstrated to yield good performances on *M. tuberculosis* detection. The disadvantage of these methods is that they are expensive and still require growth cultures, which are time consuming. The other disadvantage is that they also rely on good laboratory practise (GLP) to avoid contamination and therefore appropriate expertise for successful implementation (Zheng and Roberts, 1999). This makes them difficult to implement in major parts of the developing countries.

1.9.2.5 The demand for a serodiagnostic tuberculosis test

There has been a strong demand for a reliable and rapid serologic diagnostic method. Several methods for serologic diagnosis *M. tuberculosis* have been reported using mycobacterial antigens such as polypeptides, polysaccharides, phospholipids and other cell-wall components (Young *et al.*, 1987; Oswald *et al.*, 1997). Serodiagnostic tests are usually fast and affordable, but are seldomly sensitive and specific. This is because co-infection with HIV oppresses antibody production in tuberculosis patients while cross-reactivity of antibodies with other antigens can occur.

1.10 Mycobacterial mycolic acids in tuberculosis resistance

Andersen (1929[a] and 1929[b]) isolated an acid called mycolic acid from *M. tuberculosis* waxes by prolonged saponification. Mycolic acids represent the most abundant-cell wall component of *M. tuberculosis*. Mycolic acids were found to be immuno-regulatory as they stimulated proliferation of DN T-cells in a MHC-independent manner through CD1b molecules (Beckman *et al.*, 1994).

Like the MHC I molecule, the CD1 protein consist of α 1, α 2 and α 3 extracellular domains associated with β_2-microglobulin. Binding of lipids to CD1 probably occurs through hydrophobic interactions, as the α 1 and α 2 domains are unusually hydrophobic. The α 1 and α 2 domains of CD1 molecules render them remotely homologous to MHC. The CD1 molecules are divided into two groups: Group 1 consists of CD1a, -b and -c that are expressed on human professional antigen presenting cells, whereas group 2 consists of CD1d and –e in human APCs and CD1d1 and CD1d2 in murine APCs (Calabi *et al.*, 1989; Calabi and Bradbury, 1991; Bendelac, 1995; Porcelli *et al.*, 1995). The expression of CD1 on APCs requires activation by granulocyte MΦ colony stimulatory factor (GM-CSF), a non-specific cytokine expressed by both Th1 and Th2 cells, and IL-4 that is associated with a Th2 response (Kasinrerk *et al.*, 1993; Thomssen *et al.*, 1995[a]; Thomssen *et al.*, 1995[b]).

1.11 Perspectives of this study

The observations that subsets of T-cells are activated when CD1 presented mycolic acids are recognised, has led to the assumption that mycolic acids can also have an influence on the immune system in *M. tuberculosis* infected animals. Studies preceding this one have made attempts to elucidate whether mycolic acids would add to the pathology of tuberculosis or to resistance. Siko (1999) and Pretorius (1999) found that mycolic acids helped to control tuberculosis in experimental animals, and that the protective response manifested in the cytokines in the lung and not the spleen of Balb/c mice. Stoltz (2002) showed that mycolic acids have pronounced effects on macrophages.

The first phase of this study was then focused on further elucidating these observations by investigating the cytokine response when enhanced protection is achieved. In previous studies (Siko, 1999 and Pretorius, 1999) infection with *M. tuberculosis* was performed intravenously, which lead to systemic infection in which the protective effect of mycolic acids on survival was minimal. In this study the objective was to localize infection in the lungs through intranasal infection as the response to mycolic acids administration manifested itself in the lungs. The resulting cytokine profiles induced by mycolic acids were then investigated by comparing traditional semi-quantitative endpoint polymerase chain reaction (PCR) (used in previous studies to analyse cytokine expression) with real-time quantitative PCR.

Previous studies indicated that mycolic acids pre-treatment of C57Bl/6 mice had no effect and that the mycolic acids merly improved the resistance of Balb/c mice to that of the level of resistant C57Bl/6 mice (Siko, 1999). Pretorius (1999) suggested that the resistance of C57Bl/6 mice was due to a higher base level of IL-12 in the lungs. The second phase of this study attempted to exploit this by impairing the immune system of C57Bl/6 mice with an immunedeficiency virus and then pre-treating them with mycolic acids before *M. tuberculosis* infection. This was done with the objective of elucidating if

mycolic acids would assist in the control of tuberculosis in a situation reminiscent of co-infection with HIV in humans.

For tuberculosis chemotherapy to be effective, tuberculosis patients have to take large doses of drugs for at least 6 months leading to non-compliance and relapse. This calls for an alternative approach. Immunotherapy was looked into as an adjunct treatment to shorten the duration of chemotherapy. Chemotherapy combined with IL-12 DNA vaccine immunotherapy was previously found to be successful in clearing *M. tuberculosis* in Balb/c mice (Lowrie *et al.*, 1999). Mycolic acids have been found to elicit expression of IL-12 in *M. tuberculosis* infected and uninfected Balb/c mice (Pretorius, 1999). The third phase of this study therefore assessed the immunotherapeutic value of mycolic acids in combination with chemotherapy. This was done with the objective of administering mycolic acids to mice that have undergone chemotherapy to establish if mycolic acids will provide *M. tuberculosis* clearance in the lungs and the spleens.

Studies have indicated that antibodies to trehalose 6,6 dimycolate (cord factor) can be detected in active (smear-and culture positive) and inactive (smear and culture negative) tuberculosis patients. The antigenic epitope of cord factor was found to be mycolic acids and specifically oxygenated mycolic acids by Pan and co-workers who suggested that antibodies to mycolic acids could serve as surrogate markers of infection (Pan *et al.*, 1999; Fujiwara *et al.*, 1999). Pretorius (1999) had also suggested this through determining the specificity of anti-mycolic acids antibody signal by inhibition of binding with mycolic acids pre-incubation of serum. Schleicher *et al.* (2002) showed that the anti-mycolic acids antibodies were also present at similar levels in TB patients, irrespective of co-infection with HIV, but that the predictiveness of the test was poor. The fourth phase of this study envisaged developing a biosensor system to opportune further characterisation of anti-mycolic acids antibodies in an effort to explore the possibility of developing a serodiagnostic test of anti-mycolic antibodies based on their real-time binding properties.

CHAPTER 2

Mycolic Acids-Induced Cytokines in *M. tuberculosis*-Infected Mice

2.1 Introduction

The inability to control tuberculosis represents one of the greatest health problems currently experienced worldwide, as it is generally considered to be a curable disease. The pathogenesis, transmission, diagnosis, treatment and prevention of the disease have been known for decades already (Reichman, 1997). Chaparas (1982) recognised the need to understand the nature of subcomponents of *M. tuberculosis* and the manner in which they evoke their respective effects, to be able to comprehend the biological, pathological, and immunological consequences of infection. This is because the intact cells of killed *Mycobacterium* induce a complex average response that is not easy to understand, whereas, the component parts may elucidate the roles that each play when the cell is broken down by the host immune response (Chaparas, 1982).

Mycobacterial cell wall components such as cord factor have been implicated in eliciting immune response in the form of cytokine expression (Guillemard *et al.*, 1998). Cytokines produced by the macrophages affect a variety of immune processes and also regulate production of other cytokines and T cell subsets (Flynn *et al.*, 1995).

2.1.1 Cytokine profiling

It is difficult to profile cytokine response to disease mainly due to a wide spectrum of cytokines produced. Cytokine detection is also difficult due to the very low concentration of these substances (Londei *et al.*, 1991; Londei *et al.*, 1989). This is because cytokines are expressed transiently upon an immune challenge. Detection of cytokines *in vitro* can be achieved either by immunoassay [radioimmunoassay (RIA),

enzyme-linked immunosorbent assay (ELISA), immunoradiometric assay (IRMA)] or by bioassay (Hamblin, 1993). Immunoassays are quick, sensitive and reliable, but have the disadvantage of producing falsely positive results. The shortcoming of bioassays on the other hand, is that the may respond to more than one cytokine as they are often less specific (Contreras *et al.*, 1991).

2.1.2 Quantitative cytokine analysis

Quantitative detection of cytokines is now mainly done by reverse transcriptase polymerase chain reaction (RT-PCR). Before the development of the polymerase chain reaction (PCR) technique, the gene quantification has been hampered by lack of fast, reliable and accurate methods. Northern-blotting (Alwine *et al.*, 1977) could work well, but it requires a large amount of RNA and is also time consuming. The polymerase chain reaction (PCR) has proved useful by the ability to amplify specific mRNA that is converted to cDNAs, especially those present in low copy numbers (Taniguchi *et al.*, 1993).

The combination of reverse transcription and PCR revolutionised mRNA quantification (Becker-Andre and Hahlbrock, 1989; Gilliland *et al.*, 1990; Wang *et al.*, 1989). With the advent of reverse transcriptase (RT) PCR, quantitative (Q) PCR techniques could now be employed to quantify expressed mRNA from cells and tissues. To obtain close to absolute values, competitive-qualitative QPCR was used employing a competitor standard-sample in a reaction vessel. This procedure is very laborious and time-consuming, which makes it unsuitable for large sample groups.

Semi-quantitative RT PCR is more applicable for large sample groups. This technique applies analysis of amplified cDNA against a constitutively produced mRNA such as β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAP-DH). This end-point technique is also laborious, as it requires that PCR products be retrieved at specific points at the linear stage of the DNA amplification. The linear stages of amplification are determined by plotting quantity of DNA amplified against the number of cycles. This form of quantitative PCR analysis, called "kinetic analysis," was first described

using ethidium bromide, a non-sequence-specific fluorescent dye, to detect the PCR product.

Fluorimetry has since been combined with PCR to report events of every amplification cycle and thereby eliminating the need to determine the linear phase of amplification for every test sample in comparison to a standard in different reaction vessels. This was then the advent of real-time PCR. The availability of sequence specific reporter dyes made it possible to amplify and detect the target amplicon and the endogenous control amplicon in the same tube (multiplex-PCR) (Higuchi *et al.*, 1992). The PCR reaction exploits the 5′nuclease activity of DNA polymerase to cleave a probe during PCR. The probe contains a reporter dye at the 5′ end of the probe and a quencher dye at the 3′ end of the probe. During the reaction, cleavage of the probe separates the reporter dye and the quencher dye, which results in increased fluorescence of the reporter (Holland *et al.*, 1991; Higuchi *et al.*, 1993; Jordan, 2000). Figure 2.1 shows how the 5′ to 3′ nuclease of AmpliTaq Gold™ DNA polymerase enzyme acts during PCR (Applied Biosystems, 2000).

The accumulation of PCR products can be detected directly by monitoring the increase in fluorescence of the reporter dye. Figure 2.2 depicts, the graph of normalized reporter (Rn) versus cycle number during PCR. This process has three stages: during the first phase at lower cycles Rn is represented by a flat line (Phase 1), as the fluorescent signal is below the detection limit; the second phase is depicted by an increasing signal that is directly proportional to the increasing PCR product (Phase 2); finally in the third phase, the ratio of polymerase enzyme to PCR product decreases (Phase 3) and the product ceases to grow exponentially (Applied Biosystems, 2000).

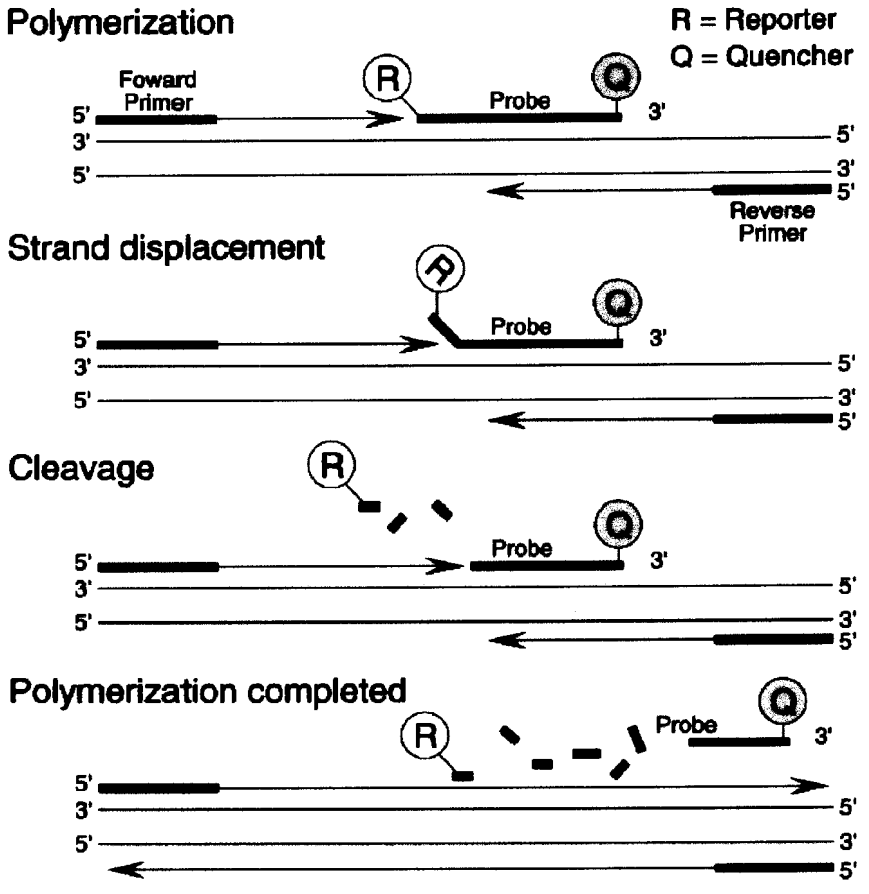


Figure 2.1: The 5' nuclease activity of the DNA polymerase enzyme exploited to detect amplified DNA. (Applied Biosystems, 2000)

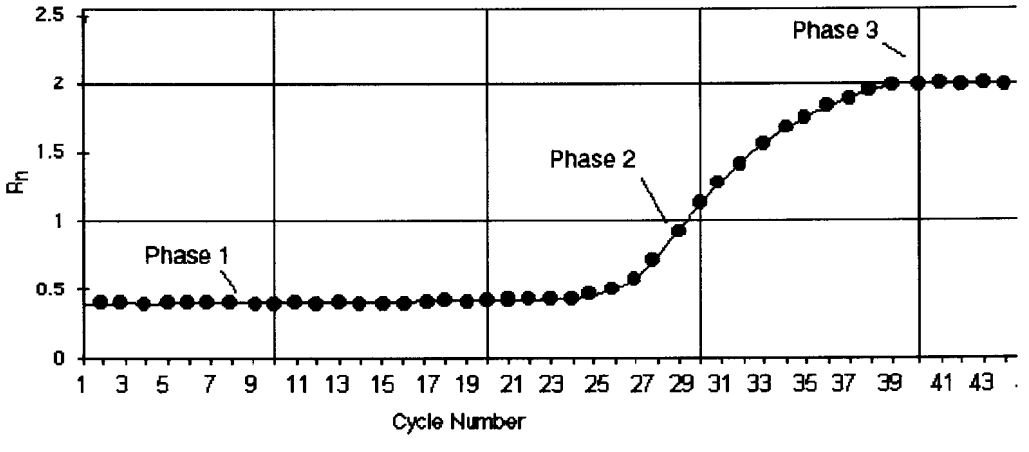


Figure 2.2: Schematic representation of normalised reporter (Rn) versus cycle number. (Adapted from Applied Biosystems, 2000).

2.2 Study Approach

This study has been motivated by previous investigations (Siko, 1999), where mycolic acids have been found to elicit an immune response in Balb/c mice. Mice that were pre-treated with mycolic acids before intravenous infection with *M. tuberculosis* were found to have a prolonged life span. This protection correlated with an enhanced expression of cytokines such as IL-12 and IFN-γ in the lungs suggestive of mycolic acid-induced Th1-bias (Pretorius, 1999). Further investigations indicated that this protection could be enhanced when the infection was intranasally introduced to the lungs, which are the normal port of *M. tuberculosis* entry. Mycolic acids were then shown to significantly enhance survival in Balb/c mice that were intranasally infected with *M. tuberculosis* (Lombard, 2002).

By using the semi-quantitative RT-PCR, Lombard (2002) was unable to assess accurately the cytokines that would test the hypothesis that mycolic acids pre-treatment improved the resistance of the mice by inducing a Th1-bias. This study focused on using the more sensitive technique of real time quantitative (RQ) RT-PCR, to analyse the cytokines and reassess the observations made with end point semi-quantitative RT-PCR and also predict an immune mechanism involved.

2.3 Aims:

1. Demonstrating the advantage of RQ-RTPCR over end-point SQ-RTPCR.
2. Elucidating the immune mechanism of protection provided by mycolic acids pre-treatment in Balb/c mice before intranasal infection with *M. tuberculosis*.

2.4 Materials

2.4.1 Bacteria

Mycobacterium tuberculosis H37Rv ATCC 27294 - type strain: A virulent strain, originally isolated from an infected human lung. The culture was purchased in lyophilized form from the American Type Culture Collection (ATCC), Maryland, USA. A detailed composition of the ingredients necessary for the preparation of the media as well as the conditions recommended for their sterilization, are given in the Laboratory Manual of Tuberculosis Methods, Tuberculosis Research Institute of the SA Medical Research Council (1980, Chapter 6, pp 83-105; Second Edition, revised by E E Nel, H H Kleeberg and E M S Gatner).

2.4.2 Animals

Inbred female Balb/c mice were bought from the South African Institute for Medical Research (SAIMR-Johannesburg). The mice used in this study were of the age 6 to 10 weeks. The animals were kept in a glove isolator in a temperature and humidity controlled room.

2.4.3 Reagents

Reagents used in the Semi-Quantitative Competitive Reverse Transcriptase Polymerase Chain Reaction (QC-RT-PCR) or end-point PCR and Real-time Quantitative Reverse Transcriptase Polymerase Chain Reaction (RQ-RT-PCR):

Ethidium bromide (Boehringer Mannheim, Germany)

Formamide and formaldehyde (BDH, Poole UK)

Tris (Hydroxymethyl)-aminomethane (Merck, Darmstadt Germany)

EDTA (Ethylenediaminetetra-acetic acid) (Merck, Darmstadt Germany)

Sodium acetate (Merck, Darmstadt Germany)

TRI-reagent (Molecular Research Centre Inc, USA)

Formazol (Molecular Research Centre Inc, USA)

MOPS (3-(*N*-morpholino) propanesulphonic acid) (Sigma Chemicals, St Louis USA)

Diethyl pyrocarbonate (DEPC) (Sigma Chemicals, St Louis USA)

Oligo dT primers (Life Technologies Inc., Scotland)

Superscript™ RNase H Reverse Transcriptase (Life Technologies Inc., Scotland)

Recombinant RNasin (Promega Corporation, Woods USA)

Amplitaq Gold™ (Roche Molecular Systems, New Jersey USA)

Qiagen mini preparatory column Kit (Qiagen GmbH, Hilden Germany)

Tris EDTA buffer: Tris base 10 mM disodium ethylene diamine tetraacetate.$2H_2O$, pH adjusted to pH 8,3.

For the preparation of the reagents used for the extraction, derivatization and High-Performance Liquid Chromatography (HPLC) analysis of mycolic acids, HPLC Grade methanol (BDH) and double-distilled deionized water were used.

2.5 Methods

2.5.1 Preparation of mycobacteria and Preparation of mycobacterial suspensions

Mycobacterium tuberculosis H37Rv (ATCC 27294) was cultured on Löwenstein-Jensen (LJ)-slants at 37°C for 3 to 6 weeks. Cells of total count varying between 10^4 and 10^6 per ml were suspended in 0.89 % sterile saline, washed twice by centrifugation (2000 x g for 15 min), and re-suspended in 0.89 % saline. Bacterial titers (viable counts) of the samples were determined by counting colony forming units (CFU) in 1:10 to 1:1 000 000 dilutions on Middlebrook 7H10 agar after incubation at 37 °C for 4 to 6 weeks.

Total count was determined directly by using a Neubauer counting chamber and various dilutions of bacterial suspensions. Staff from the National Tuberculosis Institute of the Medical Research Council of South Africa, Pretoria, prepared the media. The sterility of all the media was confirmed before they were used in the experiments by observation after incubating them at 37°C for 24 h.

The harvested bacteria were washed with sterile 0,9% m/v NaCl (Chemically Pure, Saarchem, RSA). Medium used for the preparation of serial dilutions, preceding the determination of viable counts of *M. tuberculosis* was prepared by dissolving Tween 80 (Chemically Pure, Merck) in 0,9% m/v NaCl (Saarchem, Chemically Pure) to a concentration of 0,01% v/v and distributing it in 9,0 ml aliquots into test-tubes. The autoclaved media were stored at 4°C.

2.5.2 Mycolic acids preparation

M. tuberculosis -derived mycolic acids (MA) were extracted, purified and derivatised as described by Butler *et al.* (1991), and purified according to Siko (1999). Briefly, bacteria were scraped from LJ slants and resuspended in 25 % potassium hydroxide in methanol-water (1:1). The suspension was vortexed in the presence of glass beads

and homogenized. The cells were subsequently saponified by autoclaving at 120 °C for 30 min. This was followed by the addition of 1.5 ml 50 % (v/v) HCl per 2 ml sample to adjust the pH to a reading of 1. The lower phase was transferred to a new vial, after two times chloroform-water (1:1) extractions. The chloroform was evaporated at 85 °C in a heat-block evaporator, under a flow of nitrogen. A volume of 100 µl of 2 % K_2CO_3 dissolved in methanol-water (1:1) was added and the sample was evaporated to dryness.

This crude saponified extract was used to purify MA by countercurrent separation (King and Craig, 1962). A biphasic, tri-component solvent system consisting of 42 % (v/v) chloroform, 39 % (v/v) methanol and 19 % (v/v) 0.2 M NaCl was used.

Purity analysis was done by HPLC after the MA sample was derivatised with para-bromophenacylbromide (Pierce, Rockford, Illinois, USA) in acetonitrile and crown ether (100 µl per 2 ml sample).

The samples were vortexed and heated for 20 min at 85 °C, followed by the addition of concentrated HCl-methanol-water (1:2:1). The bottom layer was removed and evaporated to dryness at 85 °C under a stream of nitrogen. The dried residues were re-suspended in methylene chloride and quantified by HPLC in comparison to an internal standard.

Saponification of MA: The purified MA were saponified before conjugation with mouse serum with 25 % potassium hydroxide in methanol-water (1:1) at room temperature for 15 min and were thereafter extracted with chloroform, after the excess potassium hydroxide was neutralized with concentrated HCl. Excess salts were removed by several chloroform water (1:1 v/v) extractions (Butler *et al.*, 1991).

Preparation of MA-serum conjugate and control serum: Blood was obtained by bleeding male Balb/c and C57BL/6J mice from the dorsal tail vein. Serum was obtained after the blood was allowed to clot at 4 °C for 60 min, followed by

centrifugation (12000 g for 10 min). The sera of each strain were pooled and were stored at -20 °C until used.

Conjugates of MA and serum were obtained by adsorption. MA were analytically weighed and dissolved in chloroform. The volume of chloroform that was added never exceeded 2 % of the final volume of serum into which the MA were absorbed. The chloroform/MA were added to the serum and were sonicated on a Branson sonifier B-30 for 50 pulses at an output control of 2 and a 20 % duty cycle at room temperature.

Control serum was prepared by adding 2 % pure chloroform (v/v) to the serum before the mixture was sonicated as described above for the preparation of MA-serum conjugate. The chloroform was removed by blowing nitrogen over the surface of the vigorously agitated mixture. The serum was left to stand at room temperature for at least one hour to allow air bubbles to escape, before it was injected in the mice.

The MA-serum conjugate containing the highest concentration of MA was prepared by the sonication method as described above. Lower concentrations were prepared by dilution of the highest concentration using chloroform-treated mouse serum. To verify the presence of the MA in the mouse serum conjugate, HPLC analysis was performed on the highest concentrations according to the method described above.

2.5.3 Treatment, infection and preparation of the organs

Mice were divided into groups of at least 8 mice per group. The mice were inoculated by injection of 2.5 x 10^5 colony forming units (CFU) of *M. tuberculosis*, intranasally (i.n.) with 100 µl 0.9 % NaCl, or 100 µl 0.9 % NaCl. One week prior to infection, mice from the pre-treatment groups were injected with the MA-serum conjugate (5 µg, or 25 µg) or with chloroform-treated serum alone. The organs originating from *M. tuberculosis*-infected and uninfected mice, used for the Real-Time PCR analysis, were the lungs. Mice were sacrificed by rapid cervical dislocation. The organs were

removed from each mouse aseptically and kept at -72 °C after snap freezing in liquid nitrogen.

2.5.4 RNA extraction and quantification

Total RNA of the organs was extracted using TRI-reagent (Molecular Research Centre Inc, Cincinnati, USA), based on a method developed by Chomczynski and Sacchi (1987). Before the RT reaction, the RNA was co-precipitated with primer as described by Maniatis (1982). Total RNA (6µg) was precipitated overnight at -20 °C in the presence of 3 pmol Oligo(dT)12-18 (Gibco BRL, Gaithersburg, MD, USA) with 0.1M NaOAc, pH 5.5 and absolute ethanol (1:2). After centrifugation, the pellet was washed with 70 % ethanol-DEPC treated H_2O and allowed to dry briefly for about 10 to 15 minutes. Subsequently, the RNA was dissolved in a resuspension buffer [80 mM Tris-HCl pH 8.3, 90 mM KCl and 40 U RNasin (Promega, Madison, WI, USA)], heated to 70°C for 10 minutes and followed by a 3 hour incubation at 37 °C. RNA was quantified using the GENEQUANT RNA/DNA calculator (Pharmacia) and the integrity of the RNA visualised on a 1 % denaturing agarose gel. From that cDNA was synthesised using the Superscript™ reverse transcriptase enzyme (Gibco BRL, Gaithersburg, MD, USA) as recommended by the manufacturer.

2.5.5 End-point Semi-quantitative reverse transcriptase polymerase chain reaction (SQ-RTPCR)

To reach non-saturating concentrations of amplification product in the RT-PCR reaction, three different dilutions of cDNA for each sample were assayed. The 20 µl reaction mixtures of all the samples contained 0.2 mM each of dATP, dCTP, dGTP and dTTP and 0.04U/µl of Amplitaq Gold™ enzyme. The magnesium chloride ($MgCl_2$) was 2mM and the primer concentrations 5ng/µl for β-actin. The $MgCl_2$ for IL-12, IFN-γ, TNF-α, IL-10 and IL-4 was 1.5mM. The primer concentrations for IL-12 were 5ng/µl; 12.5ng/µl for IFN-γ, TNF-α and IL-4 and 6.25ng/µl for IL-10. The primer sequences for β-actin were described by Ma *et al.* (1994); those for IL-12 by

Chong *et al.* (1996); those for TNF-α and GM-CSF by Benavides *et al.* (1995) and those for IFN-γ, IL-10 and IL-4 by Reiner *et al.* (1994). For each cytokine all samples were amplified during the same PCR in a Perkin Elmer thermal cycler. The Amplitaq Gold enzyme was activated by incubation at 94°C for 10 minutes. The second cDNA strands were synthesised by denaturing at 94°C for 45 seconds, annealing at 60°C (β-actin, IL-12 and IFN-γ) or 48°C (TNF-α) for 75 seconds and extension for 105 seconds at 72°C.

The subsequent amplification cycles were as follows: denaturing at 94°C for 35 seconds, annealing at 60°C (β-actin, IL-12 and IFN-γ) or 48°C (TNF-α) for 45 seconds and extension for 75 seconds at 72°C. For each cytokine, preliminary experiments were performed to define the optimal number of cycles such that aliquots would be obtained during the linear phase of the PCR reaction. β-actin samples were amplified for 27 cycles, IL-12 samples for 33 cycles, IFN-γ samples for 28 cycles, TNF-α samples for 28 cycles.

In all experiments negative controls without cDNA always gave negative results. An equivalent amount of RNA of each sample was amplified with β-actin primers but no products could be detected. Amplification products were analysed by electrophoresis in 2% agarose gels and visualised by ethidium bromide staining. The amount of target cytokine mRNA was determined by measuring the signal intensities using NIH Image densitometry. Linear regression was used to determine that the volume of each sample contained the same amount of cytokine mRNA as a chosen reference sample. The β-actin signal was taken as an indicator of the actual amount of cDNA used in each PCR reaction. Therefore, the values for all the other cytokines were normalised for β-actin and expressed in relative units.

2.5.6 Real-time quantitative PCR

Real-time PCR was performed using TaqMan™ Universal PCR Master Mix™ protocols with pre-developed TaqMan™ assay reagents i.e. rodent cytokines, house-

keeping gene (GAP-DH) primers and probes (Applied Biosystems, Norwolk, CT, USA). Each sample was analysed in triplicate on a 96 well optical plate made out of 50µl of the sample reagent (Table 2.1). Analyses were made on ABI PRISM 7700 sequence detection system over 50 thermal cycles. The Real-time PCR system of the TaqMan™ PCR Core Reagents Kit™ performs 5' nuclease assay using TaqMan™ probes with sample template cDNA. The PCR product is directly detected with processing within minutes of PCR completion by monitoring the increase of fluorescence of a dye-labelled DNA probe. The statistical significance of the results was tested using the Student t-test.

Table 2.1: Murine Pre-Developed TaqMan™ Assay reagent plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	1				2.2			5.1				
B	0.1				2.3			5.2				
C	0.02				3.1			5.3				
D	0.01				3.2			6.1				
E		1.1			3.3			6.2				
F		1.2			4.1			6.3				
G		1.3			4.2		NTC					
H		2.1			4.3		NAC					

NTC = No template control (No cDNA), NAC = No amplification control (No enzyme)
The shaded block A1 to D3 represents the positive control dilution triplicates and numbers 1.1 to 6.3 represents triplicates of lungs from different groups with the first digit representing the group number and last digit the mouse number.
The areas between the jagged lines were activated for sample detection during the cycles.

2.6 Results

Pre-treatment of Balb/c mice with mycolic acids provided significant protection ($P<0.005$) against tuberculosis when compared to serum-only pre-treatment (Figure 2.3). Pre-treatment of Balb/c mice with 25 µg mycolic acids before intranasal infection with *M. tuberculosis* (open squares on the graph) was able to maintain 90% percent survival for 40 weeks, as compared to the 20% observed with 5 µg mycolic acids pre-treatment (open circles on the graph) and serum pre-treated (open upside-down triangles). The effects of mycolic acids pre-treatment on cytokine expression in the *M. tuberculosis* infected Balb/c mice were determined for this experiment by real-time quantitative RT-PCR and IL-12 and IFN-γ value were compared with those obtained by endpoint semi-quantitative RT-PCR.

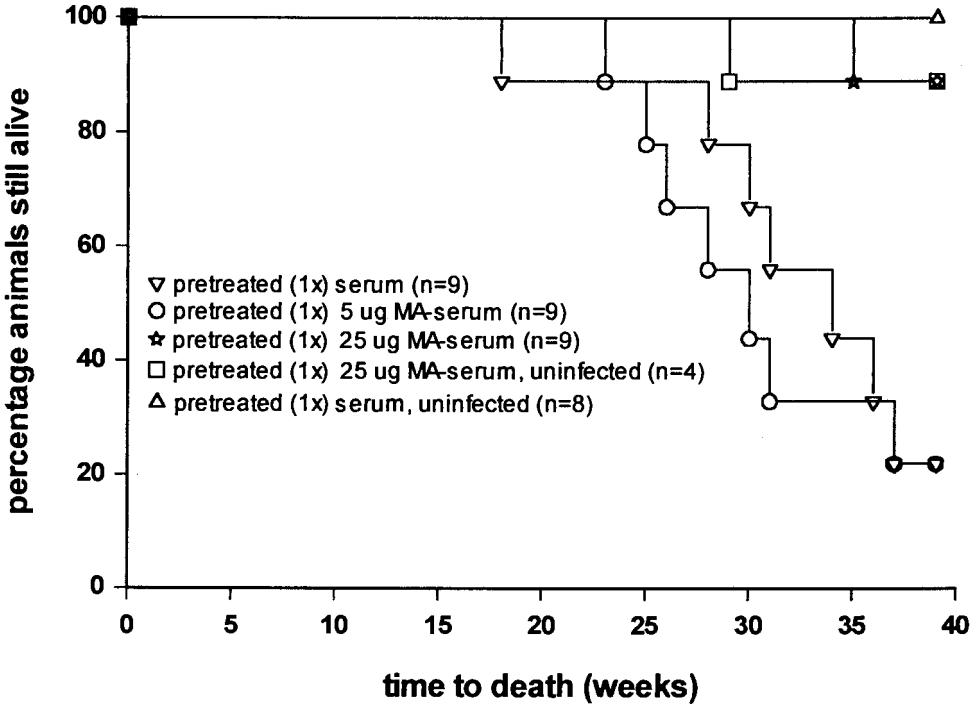
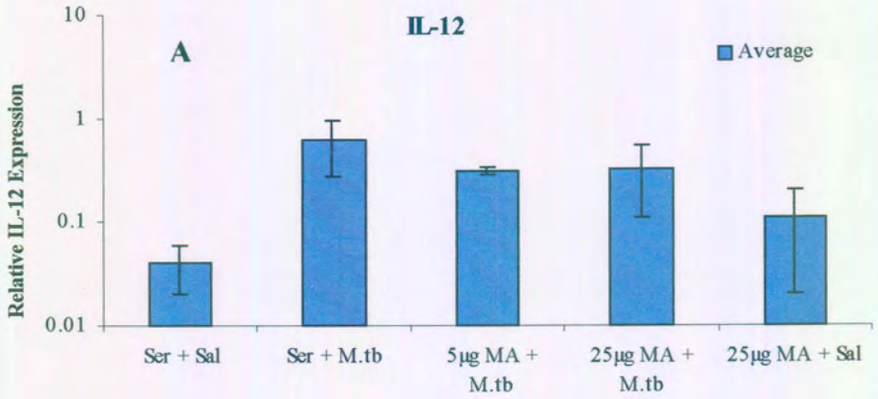


Figure 2.3: Survival of BALB/c mice infected intranasally with 2.5×10^5 *M. tuberculosis* H37Rv after pretreatment with purified mycobacterial mycolic acids (MA) conjugated onto normal mouse serum.

2.6.1 Interleukin-12 (IL-12) mRNA expression

The expression of IL-12 mRNA measured with the traditional end-point PCR method was here compared to the expression of IL-12 measured with the real-time quantitative PCR (RQ-PCR) method. Measurements made with both techniques displayed similar IL-12 mRNA expression patterns, with RQ-PCR being the more sensitive technique compared to endpoint PCR. From both plots (Fig. 2.4A and 2.4B), the level of IL-12 mRNA expression in Balb/c mice pre-treated with 5µg mycolic acids and infected with *M. tuberculosis* (5µg MA + M.tb) showed a significant (end-point PCR, $P<0.05$ and RQ-PCR, $P>0.025$) decrease in IL-12 mRNA expression compared to *M. tuberculosis* infected control mice (Ser + M.tb).

Mice infected with *M. tuberculosis* exhibited an elevation of IL-12 mRNA expression as compared to uninfected-untreated mice (Ser + M.tb compared to Ser + Sal, $P<0.05$). Mice treated with 25µg mycolic acids with no infection (25µg MA + Sal) produced a significantly ($P<0.005$) enhanced IL-12 mRNA expression as compared to untreated controls (Ser + Sal) only with RQ-PCR. In RQ-PCR only, pre-treatment with mycolic acids (25 µg) appeared to induce IL-12 expression in mice that were infected with *M. tuberculosis* as compared to pre-treatment with a lower dose (25µg MA + M.tb compared to 5µg MA + M.tb, $P<0.025$). In both methods the enhancement of IL-12 expression due to 25µg mycolic acids pre-treatment (25µg MA + M.tb) brought the expression of IL-12 to that of untreated and *M.tuberculosis* infected mice (Ser + M.tb).



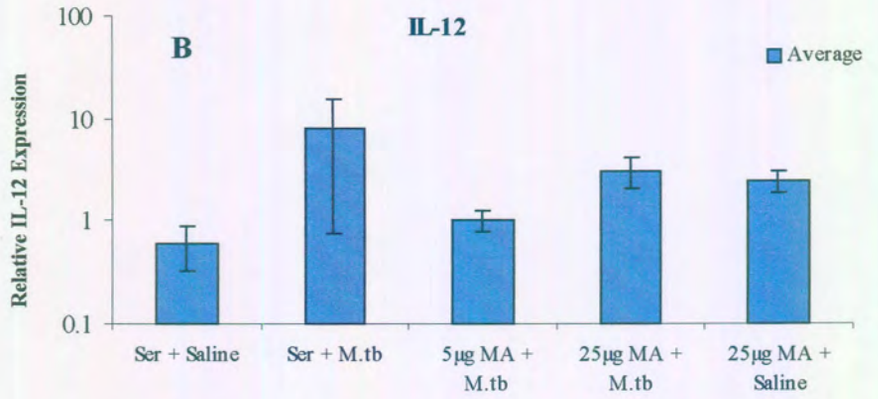


Figure 2.4: Relative IL-12 levels in the lungs of Balb/c mice removed five weeks after *M. tuberculosis* infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using the end-point PCR method (A) and the TaqMan™ Real-time Quantitative PCR method (B). The bars on the graph represent an average of three mice per group obtained by random selection. M.tb = *M. tuberculosis* in saline infection, Saline = saline administration Ser = serum pre-treatment and MA = mycolic acids-serum conjugates pre-treatment.

2.6.2 Interferon gamma (IFN-γ)

The expression patterns of IFN-γ measured with endpoint PCR (Figure 2.5A) was similar to that obtained with RQ-PCR technique (Figure 2.5B). Again here, as expected, RQ-PCR was more sensitive than end-point PCR. With traditional endpoint PCR lower levels of expression of IFN-γ mRNA in uninfected animals (Ser+ Saline and 25μg MA + Saline) could not be detected. Infection with *M. tuberculosis* resulted in an enhanced expression of IFN-γ mRNA. This was indicated using both the end-point PCR and RQ-PCR methods. Pre-treatment of animals with mycolic acids followed by *M. tuberculosis* infection, appeared to down-regulate IFN-γ expression as compared to *M. tuberculosis* infection without mycolic acids pre-treatment (5μg MA + M.tb compared to Ser + M. tb; $P<0.005$). This was only observed with the RQ-PCR method.

Administration of a higher dose of mycolic acids in mice that were infected with *M. tuberculosis* resulted in an enhanced expression of IFN-γ as compared to the lower dose of mycolic acids (25μg MA + Mtb compared to 5μg MA + M.tb; $P<0.025$) (Fig. 2.5B). The significance of this difference was not illustrated with end-point PCR analysis (Fig. 2.5A). Pre-treatment with 25 μg mycolic acids of *M. tuberculosis* infected mice did not result in enhanced expression of IFN-γ as compared to untreated *M. tuberculosis* infected mice (25μg MA + M.tb compared to Ser + M.tb).

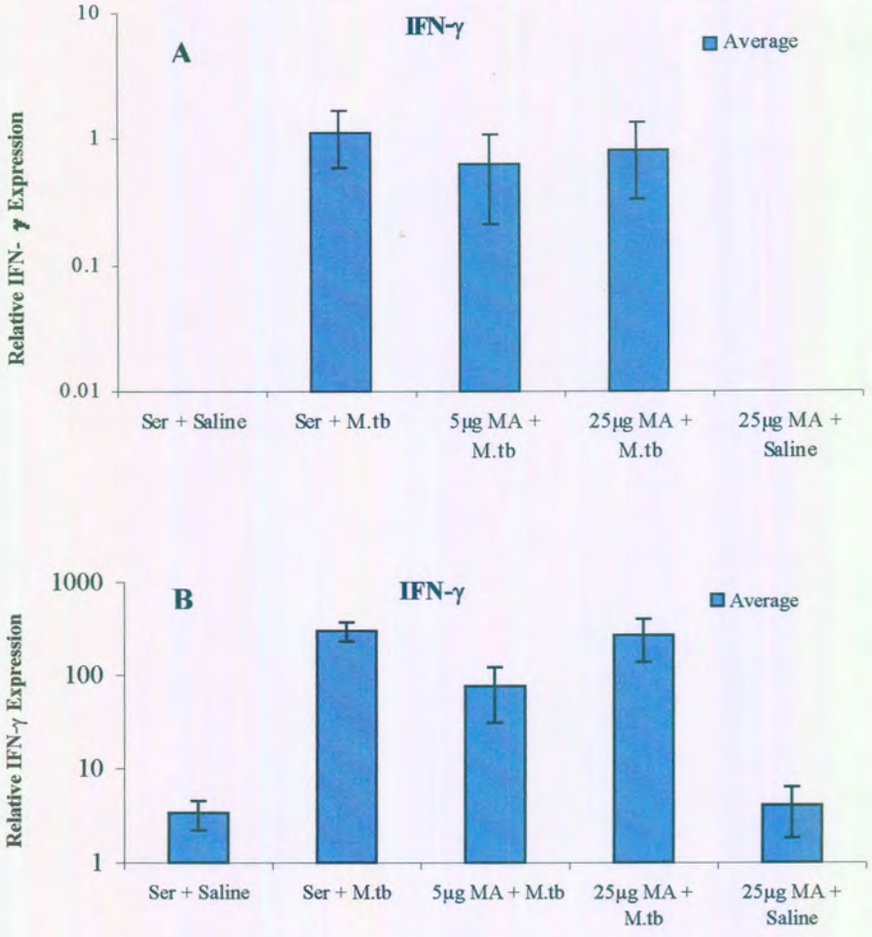


Figure 2.5: Relative IFN-γ levels in the lungs of Balb/c mice removed after five weeks of infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using the end-point PCR method (A) and the TaqMan™ Real-time Quantitative PCR method (B). The bars on the graph represent an average of three mice per group obtained by random selection. M.tb = *M. tuberculosis* in saline infection, Saline = saline administration Ser = serum pre-treatment and MA = mycolic acids-serum conjugates pre-treatment.

With the added sensitivity of RQ-PCR, an apparent down-regulation of expression of both the IL-12 and IFN-γ observed with 5µg mycolic acids administration could be distinguished. The results imply a complex relationship between survival and IL-12/IFN-γ secretion. Low concentrations of administered mycolic acids provide no

protection and are observed to down-regulate IL-12 or IFN-γ secretion in the lungs. Higher amounts of mycolic acids protect the mice but not directly due to the enhanced levels of IL-12 or IFN-γ. These cytokines are simply restored to the same levels as in non-treated *M. tuberculosis* infected controls.

2.6.3 Tumour necrosis factor-alpha (TNF-α) expression

Infection with *M. tuberculosis* resulted in an increase in the expression of TNF-α mRNA as compared to uninfected mice (Fig. 2.6). Mice infected with *M. tuberculosis* and pre-treated with 5μg mycolic acids (5μg MA + M.tb) showed an apparently reduced TNF-α mRNA expression level as compared to those that received 25 μg mycolic acids (25μg MA + M.tb, $P<0.05$) and those that were untreated (Ser + M.tb, $P<0.05$). Treatment of uninfected animals with 25μg mycolic acids did not have a significant effect on the expression of TNF-α mRNA, when compared to uninfected mice (Ser +Sal). Expression of TNF-α therefore followed a similar pattern to that of IL-12 and IFN-γ expression.

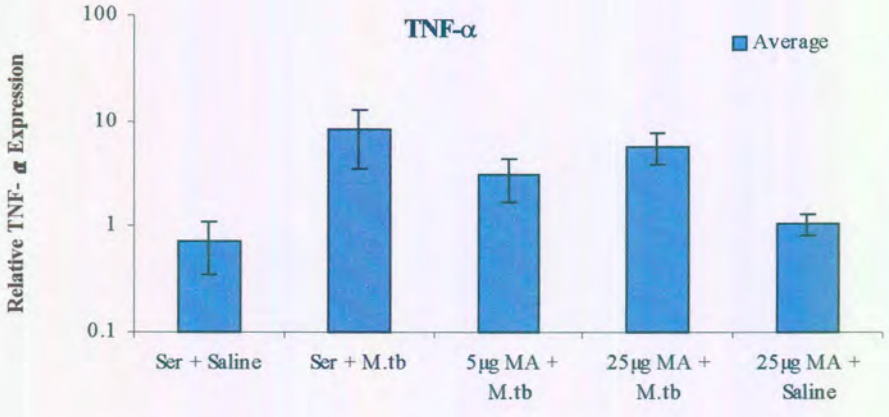


Figure 2.6: Relative TNF-α levels in the lungs of Balb/c mice removed after five weeks of infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using Taqman™ Real-time Quantitative PCR method. The bars on the graph represent an average of three mice per group obtained by random selection. Ser = serum administration, M.tb = *M. tuberculosis* infection, and MA = mycolic acids administration.

2.6.4 Interleukin-4 (IL-4) expression

The level of IL-4 in the lungs of uninfected (Ser + Sal; Fig. 2.7) Balb/c mice was low and could not be accurately determined with end-point PCR. With RQ-PCR, it appeared that *M. tuberculosis* infection did not result in the elevation of IL-4 expression in the lungs, as was observed with the pro-inflammatory cytokines IL-12, IFN-γ, and TNF-α. Mycolic acids administration to mice that did not receive infection resulted in an apparent enhancement of the expression of IL-4 (Ser + Saline compared to 25μg MA + Saline, $P<0.025$).

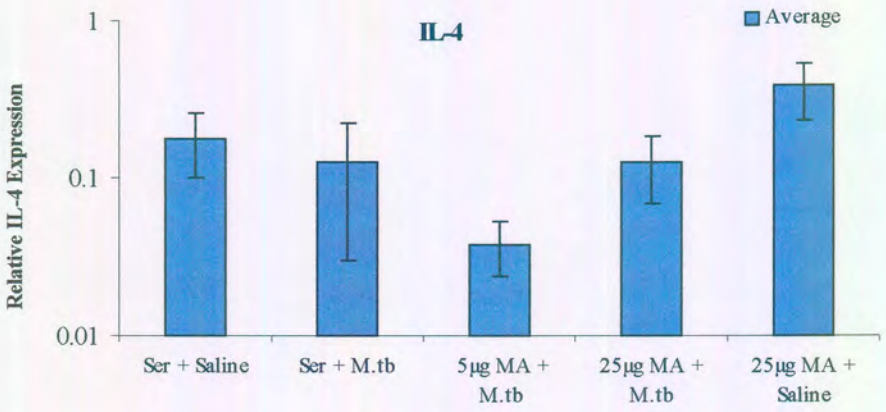


Figure 2.7: Relative IL-4 levels in the lungs of Balb/c mice removed after five weeks of infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using Taqman™ Real-time Quantitative PCR method. The bars on the graph represent an average of three mice per group obtained by random selection. Ser = serum administration, M.tb = *M. tuberculosis* infection, and MA = mycolic acids administration.

The effects of mycolic acids administration on the level of IL-4 expression was not so prominent as with the pro-inflammatory cytokines IL-12, IFN-γ, and TNF-α.

2.6.5 Granulocyte monocyte colony-stimulating factor (GM-CSF) mRNA expression

The levels of GM-CSF expression (Fig. 2.8) were observed not to be influenced significantly either by *M. tuberculosis* infection, or mycolic acids pre-treatment.

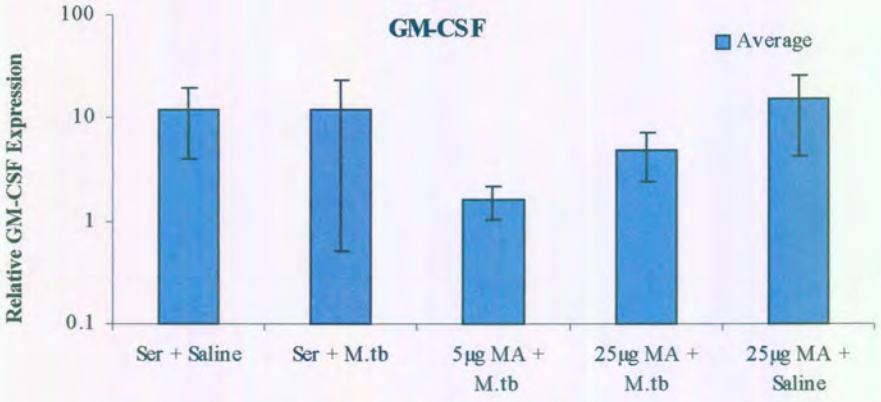


Figure 2.8: Relative GM-CSF levels in the lungs of Balb/c mice removed after five weeks of infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using Taqman™ Real-time Quantitative PCR method. The bars on the graph represent an average of three mice per group obtained by random selection. Ser = serum administration, M.tb = *M. tuberculosis* infection, and MA = mycolic acids–serum conjugate administration.

Infection with *M. tuberculosis* did not result in an enhancement or inhibition of GM-CSF mRNA expression. GM-CSF expression in response to mycolic acids administration proceeded according to the profile obtained with IL-4 expression, except that no enhanced GM-CSF expression was induced upon mycolic acids administration to non-infected mice.

2.6.6 The T-helper-1/ helper-2 bias

In order to determine whether the mycolic acids-induced resistance to tuberculosis was due to a responding change in the Th1/Th2 bias, and in order to minimize the inter-animal variations, the IL-4/IL-12 ratios per animal were determined and the results are presented in Fig. 2.9.1. It is clear that a Th1 bias was induced by *M.tuberculosis* infection, and that pre-treatment with mycolic acids had no influence on this. There is no correlation at all between IL-4/IL-12 ratios and the survival of these mice (Fig 2.3), indicating that the effect of mycolic acids is not directly on the level of T-helper lymphocytes. Mycolic acids may rather have their effect on macrophages that produce IL-12, as the non-infected controls (Ser + Saline compared to 25µg MA + Saline, Fig. 2.9.1) show a significant difference in IL-4/IL-12 ratio, correlating to an increased expression of IL-12 (Fig. 2.4B).

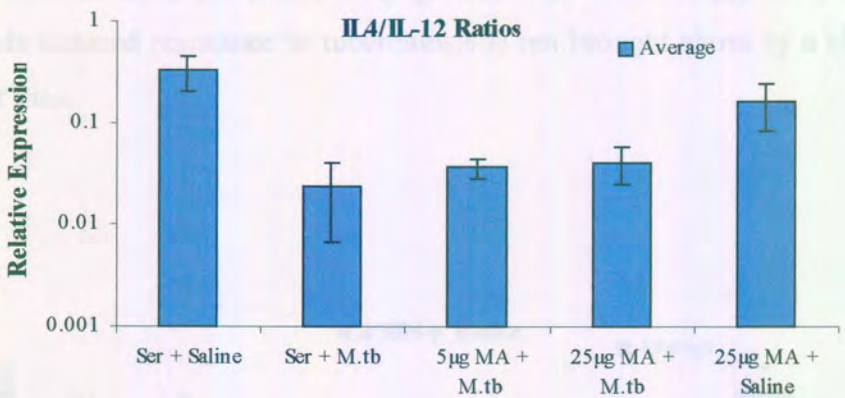


Figure 2.9.1: Relative expression of IL-4/IL-12 cytokine levels in the lungs of Balb/c mice removed after five weeks of infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using Taqman™ Real-time Quantitative PCR method. The bars on the graph represent an average of three mice per group obtained by random selection. Ser = serum administration, M.tb = *M. tuberculosis* infection, and MA = mycolic acids–serum conjugate administration.

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A stronger indication of the Th-bias is given by the IL-4/IFN-γ ratio, as T-helper cells produce both cytokines. The IL-4/IFN-γ ratios (Fig. 2.9.2) indicated that IL-4 levels, when expressed relative to the IL-12 expression levels, were significantly suppressed with *M. tuberculosis* infection (Ser + M.tb, 5μg MA + M.tb, and 25μg MA + M.tb compared to Ser + Saline; all $P<0.05$), and not affected at all by mycolic acids pre-treatment, either with or without *M. tuberculosis* infection. Again the known Th1/Th2 bias induced by *M. tuberculosis* infection could be clearly demonstrated in this experiment. It was not influenced at all by mycolic acids treatment, in spite of the very substantial improvement of resistance to *M. tuberculosis* infection that was induced by mycolic acids pre-treatment (Fig. 2.3). This convincingly shows that the mycolic acids induced resistance to tuberculosis is not brought about by a change in the T-helper bias.

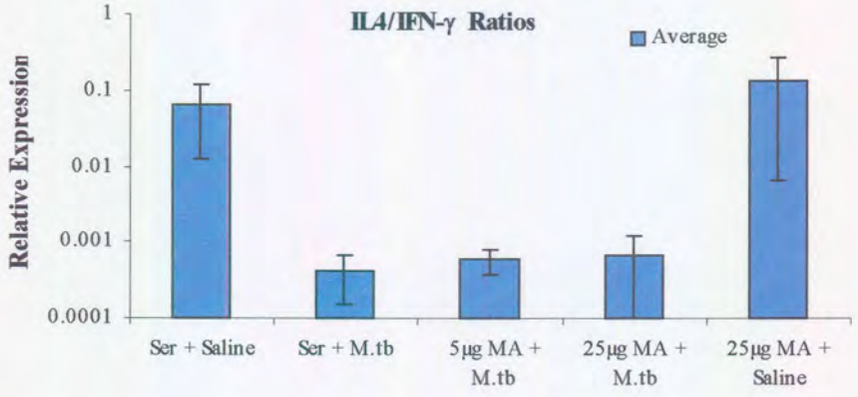


Figure 2.9.2: Relative expression of IL-4/IFN-γ cytokine levels in the lungs of Balb/c mice removed after five weeks of infection. The cytokines were assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using the Taqman™ Real-time Quantitative PCR method. The bars on the graph represent an average of three mice per group obtained by random selection. M.tb = *M. tuberculosis* in saline infection, Saline = saline administration, Ser = serum pre-treatment and MA = mycolic acids-serum conjugate pre-treatment.

2.7 Discussion

Mycobacterial cell wall products have potent immunomodulatory effects. The presence of mycobacterial cell wall products in the circulation during latent and active TB may therefore influence the course of the infection, leading either to containment or to progression of the disease. Some mycobacterial lipid and glycolipid cell wall components have been found to suppress the immune response (Moura and Mariano, 1996; Moura et al., 1997; Oswald *et al.*, 1997; Horwitz *et al.*, 1998 Riedel and Kaufmann, 2000). Other cell wall components, such as cord factor (trehalose dimycolate, containing two mycolic acid moieties esterified to one molecule of trehalose) and mannose-capped lipoarabinomannan (Man-LAM) appeared to be more proinflammatory in that they induced the expression of cytokines such as IL-12, IFN-γ and TNF-α (Riedel and Kaufmann, 2000; Oswald *et al.*, 1997).

Mycolic acids pre-treatment was previously found (Siko, 1999) to slightly prolong survival of Balb/c mice that were intravenously (i.v.) infected with *M. tuberculosis,* and to affect the cytokine profile of the lungs, rather than the spleen. In subsequent studies we have indicated that with intranasal infection, which results in the lungs being the port of infection, mycolic acids pre-treatment could increase survival of *M. tuberculosis* infected Balb/c mice to up to 90% (Lombard, 2002). The mice from this experiment were investigated here for their cytokine profiles.

The cytokine response is a major factor determining the outcome of *M.tuberculosis* infection. IL-12 is produced early in the infection and induces NK cells and T cells to produce IFN-γ. Interferon-γ together with other cytokines such as TNF-α or GM-CSF act on macrophages, potentiating anti-microbial activity and the production of NO (Flesch and Kaufmann, 1987; Flesch and Kaufmann, 1991; Dalton *et al.*, 2000; Ehlers *et al.*, 2001; Toossi, 2002). Several studies have implicated IL-12 as a protective cytokine in the immune resistance against *M. tuberculosis* (Cooper *et al.*, 1997; Fehniger *et al.*, 1999; Cooper *et al.*, 2002). Administration of IL-12 increases resistance against *M. tuberculosis* infection (Flynn *et al.*, 1995), while blocking IL-12 with anti-IL-12 antibody removed this resistance (Cooper *et al.*, 1995).

Previous studies from our laboratory indicated that when Balb/c mice were pre-treated with 25µg of mycolic acids before i.v. infection with *M. tuberculosis*, there was an enhancement of IL-12 mRNA expression in the lungs but not in the spleens. This correlated with slightly improved survival upon subsequent *M. tuberculosis* infection (Pretorius, 1999; Siko, 1999). In this study, administration of 25 µg mycolic acids upregulated the production of IL-12 mRNA in uninfected mice but not in infected mice. This time the mycolic acids protection against subsequent *M. tuberculosis* infection was much more pronounced, probably due to the intranasal, rather than intravenous, route of infection. In the previous study, [Pretorius (1999) and Siko (1999)] the mycolic acids induced upregulation of IL-12 in the lungs did not result in a concomitant increase of IFN-γ expression in the lungs, five weeks after *M. tuberculosis* infection. The improved survival due to mycolic acids administration could therefore not be attributed directly to a Th1-bias. The level of IL-12 expression in *M. tuberculosis*-infected human individuals and experimental animals has been shown to correlate with increase in the expression of IFN-γ (Chan *et al.*, 1992; Cooper *et al.*, 1993 and Chan *et al.*, 1993; Cooper *et al.*, 1997; Fehniger *et al.*, 1999). Interleukin-12 induces IFN-γ to initiate the immune defence to a Th1 mode in naïve T cells and also increase the cytotoxicity of NK cells. The T-helper bias can best be determined by determining the ratio of expression of a typical Th1 and Th2 cytokines for each mouse (Mosmann and Coffman, 1989). In the previous experiments [Pretorius (1999) and Siko (1999)], IL-4 could not be accurately determined with end-piont PCR but a profile of IL-12 and IFN-γ was measurable. Here, accurate values for IL-4 were obtained and more accurate values were also obtained with RQ-RTPCR for IL-12 and IFN-γ, the hallmark Th1 cytokine. The ratios of IL-4/IL-12 and especially IL-4/IFN-γ convincingly showed that mycolic acids had no effect on the Th-bias at both low and high doses, even though the high dose of mycolic acids pre-treatment significantly protected against *M. tuberculosis* infection.

If a mechanism for the protective effect of mycolic acids against tuberculosis is not to be found at the level of T-helper bias on TNF-α or T-cell produced GM-CSF, then macrophages provide a strong lead. Mycolic acids-induced secretion of IL-12 by macrophages correlate with the results of Oswald e*t al.* (1997), who found that

trehalose dimycolate, a mycolic acids-containing glycolipid present in the mycobacterial cell wall, induced the expression of IL-12 mRNA in mouse macrophages. In this study, the level of IL-12 expression in *M. tuberculosis* infected mice, was not affected by mycolic acids pre-treatment, but uninfected mice did show an upregulated IL-12 expression even at six weeks after a single mycolic acids administration.

Upon infection with *M. tuberculosis*, macrophages respond by secretion of IL-12, which then acts on the T-cells to produce IFN-γ (D'Andrea *et al.*, 1992; Chan *et al.*, 1992; Flynn *et al.*, 1993; Perussia *et al.*, 1992). This phenomenon was observed in this study upon infection with *M. tuberculosis*, but mycolic acids pre-treatment did not affect it. This suggest that, although IL-12 appears to respond to mycolic acids, IFN-γ is not involved in protection against tuberculosis that is derived from mycolic acids pre-treatment.

Stoltz (2002) showed that mycolic acids administration has pronounced effects on peritoneal macrophages by observing *M. tuberculosis* growth inhibition in mycolic acids treated macrophages. Stoltz also observed an enhanced production of GM-CSF concomitant with administration of mycolic acids, which led to a suggestion of an alternative activation of the macrophages. This concept was first introduced back in 1992 when IL-4 was first seen to activate macrophages in contrast to the classical IFNγ activation (Stein *et al.*, 1992). Here, mycolic acids administration did not influence the level of expression of GM-CSF in the lungs, however. The beneficial effect of mycolic acids pre-treatment in *M. tuberculosis* infected mice could not be explained by the induction of a protective cytokine response, as the levels of IL-12, IFN-γ and TNF-α mRNA in the lungs of infected mice were not increased by mycolic acids pre-treatment. Instead, the beneficial effect of mycolic acids pre-treatment on the survival of *M. tuberculosis* infected mice is more likely due to the induction of innate immune mechanisms resulting in the increased differentiation, maturation and activation of infected macrophages as outlined in more detail in Stoltz (2002).

CHAPTER 3

Mycolic Acids Cytokine Response During The Combined Murine AIDS And *M. Tuberculosis* Infection

3.1 Introduction

The tuberculosis epidemic in South Africa counts among the worst in the world, with the disease rates more than double the world average and up to 60 times higher than those currently observed in the developed world. This problem in South Africa is largely considered to be a result of historical neglect and poor management systems. Before the year 1995, when the Tuberculosis Register was introduced, cure rates were unknown. By 1997 a 54% cure rate was recorded, a number characteristic of persisting high transmission rates (Dick and Youngleson, 1994; Maher *et al.*,1997; Blomberg *et al.*, 2001).

South Africa has also one of the fastest growing HIV epidemics in the world. The Department of Health has since 1990 undertaken a series of annual unlinked, anonymous HIV surveys amongst South African women attending antenatal clinics of the Public Health Services. The 1996 survey was based on 15044 blood samples tested. From that survey it was estimated that, of all women attending these clinics, 14.17% were infected with HIV. By the end of 1997 the overall prevalence had increased to 16.01%, and by the end of 1998 to 22.8%. Levels of HIV infection had increased in eight of the nine provinces. KwaZulu/Natal turned out to be the province with the highest prevalence. It showed an increase from 19.90% in 1996, 26.92% in 1997 and up to 32.5% in 1998. This survey only reflects the situation of women at childbearing age (Steyn, 1997). In 1997 the Medical Research Council (MRC) of South Africa had estimated that the country had 180 507-tuberculosis cases of which 40,8% (59 206 cases) were co-infected with HIV (Maher *et al.*,1997; Fourie and

Onyebujoh, 1998). Tuberculosis had a consisted decline prior to 1984. The current resurgence of tuberculosis is most probably due to the concomitant HIV epidemic as countries with the highest numbers of AIDS cases also report high numbers of tuberculosis cases.

Exposure to *M. tuberculosis* does not usually develop into disease, as the immune system, particularly T-cells and macrophages (MΦ), clears or kills the mycobacteria or induces a state of dormancy of the pathogen. Human immunodeficiency virus (HIV) infection renders individuals more susceptible to *M. tuberculosis* infection by weakening immunity (Stead and Dutt, 1999). Susceptibility to tuberculosis, facilitated by concomitant HIV infection, supposedly comes about by at least two mechanisms. First, whereas protection to TB is brought about by Th1-cellular immune response, HIV orientates cellular immunity to the Th2 type. Second, the HIV kills $CD4^+$ T-lymphocytes and also Mϕ to an extent. These cells are the bulwark of the immune defence against *M. tuberculosis* (Kaplan *et al.*, 1986; Pitchenik *et al.*, 1987; Olshevsky *et al.*, 1990). Some immunity to *M. tuberculosis* may however be kept up independently of HIV co-infection. *M. tuberculosis* reactive $CD4^-8^-$ double negative (DN) CD1-restricted T-cells have been isolated from HIV-infected patients (Gong, 1998). These T-cells produced IFN-γ in response to Mϕ infected with *M. tuberculosis*, and may therefore play an important role in determining resistance to tuberculosis under conditions of co-infection with HIV. By their lack of CD4-expression, DN T-cells may escape the devastation caused by HIV infection among T-cells. This chapter concerns itself with an attempt to boost this type of immunity in an animal model of HIV/TB co-infection.

3.1.1 Animal models

The study of the interaction between HIV infection and tuberculosis requires a suitable animal model. For *M. tuberculosis* infection alone a number of models exist, but a suitable model for HIV infection remains elusive. Non-human primates are largely tolerant towards Simian immunodeficiency virus (SIV) infection. Recently, a non-human primate model was announced in which HIV was substituted with a

mutant SIV with the gene for its coat protein replaced by the HIV-type 1 coat protein gene (Himathongkham *et al.*, 2000). The latter was done to suit the basic cell tropism requirement of HIV. Although an improvement on the normal SIV models, this still remains a non-human primate model, typically associated with problems of limited availability, cost and small numbers per experimental group. This left a void for the development of other available retroviral infection models.

3.1.2 Murine AIDS

Retrovirus infections have long been associated with states of immunodeficiency in cats and mice (Bendinneli *et al.*, 1985; Anderson *et al.*, 1971; Hoover *et al.*, 1972). The murine acquired immunodeficiency syndrome (MAIDS) is induced by the Duplan strain of murine leukemia virus (MuLV). It contains a replication-competent ecotropic (PBM5-eco) helper virus, a B-tropic mink cell focus forming (MCF), murine leukemia virus (MuLV) and a replication defective virus (BM5-def) (Morse *et al.*, 1992; Tse *et al*, 1994; Gallicchio *et al*, 1995; Doherty *et al.*, 1995; Hasegawa *et al.*, 1995; Desforges *et al.*, 1996; Doherty *et al.*, 1997; Oakley *et al*, 1998; Klein *et al*, 1998). The model became known as the LP-BM5 model: Susceptible adult C57BL/6 mice inoculated intraperitoneally with this mixture of murine leukemia viruses exhibited many similarities to HIV-1 infection (Table 2), including lymphadenopathy and splenomegaly related to polyclonal activation of T and B cells to mitogenic and antigenic stimuli (Mosier *et al*, 1987; Klinman *et al.*, 1992; Palamara *et al.*, 1996).

Table3.1: Comparisons of MAIDS and AIDS

Similarities	
T-cells	*B-cells (continued)*
-Increased proliferation	-Impaired response to help from normal T-cells
-Impaired response to mitogens	-Decreased responses to helper T-cell-independent antigens
-Impaired CD4+ cell function prior to changes in CD4+cell frequency, including:	-Development of lymphomas
-Help for CD8+ cytotoxic T-lymphocyte responses to modified self antigens	*Non-T, non-B-cells*
-Response to soluble antigens	-Increased proliferation
-Autologous mixed lymphocyte reaction	-Decreased natural killer function
-Help for normal B cells	-Decreased natural killer responsiveness
B-cells	*Other*
-Polyclonal activation	-Lymphoproliferation
-Differentiation to immunoglobulin secretion	-Enhanced susceptibility to infection
-Hypergammaglobulinemia	-Effects of major histocompatibility complex polymorphisms on disease progression
-Autoantibody production	-Disease more progressive in neonates than adults
-Circulating immune complexes	-Neurodegenerative disease
-Impaired response to mitogens	
Differences	
-Lentivirus in AIDS, C-type murine leukemia virus in MAIDS	
-CD4 as the prominent receptor for HIV, not for MuLV	
-Kaposi's sarcoma in AIDS, not in MAIDS	
-Opportunistic infections are a much greater problem in AIDS	
The MuLV are simpler in genomic structure than HIV. For example, they lack the *tat*, *rev*, and *nef* regulatory genes	

3.1.3 Immune response during MAIDS

During the first 8-10 weeks after infection with the LP-BM5 virus, serum IgG concentrations increased and then declined (Morse *et al.*, 1992). Lymph node and spleen enlarged continuously for 4 - 12 weeks after infection and this paralleled the progression of disease (Mosier *et al.*, 1996; Jolicouer, 1991). These studies indicated

that in the spleens and lymph nodes of LP-BM5 MuLV infected mice the frequency of CD4+ cells decreased slightly, while the frequency of CD8+ cells decreased significantly in these organs. Previous studies (Portnoi *et al.*, 1990) indicated that CD8+ cells were reduced to 4% of splenic lymphoid cells compared to 13% in uninfected control mice.

A number of studies have demonstrated the need for T-cell and B cell interaction in the development of MAIDS (Muralidhar *et al.*, 1992; Gilmore *et al*, 1993; Klein *et al.*, 1998). $CD4^+$ T-cells appeared to be less affected by LP-BM5 MuLV infection compared to human HIV infection. It has been observed that depletion of $CD4^+$ T-cell function through $CD4^+$ knockout or by anti-CD4 antibody treatment rendered the animals resistant to disease progression characterised by splenomegaly and hypergammaglobulinemia. Reintroduction of CD4 function by bone marrow transplantation and CD4 cell introduction restored the susceptibility to LP-BM5 virus (Andrews *et al.*, 1997; Giese *et al.*, 1994; Morse *et al.*, 1995). Even though there was a proliferation of $CD4^+$ T-cells observed during the early stages of MAIDS, their function was diminished, as they became anergic. Addition of anti-CD28 antibodies, a co-stimulatory signal for $CD4^+$ T-cells, or IL-12 could restore $CD4^+$ T-cell function before total anergy was established. Once anergy was established it could not be reversed via IL-12 or addition of anti-CD28, as the cells progressed towards apoptosis (Giese *et al.*, 1995; Muralidhar *et al.*, 1996; Andrews *et al.*, 1997).

The number of $CD8^+$ T-cells was diminished severely in both the spleen and the lymph nodes during the development of MAIDS (Morse *et al.*, 1995). This is quite the opposite of what is observed during human AIDS, where it is the number $CD4^+$ T-cells are diminished. In both human AIDS and MAIDS, however, the $CD4^+$ T-cell function is lost as the disease progresses, regardless of the persistence of $CD4^+$ T-cells in MAIDS (Mosier, 1996). MAIDS is also characterised by the impairment of NK cell function, even though the mechanism is not known (Hiromatsu *et al.*, 1996; Liang *et al.*, 1996; Peacock *et al.*, 1997). The B cells significantly increase in the spleen and lymph nodes during the first week after LP-BM5 infection. As lymphoadenopathy is

the principal feature in MAIDS, the spleen and lymph node continue to grow until the animals die from 10 to 26 weeks from infection.

3.1.4 Cytokines in MAIDS

Since LP-BM5 MuLV infection leads to a disease that is characterised by immunodeficiency and lymphoproliferation associated with B-cell lymphoblast proliferation, there is reason to believe that the disease may be characterised by a shift in cytokine expression to a Th2- profile. This was supported by the observation that removal of IL-4 and IL-10 prevented disease development. Animals that are treated with anti-IL-4 or anti-IL-10 monoclonal antibodies were found to be resistant to the disease. Once the disease had progressed, the viral titre could be reduced with IL-4 inhibition (Doherty *et al.*, 1997; Morawetz *et al.*, 1996; Oakley *et al.*, 1998). Suppressing the Th1 cytokine profile with anti-IFN-γ and anti-IL-12 antibody treatment exacerbated the disease development, similar to what was found with murine tuberculosis (Flynn *et al.*, 1995; Peacock and Price, 1999; Morawetz *et al.*, 1996).

Previous studies in this laboratory showed that treatment of tuberculosis susceptible Balb/c mice with mycolic acids induced resistance against *M. tuberculosis* infection that corresponded with an enhanced expression of the Th1 cytokines IL-12 and IFN-γ mRNA, and suggesting Th1 induction (Pretorius, 1999; Lombard, 2002). Mycolic acids raised the level of resistance to intravenous administration *M. tuberculosis* infection in Balb/c mice to that of the tuberculosis resistant C57Bl/6 strain of mice (Fig 3.1).

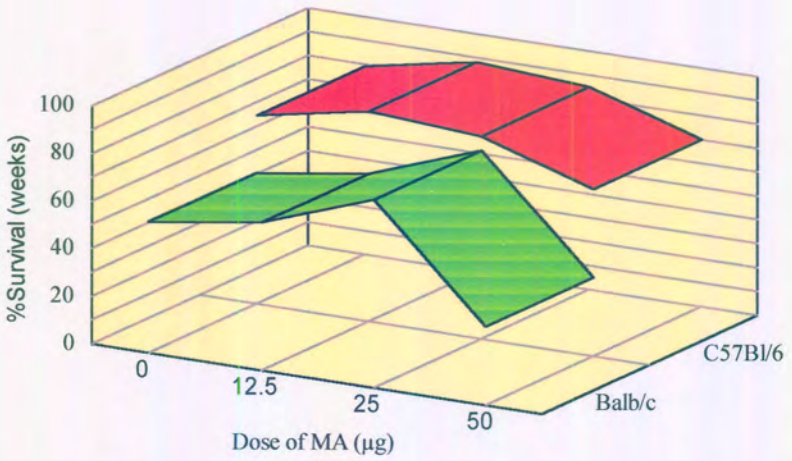


Figure 3.1: Comparison of Balb/c and C57Bl/6 mice that were pre-treated with different dosages of mycolic acids prior to infection with *M.tuberculosis*. Mice were kept in a glove isolator and in groups of 8 while being daily monitored and maintained for 40 weeks of infection. (Adapted from Siko, 1999)

In this study C57Bl/6 mice are infected with LP-BM5 virus and then infected with *M. tuberculosis* to emulate the situation in human beings where increased susceptibility to *M. tuberculosis* infection is observed in HIV infected individuals. This study then tests whether the resistance of virally immune-compromised C57Bl/6 mice to *M. tuberculosis* infection can be restored with administration of mycolic acids. It was assumed that the resilience that C57Bl/6 mice exhibited towards mycolic acids (Fig 3.1) will be annulled by the LP-BM5 virus infection.

3.2 Aims

The current study aims at testing the hypothesis that administration of MA can enhance innate immunity in LP-BM5 MuLV induced immuno-compromised mice to enhance protection against tuberculosis. It is regarded as an exploratory investigation that may eventually be extrapolated to the feasibility of immunotherapy of tuberculosis in HIV infected humans.

The study comprised first the optimisation of an infective dose of LP-BM5 MuLV that would be able to produce MAIDS symptoms in C57Bl/6 mice after a sufficiently long incubation period to allow subsequent co-infection with *M. tuberculosis* H37Rv. Secondly, the treatment of the virally infected mice with an optimised dosage of purified mycolic acids from *M. tuberculosis* H37Rv to test if mycolic acids could influence the onset and duration of tuberculosis. Finally, the correlation of the survival results to cytokine profile in the spleen and lungs of these mice was done, using Real-time quantitative reverse transcriptase polymerase chain reaction (RQ-RT-PCR), in an attempt to find clues to the mechanism of the biological activity of mycolic acids.

3.3 Materials

3.3.1 Bacteria

Mycobacterium tuberculosis H37Rv ATCC 27294 - Type strain: A virulent strain, originally isolated from an infected human lung. The culture was purchased in lyophilized form from the American Type Culture Collection (ATCC), Maryland, USA. A detailed composition of the ingredients necessary for the preparation of these media as well as the conditions recommended for their sterilization, are given in the Laboratory Manual of Tuberculosis Methods, Tuberculosis Research Institute of the SA Medical Research Council (1980, Chapter 6, pp 83-105; Second Edition, revised by E E Nel, H H Kleeberg and E M S Gatner).

3.3.2 Virus

Murine leukemia virus (MuLV) LP-BM5 stock derived from a bone marrow stromal cell line (SC-1) harvested from animals infected with mink cell focus forming MuLV was kindly provided by Dr Vincent Gallichio (University of Kentucky, Lexington USA). This stock virus was kept at -70^0C until used.

3.3.3 Animals

Inbred female C57Bl/6 mice were bought from the South African Institute for Medical Research (SAIMR-Johannesburg). The mice used in this study were of the age 6 to 10 weeks. The animals were kept in a P3 laboratory in a glove isolator in a temperature and humidity controlled room.

3.3.4 Reagents

Reagents used in the Real-time Quantitative Reverse Transcriptase Polymerase Chain Reaction (RQ-RT-PCR):

Ethidium bromide (Roche Molecular Systems, New Jersey USA)

Tris (hydroxymethyl)-aminomethane (Merck, Darmstadt Germany)

EDTA (Ethylenediaminetetra-acetic acid) (Merck, Darmstadt Germany)

Sodium acetate (Merck, Darmstadt Germany)

RNAZol ™-reagent (Biotech, Italy)

Diethyl pyrocarbonate (DEPC) (Sigma Chemicals, St Louis USA)

Oligo dT primers (Life Technologies Inc., Scotland)

Superscript RNase H Reverse Transcriptase (Life Technologies Inc., Scotland)

Recombinant RNasin (Promega Corporation, Woods USA)

Pre-developed TaqMan™ Kit consisting of: TaqMan™ Universal PCR Master Mix and murine cytokine primers and probes

Housekeeping gene (GAPDH) primers and probes

For the preparation of the reagents used for the extraction, derivatization and High-Performance Liquid Chromatography (HPLC) analysis of mycolic acids, HPLC Grade methanol (BDH) and double-distilled deionized water were used.

3.4 Methods

3.4.1 Virus preparation

The viral titre of LP-BM5 MuLV was obtained by growing murine stromal cells already infected with LP-BM5 virus in *in vitro* cell culture. The cells were thawed and grown to approximately 90-95% confluence in four days in 5% FCS in RPMI without changing the medium. After 13 passages, the supernatant from each flask was decanted, all the cellular debris removed by spinning at 2000 rpm for 10 minutes and the virus containing cell supernatant then passed through 0.45 micron Millipore filters. C57BL/6 female mice were infected by means of intraperitoneal injections of 0.4 ml LP-BM5 cell supernatant, which was filtered through the 0.45 micron filters.

3.4.2 Bacterial culture

Mycobacterium tuberculosis H37Rv (ATCC 27294) was cultured on Löwenstein-Jensen (LJ)-slants at 37°C for 3 to 6 weeks. Bacterial samples with a total count varying between 10^4 - 10^6 bacilli per ml were suspended in 0.9 % sterile saline, washed twice with saline by centrifugation (2000 x g for 15 min), and re-suspended in 0.9 % saline. Bacterial titres (viable counts) of the samples were determined by counting colony forming units (CFU) in 1:10 to 1:1 000 000 dilutions on Middlebrook 7H10 agar after incubation at 37 °C for 4 to 6 weeks.

Total bacterial count was determined directly using a Neubauer counting chamber and various dilutions of bacterial suspensions. Staff from the National Tuberculosis Institute of the Medical Research Council of South Africa, Pretoria, prepared the media. The sterility of all the media was confirmed by eye after incubating them at 37°C for 24 h, before they were used in the experiments.

The harvested bacteria were washed in sterile 0,9% m/v NaCl (Chemically Pure, Saarchem, RSA). The diluent for the preparation of serial dilutions, preceding the

determination of viable counts of *M. tuberculosis* was prepared by dissolving Tween 80 (Chemically Pure, Merck) in 0,9% m/v NaCl (Chemically Pure, Saarchem) to a concentration of 0,01% v/v and distributing it in 9,0 ml aliquots into test-tubes. The autoclaved media were stored at 4°C.

3.4.3 Mycolic acids preparation

M. tuberculosis -derived mycolic acids (MA) were prepared according to a method described in chapter 2.

3.4.4 Infection and treatment

Female inbred C57Bl/6 mice were first inoculated with an optimised dosage of LP-BM5 murine leukemia virus (Mu LV) or RPMI medium. Four weeks later they were intravenously treated with mycolic acids serum conjugate. The mice were then intra-nasally inoculated with *M. tuberculosis* H37Rv or saline as a negative control, a week after pre-treatment with mycolic acids.

3.4.5 Organ preparation and survival study

The organs originating from *M. tuberculosis* infected and uninfected mice, used for the Real-Time PCR analysis, were the lungs and spleens. Mice from each group were sacrificed by rapid cervical dislocation three weeks after *M. tuberculosis* infection. The organs were removed from each mouse aseptically and kept at -72 °C after snap freezing in liquid nitrogen. The remaining mice (eight mice per group) were kept for survival studies for 18 weeks after *M. tuberculosis* infection. These mice were monitored everyday and their mass gain or loss recorded every week for disease progression. The significance of the effects of infection and mycolic acids pre-treatment on survival of C57Bl/6 mice was tested using the one tailed Wilcoxon's Rank-Sum test.

3.4.6 RNA preparation

Total RNA of the organs was extracted using Rnazol, based on a method developed by Chomcszynski and Sacchi (1987). Before the reverse transcriptase (RT) reaction, the RNA was co-precipitated with primer as described by Ausubel *et al.* (1992). Total RNA (6 µg) was precipitated overnight at -20 °C in the presence of 3 pmol Oligo(dT)12-18 (Gibco BRL, Gaithersburg, MD, USA) with 0.1 M NaOAc, pH 5.5: absolute ethanol (1:2). After centrifugation the pellet was washed with 70 % ethanol-DEPC treated H_2O and allowed to dry for about 10 to 15 minutes. Subsequently, the RNA was dissolved in a resuspension buffer [80 mM Tris-HCl pH 8.3, 90 mM KCl and 40 U RNasin (Promega, Madison, WI, USA)], heated to 70 °C for 10 minutes and followed by a 3 hour incubation at 37 °C. RNA was quantified using the GENEQUANT RNA/DNA calculator (Pharmacia) and the integrity of the RNA visualised on a 1% denaturing agarose gel. cDNA was synthesised using the Superscript reverse transcriptase enzyme. The RT reaction was performed with Superscript™ RNase H Reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA) as recommended by the manufacturer.

3.4.7 Real-time PCR

Real-time PCR was performed using TaqMan™ Universal PCR Master Mix™ protocols as described in chapter 2.

3.5 Results

The effects of mycolic acids pre-treatment in LP-BM5 MuLV-induced immune-compromised animals with or without co-infection with *M. tuberculosis* is described. Data presented here first show the effects of mycolic acids on survival of infected and co-infected C57Bl/6 mice and is then followed by their cytokine profiles.

3.5.1 Survival study

Mice were infected with LP-BM5 virus five weeks before infection with *M. tuberculosis* and four weeks before treatment with serum or mycolic acids-serum conjugates. The infection of mice with either LP-BM5 virus (M) or *M. tuberculosis* (Serum + TB) alone did not induce fatalities in the mice up to week 12 after the *M. tuberculosis* infection date, compared to deaths already occurring at week 6 in LP-BM5 MuLV/*M. tuberculosis* co-infected mice (M+Serum+TB and M+MA+TB, Fig. 3.2). This confirms the mutually enhancing effect of the mycobacterial and leukemia virus co-infections. Co-infection significantly reduced the survival of the animals compared to mono-infection with *M. tuberculosis* when only serum treatment was done ($P<0.01$). The significance of the difference in susceptibility between co-infection (M+MA+TB) and mono-infection (MA+TB) fell away with mycolic acids administration.

As expected mycolic acids pre-treatment had no significant effect on the survival of *M. tuberculosis* mono-infected C57Bl/6 mice at the dose (25 μg) that was found to be optimal to protect Balb/c mice against tuberculosis. This confirms the resilience of the tuberculosis resistant C57Bl/6 mice towards mycolic acids. Co-infection with the virus did however break the natural resistance of the mice to tuberculosis.

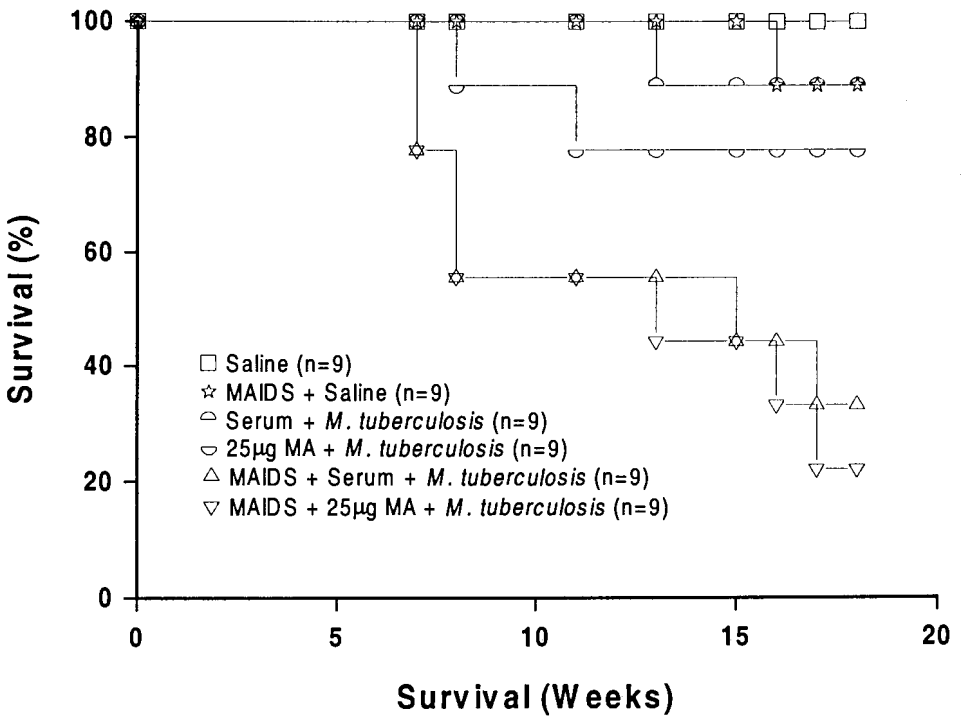


Figure 3.2: Relative survival of C57Bl/6 mice infected with LP-BM5 murine leukemia virus and *M. tuberculosis*. Mice were divided into groups of eight. M=LP-BM5 MuLV infection, Serum= serum treatment, MA= treatment with mycolic acids-serum conjugates and *M. tuberculosis*= infection with *M.tuberculosis*.

The survival results argue against the hypothesis that mycolic acids may uphold a protection to *M. tuberculosis* infection through its effect on innate immunity under conditions of viral immune-compromise.

3.5.2 Mycolic acids induced cytokine responses during tuberculosis in immuno-compromised animals

In this study, the effects of mycolic acids pre-treatment on the immune system of LP-BM5 MuLV immuno-compromised and *M. tuberculosis* infected mice were investigated. Both IFN-γ and IL-12 are known to be protective against *M. tuberculosis*, whereas IL-4 and IL-10 are known to be upregulated during LP-BM5 viral disease progression. The cytokines investigated were IL-12, IL-10 and IFN-γ, while IL-4 turned out to be very difficult to determine without prior enrichment of the IL-4 mRNA, which was not done.

3.5.2.1 Interleukin-12 (IL-12) mRNA expression

Within the limitations imposed by the relatively high degree of variation of the IL-12 measurements in the lungs among the mice of each group, no significant difference of IL-12 expression could be detected between mycolic acids treated ("MA + TB"; "M + MA + TB") and non-treated ("Ser + TB"; "M + Ser + TB") mice (Fig. 3.3) with Real-time PCR. Mycolic acids pre-treatment did therefore not have any significant effect on either the survival of the mice, or the expression of IL-12 in the lungs. One did, however, expect a difference in IL-12 expression in animals that were co-infected with LP-BM5 virus and *M. tuberculosis* and succumbed soon, in comparison with the animals that were infected with either the virus or *M. tuberculosis* alone and that endured much longer. No such difference could be observed with an acceptable degree of confidence (Fig 3.3). From these results it can be concluded that the increased susceptibility to TB by co-infection with LP-BM5 virus did not appear to manifest itself through the degree of expression of IL-12 in the lungs.

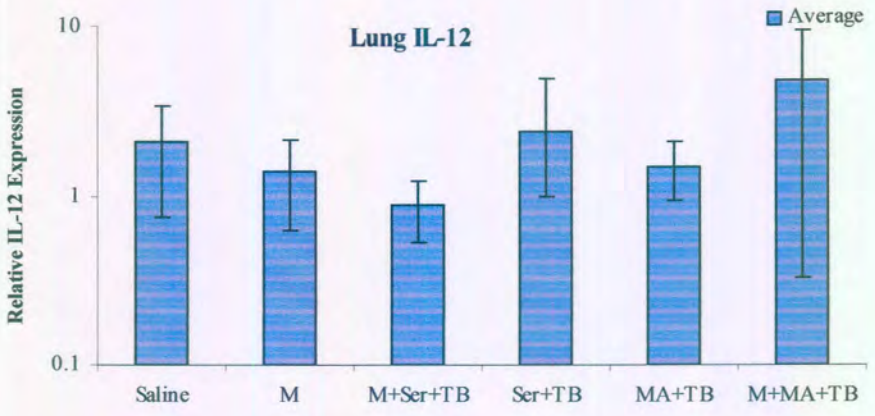


Figure 3.3: Relative IL-12 levels in the lungs of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed by the TaqMan™ Real time PCR method. The bars on the graph represent an average of the three mice per group obtained by random selection. M=LP-BM5 MuLV infection, Ser= serum treatment, MA= treatment with mycolic acids-serum conjugates and TB= infection with *M.tuberculosis*.

In the spleen (Fig. 3.4) there was a significant ($P<0.05$) induction of IL-12 mRNA expression in animals that received mycolic acids and tuberculosis (M+MA+TB) compared to the animals that received LP-BM5 MuLV, serum and tuberculosis (M+Ser+TB). A significant ($P<0.05$) stimulatory effect of mycolic acids on IL-12 expression was also observed in mice mono-infected with *M. tuberculosis* and treated with mycolic acids serum conjugates (MA+TB) compared to those treated with serum only (Ser+TB). LP-BM5 MuLV infection alone (M) appeared to significantly ($P<0.025$) down regulate IL-12 expression in comparison to non infected control mice (Saline).

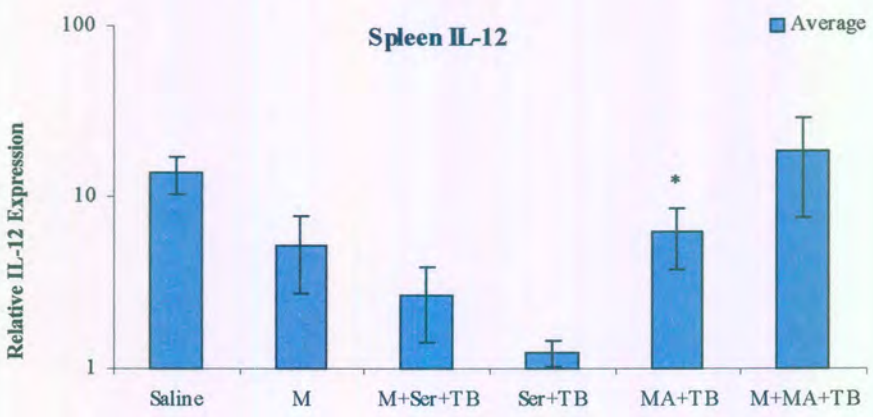


Figure 3.4: Relative IL-12 levels in the spleens of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using TaqMan™ Real time PCR method. The bars on the graph represent an average of three mice per group obtained by random selection, while * denotes an average of two, M=MAIDS induction, Ser= treated serum, MA= mycolic acids conjugates and TB= *M. tuberculosis*, presented in the order of administration.

These results indicate that mycolic acids pre-treatment restored most or all of the IL-12 down regulation effected by infection with either *M. tuberculosis* or LP-BM5 MuLV or co-infection with both in the spleens of mice. This effect could not be correlated with observations made in the survival studies.

3.5.2.2 Interferon-gamma (IFN-γ) mRNA expression

There appeared to be no significant effect on the expression of IFN-γ in the spleens due to infection with either LP-BM5 virus or *M. tuberculosis* or co-infection with both. Mycolic acids did however, significantly upregulate IFN-γ expression ($P<0.05$) in mice that were mono-infected with *M. tuberculosis* (MA+TB) as compared to those that were mono-infected with *M. tuberculosis* and treated with serum (Ser+TB). This was not the case with co-infection as there was no enhancement of IFN-γ expression with treated (M+MA+TB) compared to untreated groups of mice (M+Ser+TB) [Fig. 3.5].

In contrast to the spleen, the lungs responded to infection with LP-BM5 Mu LV or *M. tuberculosis* or co-infection with both by a significantly enhanced expression of IFN-γ ($P< 0.01$) [Fig. 3.6]. In addition, pre-treatment with mycolic acids had no effect on IFN-γ expression in mono-infected mice (MA+ TB versus Ser + TB), and in mice that were co-infected with LP-BM5 Mu LV and *M. tuberculosis* (M+MA+TB versus M+Ser+TB).

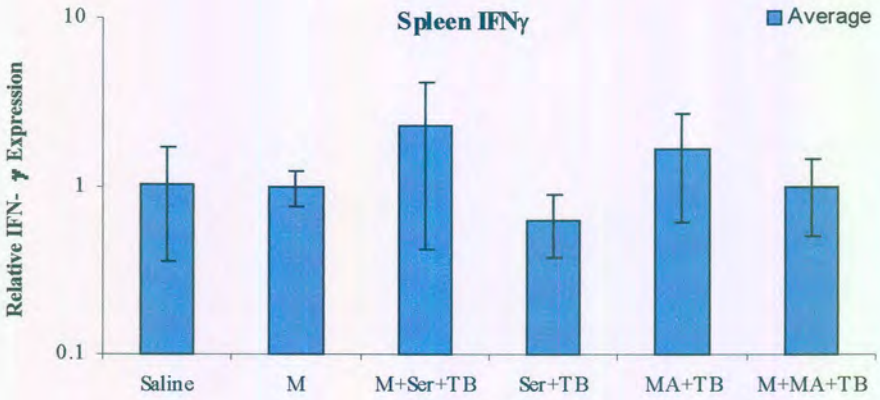


Figure 3.5: Relative IFN-γ mRNA levels in the spleens of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using TaqMan™ Real time PCR method. The bars on the graph represent an average of the three mice per group obtained by random selection. M=MAIDS induction, Ser= treated serum, MA= mycolic acids conjugates and TB= *M. tuberculosis*, presented in the order of administration.

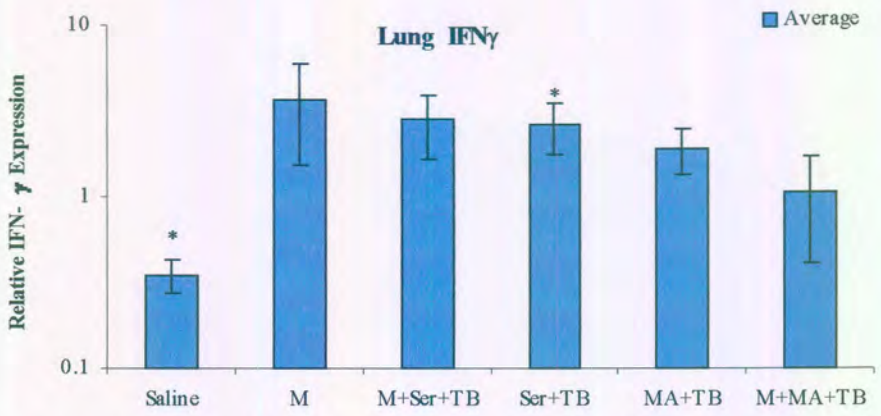


Figure 3.6: Relative IFN-γ mRNA levels in the lungs of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using TaqMan™ Real time PCR method. The bars on the graph represent an average of three mice per group obtained by random selection while * denotes an average of two. M=MAIDS induction, Ser= treated serum, MA= mycolic acids conjugates and TB= *M. tuberculosis*, presented in the order of administration.

The active IFN-γ response of the lungs to infection appeared to be stemmed by mycolic acids administration, whereas the spleen appeared not to respond to infection, but showed a tendency towards upregulation with mycolic acids. The inhibitory effects of mycolic acids on expression of IFN-γ in co-infected mice did not correlate with observations made in the survival study.

3.5.2.3 Interleukin-10 (IL-10) mRNA expression

Similarly to IFN-γ, infection with LP-BM5 MuLV or *M. tuberculosis* or co-infection with both resulted in an enhanced expression of IL-10 in the lungs (Fig. 3.7). In the spleen he response to infection was weaker but still significant (Fig. 3.8). Mycolic acids administration appeared to suppress the level of IL-10 expression in the lungs of *M. tuberculosis* mono-infected mice ($P<0.025$). In the spleen significant suppression was observed with mycolic acids pre-treatment in mice that were co-infected with LP-BM5 Mu LV and *M. tuberculosis* (M+MA+TB versus M+Ser+TB; $P<0.025$) as well as in mice that were mono-infected with *M. tuberculosis* (MA+TB and Ser+TB).

Mycolic acids inhibited IL-10 expression in the spleen to the level of uninfected control mice (Saline).

Within the limits of the variation among mice of the IL-10 expression data, one can conclude that mycolic acids appeared to suppress the infection-induced IL-10 expression in both lungs and spleen, thereby in general exerting a pro-inflammatory effect against LP-BM5 MuLV and/or *M. tuberculosis* infection.

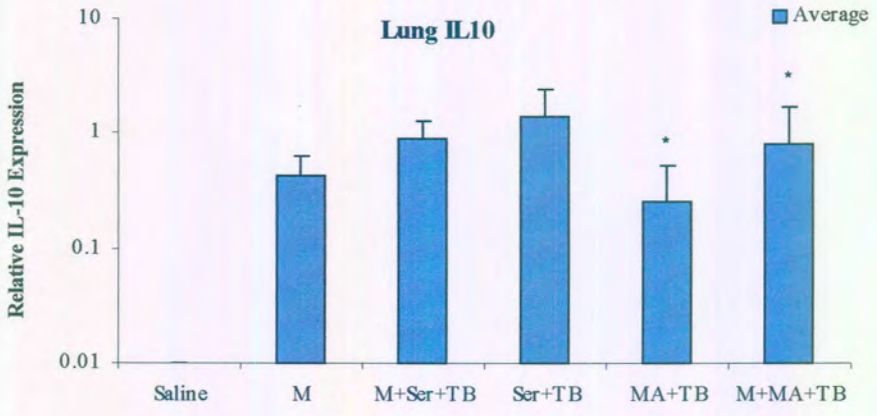


Figure 3.7: Relative IL-10 levels in the lungs of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using TaqMan™ Real time PCR method. The bars on the graph represent an average of the three mice per group obtained by random selection, while *denotes an average of two. M=MAIDS induction, Ser= treated serum, MA= mycolic acids conjugates and TB= *M.tuberculosis*, presented in the order of administration.

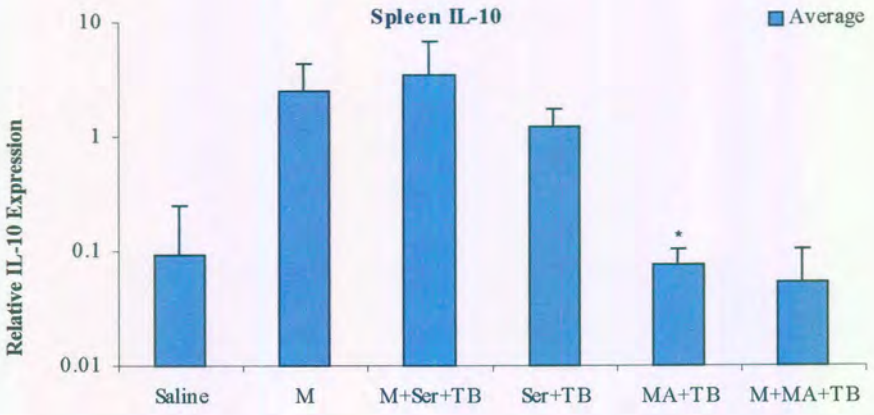


Figure 3.8: Relative IL-10 levels in the spleens of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using TaqMan™ Real time PCR method. The bars on the graph represent an average of the three mice per group obtained by random selection while * denotes an average of two. M=MAIDS induction, Ser= treated serum, MA= mycolic acids conjugates and TB= *M.tuberculosis*, presented in the order of administration.

Mycolic acids pre-treatment in this study had no significant advantage towards increased survival of either the TB mono-infected or virus and TB co-infected mice. The mice that were co-infected with LP-BM5 Mu LV and *M. tuberculosis* died much earlier than those that received either the viral or the mycobacterial type of infection. The level of IL-12, IL-10 and IFN-γ expression in the spleens gave an indication that mycolic acids shifted the immune system towards a pro-inflammatory Th1-type state, although this did not correlate with the survival study. The pro-inflammatory cytokines IL-12 and IFN-γ were upregulated in the spleens of MA-treated and infected mice but a mixed message was obtained from the lungs of the same mice. In order to reduce the variability of cytokine expression among animals, the data was reworked to represent the IL-10/ IL12 and IL-10/IFN-γ ratios of each individual animal in each group to determine how the pro-inflammatory response shifted in the mycolic acids treated and infected mice.

3.5.2.4 Anti-inflammatory IL-10/ Pro-inflammatory IL-12 and IFN-γ ratios

Looking at the IL-10/IL-12 ratios in the lungs (Fig. 3.9), much more accurate values were obtained compared to values for single cytokine production. In the lungs, mycolic acids appeared to shift the immune system towards a pro-inflammatory state (Figure 3.9) in mice that were co-infected with LP-BM5 MuLV and *M. tuberculosis* (M+MA+TB versus M+Ser+TB, $P<0.005$) or in mice that were mono-infected with *M. tuberculosis* (MA+TB versus Ser+TB.

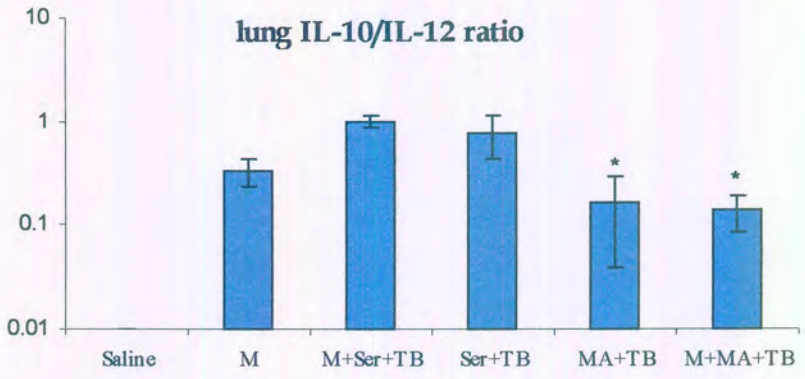


Figure 3.9: The ratios of IL-10 and IL-12 cytokine expression levels in the lungs of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The bars on the graph represent average ratios of the three mice per group obtained by random selection, while * denotes an average of two. M=MAIDS induction, Sera= treated serum, MA= mycolic acids conjugates and TB= *M.tuberculosis*, presented in the order of administration.

In the spleen (Figure 3.10), mycolic acids also shifted the immune system towards a pro-inflammatory state in mice that were either co-infected with *M. tuberculosis* and LP-BM5 MuLV (M+Serum+TB and M+MA+TB, $P<0.01$), or mice that were mono-infected with *M. tuberculosis* mycolic acids (MA+TB and Serum+TB, $P<0.01$).

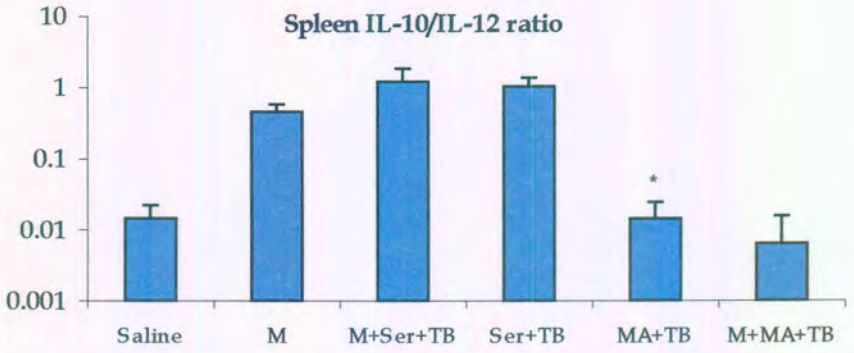


Figure 3.10: The ratios of IL-10 and IL-12 cytokine expression levels in the spleen of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The bars on the graph represent average ratios of the three mice per group obtained by random selection, while * denotes an average of two. M=MAIDS induction, Sera= treated serum, MA= mycolic acids conjugates and TB= *M.tuberculosis*.

There appeared to be no significant shift in the IL-10 to IFN-γ ratios due to mycolic acids in the lungs of these mice (Fig. 3.11), in contrast to that found in the spleens (Fig. 3.12) where mycolic acids once again caused a significant shift towards a pro-inflammatory IFN-γ (Serum+TB versus MA+TB, $P<0.01$ and M+Ser+TB versus M+MA+TB, $P<0.05$).

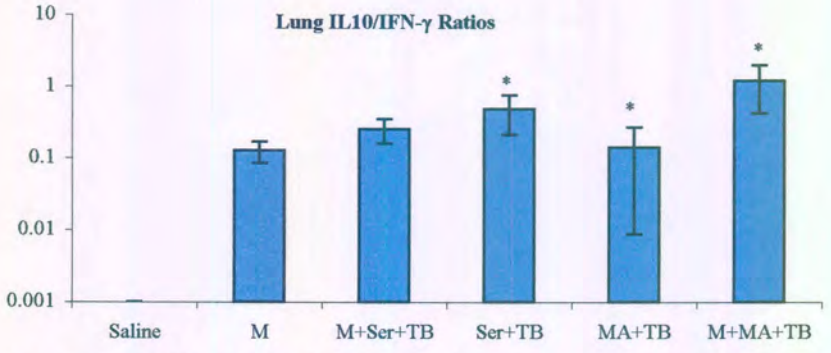


Figure 3.11: The ratios of IL-10 and IFN-γ cytokine expression levels in the lungs of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The bars on the graph represent average ratios of the three mice per group obtained by random selection, while * denotes an average of two. M=MAIDS induction, Sera= treated serum, MA= mycolic acids conjugates and TB= *M. tuberculosis*.

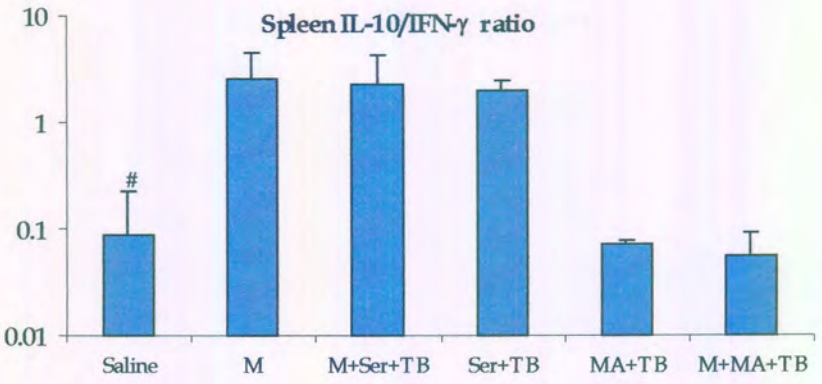


Figure 3.12: The ratios of IL-10 and IFN-γ cytokine expression levels in the spleens of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The bars on the graph represent average ratios of the three mice per group obtained by random selection. M=MAIDS induction, Sera= treated serum, MA= mycolic acids conjugates, TB= *M.tuberculosis*. # indicates a large error.

The anti-inflammatory IL-10 to pro-inflamatory IL-12 and IFN-γ ratios in both the spleens and lungs of mice indicated that mycolic acids did induce expression of IL-12 but that IFN-γ expression was restrained in the lungs. Mycolic acids administration therefore induces expression of pro-inflammatory IL-12 concomitant with the observed inhibition of anti-inflammatory IL-10 in both the lungs and the spleens. In the spleens IFN-γ is upregulated as well, but not in the lungs. This was however, not enough to provide increased survival, as the survival studies indicated that mycolic acids had no effect.

3.6 Discussion

C57Bl/6 mice are more resistant to *M tuberculosis* infection than Balb/c mice, apparently due to a higher base level of IL-12 expression (Flynn *et al.*, 1995; Flynn *et al.*, 1996). Flynn *et al.* (1995) showed that protection against tuberculosis could be effected by administration of IL-12 in Balb/c mice, but not in C57Bl/6 mice. Pretorius (1999) showed that the difference in basal IL-12 expression between the two strains of mice mainly manifested itself in the lungs. C57Bl/6 mice are however extremely susceptible to infection by LP-BM5 murine leukaemia virus, which renders the animals immune deficient (Morse *et al.*, 1992).

The protection provided by mycolic acids against tuberculosis in Balb/c mice observed in previous studies correlated with the induction of IL-12 in the lungs (Pretorius, 1999), but not in the spleen (Siko, 1999). Siko (1999) also found that C57Bl/6 mice were resilient to pre-treatment with mycolic acids before *M. tuberculosis* infection, in contrast to what was observed in Balb/c mice. Evidence from cytokine studies (Pretorius 1999) suggested that mycolic acids induced a pro-inflammatory IL-12 upregulation in the lungs of the *M tuberculosis* infected mice, that correlated with marginally improved survival.

The murine leukaemia virus (MuLV), LP-BM5 causes murine AIDS (MAIDS) in C57Bl/6 mice and co-infection with *Mycobacterium avium* accelerates dissemination of mycobacteria (Orme *et al.*, 1992). This study went further to investigate if mycolic acids will provide the immune boost needed to control *M. tuberculosis* infection in virally induced immune-compromised C57Bl/6 mice. This may hold true if MAIDS mice lost their capacity to express basal levels of IL-12 in the lungs.

The survival rate of mice co-infected with *M. tuberculosis* and LP-BM5 MuLV was significantly reduced, as expected. This was in agreement with previous reports that co-infection with LP-BM5 MulV and mycobacteria, such as *M. avium* (Orme *et al.*, 1992) and *M. bovis* (Umemura *et al.*, 2001), reduced the survival rate. Here, mycolic acids pre-treatment was found to be unable to prevent disease exacerbation under conditions of co-infection with LP-BM5 MuLV and *M. tuberculosis*.

Infection with LP-BM5 MuLV did not induce any suppression of IL-12 in the lungs, but only suppressed IL-12 in the spleens. This could be the main reason why mycolic acids pre-treatment could not induce any protection against *M. tuberculosis* infection in C57Bl/6 mice immunocompromised with LP-BM5MuLV infection. Previous studies (Pretorius, 1999) indicated that C57Bl/6 mice have high IL-12 base levels in the lungs and infection with *M. tuberculosis* could not enhance this as in Balb/c mice. Mycolic acids in this study were found to induce a significant cytokine response in the spleen and not in the lungs as seen in Balb/c mice (Pretorius, 1999). This may explain why mycolic acids administration could not assist in controlling *M. tuberculosis* infection in this study. Studies in Balb/c mice indicated that mycolic acids induced protection against *M. tuberculosis* did not correlate to cytokine expression in the spleen (Siko, 1999). Hence in this study, where mycolic acids only significantly influenced cytokine expression in the spleen, there was no mycolic acids-induced protection against either infection with *M. tuberculosis* or combined LP-BM5 MuLV and *M. tuberculosis* infection.

Mycolic acids did suppress the expression of IL-10 in immune compromised animals that were infected with *M. tuberculosis*. This was more prominent in the spleens than in the lungs. In the lungs however, mycolic acids did not suppress IL-10 expression in immune-compromised mice that were co-infected with *M. tuberculosis*. Other studies in C57Bl/6 mice suggested that mycolic acids actually induce IL-10 in peritoneal macrophages (Stoltz, 2002). Stoltz speculated that mycolic acids induced alternative activation of peritoneal macrophages that is characterised by IL-10 as one of the principal indicators. Stolz suggested that mycolic acids induced protection against *M. tuberculosis* infection might proceed by alternative activation of macrophages, rather than classical activation through IL-12 to supports a Th1-type cytokine profile. Evidence in this study suggest otherwise as it was observed that mycolic acids did not have any effect on IL-10 expression in lungs of immune-compromised mice and even suppressed IL-10 expression in the lungs of mice that were only infected with *M. tuberculosis*.

Mycolic acids administration has been suggested to enhance a Th1-type bias in *M. tuberculosis* infected Balb/c mice (Pretorius, 1999). This suggestion was based on the observation that mycolic acids administration induced IL-12 and IFN-γ in the lungs, but not in the spleens of *M. tuberculosis* infected and uninfected Balb/c mice (Siko, 1999). These observations correlated with those from others, who observed that administration of IL-12 impaired growth of *M. tuberculosis* in the lungs but not the spleens of mice (Flynn et al., 1995; Cooper et al., 1995).

The results in this study in terms of ratios of anti-inflammatory and pro-inflammatory cytokines indicated that mycolic acids induce a pro-inflammatory cytokine response. Whereas mycolic acids appeared generally to have no significant effect on the individual IL-12 and IL-10 expression in the lungs when measured individually, the IL-10/IL-12 ratios, which reduced mouse to mouse variation indicate that mycolic acids are pro-inflammatory in the lungs as well as in the spleens. The IL-10/IFN-γ ratios in the lungs displayed resilience to mycolic acids administration, in contrast to spleens. Protection due to mycolic acids pre-treatment against tuberculosis was previously observed to rely not only on IL-12 expression, but also to the concomitant expression of IFN-γ in the lungs (Flynn *et al.*, 1995; Pretorius, 1999). This then correlates to the observations made in this study that there was no protection observed against tuberculosis either in mice that were infected with *M. tuberculosis*, or in mice infected with combined LP-BM5 MuLV and *M. tuberculosis*. Although the cytokine profiles complied to the induction of IL-12 upon mycolic acids administration, IFN-γ was concomitantly expressed only in the spleens and not the lungs, where it is primarily required. Mycolic acids did not, therefore protect against tuberculosis in MAIDS-induced immune-compromised C57Bl/6 mice.

The data obtained in this study confirms that mycolic acids are involved in the induction of immune activity but not of the kind that can compensate for LP-BM5 MuLV induced immunodeficiency. Mycolic acids may have their effect through cellular mechanism such as NK cells, or macrophages, rather than the T helper cells, as was shown in the previous chapter (Chapter 2).

Cellular candidates in this unclear mechanism may involve cells that express CD1 molecules. In humans, CD1b is known to present mycolic acids to T-cells (Beckman, 1994). In mouse systems only CD1d is found, but its role in the presentation of mycolic acids is still unresolved. The structure of CD1d molecules suggests that they present hydrophobic molecules, not excluding mycolic acids as a possible candidate. Studies have indicated that blocking of this molecule with antibodies enhances *M. tuberculosis* infection and its spread (Szalay *et al.*, 1999), although it was also indicated that mutant deletion of CD1d had no effect on the spread of *M. tuberculosis* infection (Behar *et al.*, 1999).

There seems to be more at play than simply the enhanced constitutive expression of IL-12 in the lungs of C57Bl/6 mice that makes them more resistant to tuberculosis than Balb/c mice. Here the level of expression of IL-12 relative to IL-10 in the lungs of infected C57Bl/6 mice, could be significantly enhanced by mycolic acids administration. This argues in support of the boosting of innate immunity to resist tuberculosis under conditions of immune-compromise. This did not happen, however, because IFN-γ expression did not follow on the IL-12 stimulation in the lungs. Future research could profitably be focused on the comparative linking mechanisms between IL-12 and IFN-γ expression in the lungs of Balb/c and C57Bl/6 mice to elucidate the possible mechanistic action of mycolic acids induced protection against tuberculosis.

CHAPTER 4

Combination of Chemotherapy with Mycolic Acids During Treatment of *M. tuberculosis* Infected Balb/c Mice

4.1 Introduction

Tuberculosis therapy has in the past three decades improved due to a better understanding of the mechanism of interaction between the host, *Mycobacterium tuberculosis* and the drugs (Stead and Dutt, 1988; Grosset, 1995). The bacterial load in the lung cavities is high because *M. tuberculosis* is an obligate aerobe growing in proportion to the oxygen concentration of the environment. For most drugs to be effective, the bacteria they are targeting must be actively growing. Because *M. tuberculosis* grows slowly and exhibits a remarkably high mutation rate, the duration of daily chemotherapy usually needs to exceed six months (Mitchison, 1969; Chopra and Brennan, 1997; Mitchison, 1998; Cynamon *et al.*, 1999).

4.1.1 Anti-tuberculosis drugs

The first antibiotic that proved to be effective towards *M. tuberculosis* was streptomycin (SM), which became available 61 years after the discovery of *M. tuberculosis*. More drugs have since been discovered, some of which are more effective than streptomycin. The standard therapy for tuberculosis was defined a number of years ago, and includes the usage of isoniazid (INH) and ethambutol (EMB), with or without SM. These drugs target different populations of growing *M. tuberculosis* (Fig 4.1).

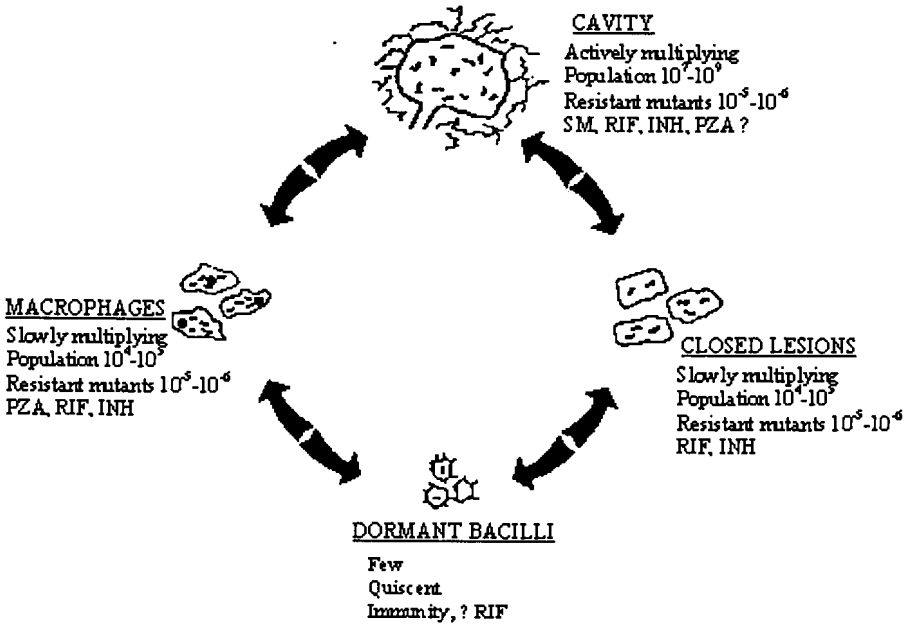
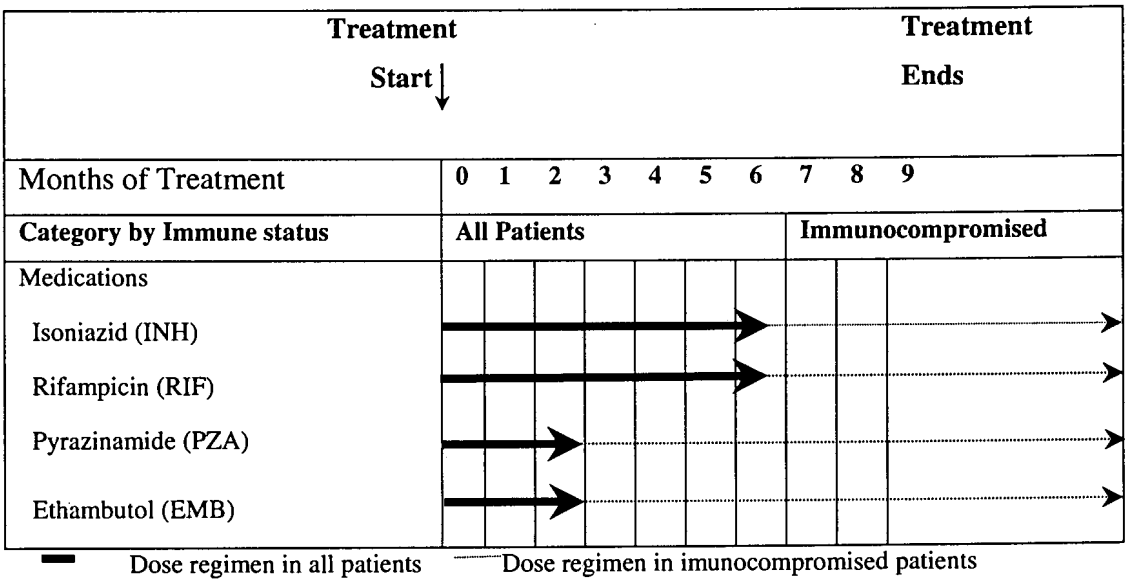


Figure 4.1: Bacterial populations during progression of tuberculosis. (Stead and Dutt, 1988)

This therapy was found to be effective on condition that it is continued for up to 24 months (Stead and Dutt, 1988; Harding and Bailey, 1994). Lack of compliance with the 24-month therapy created complications such as relapse of the disease and mutations of *M. tuberculosis*. The discovery of rifampicin (RIF) and a new understanding of the mechanism of action of an old drug, pyrazinamide (PZA), resulted in the reduction of the treatment to 9 or even 6 months. Today the World Health Organization (WHO) strongly recommends the use of a regime of a combination of four drugs (Table 4.1). These are administered to the patient in the presence of a trained health care worker in a process referred to as the directly observed treatment short course (DOTS).

Table 4.1: Drugs recommended for tuberculosis short course treatment



4.1.2 T-cell mediated immunity and tuberculosis chemotherapy

There is plenty of evidence implicating T-cell mediated immune responses in the resistance to mycobacteria (Orme *et al.*, 1993; Porcelli *et al.*, 1995; Tsukaguchi et *al.*, 1995). Tentori *et al.* (1998) and Giuliani *et al.* (1998) went a step further and described the involvement of a cytokine-induced expression of CD1b in antigen presenting cells (APC). They also implied that double negative (DN) T-cell response might be involved in chemotherapy and resistance to tuberculosis. DN T-cells react specifically to mycolic acids derived from the cell-wall of *M. tuberculosis* and presented by professional antigen presenting cells (APC) (Beckman *et al.*, 1994).

Various rifamycins (chemical derivatives of RIF), such as rifampetine (RFP) and rifalazil (RZL), are drugs that are widely used in chemotherapy against *M. tuberculosis* infection (Lenaerts *et al.*, 1999; and Lenaerts *et al.*, 2000). Administration of chemotherapeutic

drugs against *M. tuberculosis* infection has been found to reduce B and T cell-dependent responses, including chemotaxis and DTH (Van Vlem *et al.*, 1996). The use of chemotherapy alone in the absence of an effective immune system may not be effective. This could be partly responsible for the relatively large rate of relapse and the lengthy period required to treat *M. tuberculosis* infection. Combining chemotherapy and immunotherapy has demonstrated the potential to curb this problem (Liakopoulou, 1989; Van Vlem *et al.*, 1996; Lowrie *et al.*, 1999). These observations prompted Tentori *et al.* (1998) to further investigate the effects of RIF on macrophages (MΦs), with regard to antigen presentation by CD1b molecules. Their investigations were performed on cytokine-activated monocytes (CAM) [adherent mononuclear cells (AMNC) activated with either IL-4 or GM-CSF or both] and they found that administration of RIF to these cells increased expression of CD1b (Tentori *et al.*, 1998; and Giuliani *et al.*, 1998). This suggested that the administration of RIF could be beneficial in improving mycobacterial antigen presentation, but detrimental in other forms of cell mediated immunity. From this arose the idea that the efficiency of chemotherapy may be enhanced by concomitant immunotherapy (Hernandez-Pando *et al.*, 2000; Rook and Hernandez-Pando, 1994).

Studies in animal models have suggested that immunotherapy in conjunction with chemotherapy could serve as a valuable approach to curb the spread of tuberculosis. A number of studies have shown that immunotherapy with DNA vaccination combined with chemotherapy shift the immune system from that which renders bacteriostasis to that which is bactericidal (Huygen *et al.*, 1996; Lowrie *et al.*, 1999). This has been achieved by establishment of cellular immunity that ultimately resulted in the production of cytokines such as interferon-γ (IFN-γ) and interleukin-12 (IL-12) to support the chemotherapeutic effect on tuberculosis with the appropriate bactericidal immune response. The use of IL-12 DNA "vaccine" in combination with chemotherapy has been shown to clear mycobacteria from the lungs (Lowrie *et al.*, 1999). The effects observed with DNA vaccines could also be attributed to the immunostimulatory 'adjuvant' effect of the plasmid itself (Krieg *et al.*, 1998).

4.2 Aims

The objective of this study was to assess the effect of combining chemotherapy (antibiotic treatment) and mycolic acids administration on the clearance of *M. tuberculosis*. This approach has been motivated by previous studies in which mycolic acids were observed to have the ability to prolong the survival of *M. tuberculosis*-infected mice (Chapter 2 and Siko, 1999). Pretorius (1999) indicated that mycolic acids also have the ability to induce expression of IL-12 and IFN-γ in mice infected with *M. tuberculosis*, the cytokines known to protect against tuberculosis.

The chemotherapy experiment was designed in such a way as to determine whether mycolic acids administered as a homogenous mouse serum conjugate to intranasally infected mice could shorten the duration of the antibiotic treatment to which the experimental animals were subjected.

The effect of antibiotic treatment, and of a combined treatment comprising antibiotics and mycolic acids, on the spread of *M. tuberculosis* in experimental animals was studied. The animals from the experimental groups were randomly sacrificed 4, 16, 37 and 54 weeks after the start of the *M. tuberculosis* infection, and their lungs and spleens were analysed for the number of viable mycobacteria present.

4.3 Materials

4.3.1 Cultures

Mycobacterium tuberculosis H37Rv ATCC 27294 - a virulent strain, originally isolated from an infected human lung.

The cultures were purchased in lyophilized form from the American Type Culture Collection (ATCC), Maryland, USA.

4.3.2 Growth medium used for the cultivation of *M. tuberculosis*

Middlebrook 7H-10 agar medium (in plates) was used as growth medium both for the cultivation of *M. tuberculosis*, prior to the extraction of mycolic acids and for the enumeration of *M. tuberculosis* in animal organs.

A detailed composition of the ingredients necessary for the preparation of these media as well as the conditions recommended for their sterilization, are given in the Laboratory Manual of Tuberculosis Methods, Tuberculosis Research Institute of the SA Medical Research Council (1980, Chapter 6, pp 83-105; Second Edition, revised by E E Nel, H H Kleeberg and E M S Gatner). Media were prepared by staff of the National Tuberculosis Institute of the Medical Research Council of South Africa, in Pretoria.

4.3.3 Reagents

Reagents used for: the extraction, saponification and derivatization of mycolic acids; the purification of mycolic acids; and for the HPLC analysis of mycolic acids are described in detail by Siko (1999) and Goodrum, Siko *et al.* (2001)

4.3.4 Washing and dilution of *M. tuberculosis*

The harvested mycobacteria used for infecting the experimental animals were washed in sterile 0,9% m/v NaCl (Chemically Pure, Saarchem, RSA) and adjusted to the appropriate concentration.

Diluent used for the preparation of serial dilutions, preceding the determination of viable counts of *M. tuberculosis*, was prepared by dissolving Tween 80 (Chemically Pure, Merck) in 0,9% m/v NaCl (Chemically Pure, Saarchem) to a concentration of 0,01% v/v and distributing and autoclaving it in 9,0 ml aliquots into test-tubes. The autoclaved tubes with diluent were stored at 4^0C.

4.3.5 Antibiotics

Isoniazid (Noristan-Isoniazid) - manufactured by Noristan Ltd, Waltloo, Pretoria, South Africa;
Rifampicin (Rimactane) - manufactured by Ciba-Geigy Ltd, Basel, Switzerland;
Pyrazinamide (Rolab-Pyrazinamide) - manufactured by Spartan, Kempton Park, South Africa.

4.3.6 Preparation of organ homogenates

Sterile saline (0,9% m/v NaCl, Saarchem, Chemically Pure, RSA) was used for the preparation of the organ homogenates.

Diluent for the preparation of serial dilutions of the organ homogenates, preceding the determination of the concentration *M. tuberculosis* in various mouse organs was the same as that described for dilution of *M. tuberculosis* (see 4.3.4).

4.3.6.1 Blood decontamination

N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH):

i) NaOH pellets 4 g in 100 ml distilled water;

ii) Sodium citrate 2,9 g in 100 ml distilled water.

After autoclaving at 121°C for 15 minutes, both solutions were mixed together with 1,0 g of N-acetyl-L-cysteine powder.

Phosphate buffer 0,15 M, pH 6,8:

iii) Anhydrous Na_2HPO_4 9.47 g in 1000 ml distilled water;

iv) Anhydrous K_2HPO_4 9.07 g in 1000 ml distilled water.

Solutions iii and iv (50 ml) were mixed and the pH checked and adjusted to pH 6,8 with either of the solutions.

4.3.7 Experimental animals

Eight to twelve weeks old female Balb/c (a tuberculosis-susceptible strain) mice were used in the chemotherapy experiment. The mice were inbred for at least 11 generations at the Animal Centre at the South African Institute for Medical Research in Johannesburg. Male mice of corresponding age were used for the collection of sera necessary for the preparation of mycolic acids/mouse serum conjugates. Mice cubes, manufactured by EPOL and autoclaved tap water were provided *ad libitum.*

Sanitation-Bronocide, manufactured by Essential Medicines (Pty) Ltd, was used for sanitation purposes.

4.3.8 Plasticware

The following plasticware was used: Disposable Petri dishes (Promex, RSA); disposable tissue culture dishes (Promex, RSA); Sterile, disposable, 50 ml centrifuge tubes (Corning, USA) and disposable sterile tips (Elkay, Denmark)

4.3.9 Preparation of organ homogenates

Instruments and materials used for preparation of homogenates were as follows: Sterile scissors; sterile tweezers; sterile teflon "homogenisers"; sterile tubes with heparin; sterile glass rods (used for the spreading of mycobacterial suspensions on the surface of agar plates) and crushed ice.

4.4 Methods

4.4.1 Cultivation of bacteria

The mycobacteria for the extraction of mycolic acids were cultivated at 37°C using Middlebrook 7H-10 agar medium plates.

The sterility of the media was confirmed visually before use in the experiments by incubating them at 37^0C for 24 h.

For routine extraction of mycolic acids approximately 3-4-week old *M. tuberculosis,* grown on Middlebrook 7H-10 agar medium plates were used.

For the preparation of bacterial suspensions used for the experimental induction of tuberculosis, approximately 2-week old cultures of *M. tuberculosis*, grown on Middlebrook 7H-10 agar medium plates, were used.

4.4.2 Viable bacterial counts

For the viable count determination, serial suspensions of the harvested bacteria were prepared in the diluent medium (as specified under 4.3.4) to a density corresponding to a McFarland standard 4 (approximate optical density (OD) of 1,0; using a Beckman DU 65 spectrophotometer, at 486 nm). Tenfold serial dilutions were prepared using 9 ml aliquots of the diluent medium. From the last three dilutions corresponding to 10^{-3}, 10^{-4} and 10^{-5} of the original suspension, aliquots of 0,1 ml (100 µl) were withdrawn and spread over the surface of Middlebrook 7H-10 plates. The plates were incubated at 37^0C and the developed colonies counted after two to three weeks.

Statistical analysis of the bacterial counts included the mean values of bacterial counts and standard deviations.

4.4.3 Preparation of mycolic acids from bacterial samples

Mycolic acids were prepared according to a method described in chapter 2.

4.4.4 Preparation of conjugates

The required mass of mycolic acids (2,5 mg) was dissolved in 200 µl chloroform and added to 10,0 ml of mouse serum, previously filtered through a 0,22 µm filter. Thus, the volume of dissolved mycolic acids constituted 2% of the volume of mouse serum.

The sample was sonicated using a Branson Sonifier B 30 Cell Disruptor, (at 20% duty cycle, output control of 2, for 50 pulses, at room temperature). The sample was maintained for 1 hour at room temperature, to allow air bubbles formed during sonication to escape. In order to remove chloroform, nitrogen was bubbled through the conjugate until the chloroform odour was removed. The conjugate was prepared immediately before administration to the experimental animals.

4.4.5 Preparation of bacterial suspensions

The cells of *M. tuberculosis* H37 Rv, harvested from Middlebrook 7H-10 agar medium plates, were suspended in the diluting buffer (0,01% v/v Tween 80 in 0,9% m/v NaCl) and homogenized. After centrifugation in a Beckman J-6 centrifuge for 20 min at 1 580 g, the cells were washed with a sterile solution of 0,9% m/v of NaCl and adjusted to a concentration corresponding to a McFarland standard No.4. After the confirmation of the total direct bacterial count, carried out on an autoclaved suspension in a Neubauer counting chamber, the suspension was further diluted in the sterile solution of 0,9% NaCl to obtain concentrations of *M. tuberculosis* corresponding to 10^3, 10^4 and 10^5 cells/ml.

The viable counts of the mycobacteria in the suspensions were confirmed by plating 100 µl aliquotes of the relevant dilutions onto Middlebrook 7H-10 agar medium, incubating the plates at 37^0C for two weeks and counting the number of colony forming units (CFU).

The suspensions were introduced into the experimental animals in aliquots of 100 µl or 60 µl per animal.

4.4.6 Experimental animals

Eight to twelve weeks old female Balb/c mice were accommodated in cages with a floor area of 450 cm^2, with 8 mice per cage.

Environmental conditions: Temperature and humidity in the animal facility were set at 20^0C (+/- 1^0C) and 40% (+/- 10%), respectively. Lighting was provided by means of fluorescent tubes. A light-darkness cycle of alternating 12 hour periods was set up.

Mice were housed in transparent polypropylene cages with tight fitting stainless steel lids. Wooden shavings, after autoclaving, were provided as nestling material. Animal rooms, mice cages and glass bottles were cleaned and decontaminated once a week using Bronocide. Water bottles, after washing, were autoclaved once a week.

Mice were maintained and caged in Techniplast animal isolators marketed by Labotec, South Africa. A positive pressure of 4 atm inflated the isolator. It was equipped with an air inlet pre-filter (with the pore size of 0,6 μm) through which the incoming air was filtered and an outlet HEPA (High Efficiency Particulate Air) filter (with a pore size of 0,22 μm) through which the outgoing air was filtered before leaving the isolator. The airflow rate was regulated at 7 exchanges per hour. Animal rooms, the animal cage

isolators and water bottles were cleaned and decontaminated once a week using Bronocide. Individual identification of mice was accomplished by making ear marks.

4.4.7 Inoculation with *M. tuberculosis* H37 Rv suspensions

The introduction of the *M. tuberculosis* suspensions was performed in a biosafety cabinet class III in the PIII facilities at the Tuberculosis Institute of the Medical Research Council in Pretoria. The introduction of the bacterial suspensions *via* intranasal route was carried out as follows:

Mice were anaesthetized with 5% diethylether. The bacterial suspensions were introduced into the nostrils of mice in aliquots of 60 μl per animal. The suspensions were released drop-wise into the nostrils using autoclaved pipette tips, while the animals were in dorsal recumbence. Control animals received an equivalent volume of sterile saline, *i.e.*, 60 μl introduced intranasally.

4.4.8 Introduction of MA-serum conjugate and mouse serum

The administration of the mycolic acids conjugates was carried out *via* the intravenous route, after mice were heated for 5 min in a heating box to effect vasodilation of the tail veins. The mycolic acids-mouse serum conjugate was administered by introducing 5 μg or 25 μg mycolic acids in 100 μl mouse serum per mouse. Control animals received 100 μl of mouse serum introduced in the same manner.

4.4.9 Administration of antibiotics

Three antibiotics were selected for the experiment, namely: isoniazid, rimactane (rifampicin) and pyrazinamide. Although isoniazid and pyrazinamide are water-soluble and could be administered to the experimental animals with drinking water, these

compounds are chemically unstable and their potency would vary throughout the experiment. For that reason it was decided to introduce the appropriate dosages of antibiotics mixed with crushed mouse pellets. The dosages administered to mice were calculated on the basis of the daily dosages recommended for humans, expressed in terms of the mouse metabolic weight.

Isoniazid and pyrazinamide tablets were crushed using a pepper mill, mixed with the contents of rifampicin capsules and introduced into the mouse pellets crushed to powder in such a way that each 5 g portion of mouse feed contained: 1 mg isoniazid, 1,4 mg of rifampicin and 1,3 mg of pyrazinamide. Assuming that a mouse consumes 5 g of food per day, it should consume appropriate quantities of the respective antibiotics to sterilize the lungs of infected mice. Food required for the mice in each cage was then transferred using a spatula to the compartments of the special trays prepared specially for this purpose.

4.4.10 Preparation of organ homogenates

The experimental animals to be sacrificed were selected from each experimental group at random at 4, 16, 37 and 54 weeks after the infection with *M. tuberculosis* H37Rv. All the steps were carried out in a sterile manner. The specimens for further investigations were prepared as follows:

i. The animals were sacrificed by neck dislocation. The spleens and the lungs were removed using sterile scissors and tweezers and placed into individual, sterile small tissue culture dishes, suitably marked with the mouse number and the type of the organ removed. Each organ was weighed and the mass recorded.

ii. The spleens and the lungs were transferred into sterile homogenizers into which aliquotes of 300 μl sterile saline were introduced. After satisfactory homogenates

were prepared, the suspensions were diluted with the sterile saline to reach the concentration of approximately 1 mg/ml.

iii. The homogenates were decontaminated according to the method specified in section 4.4.11 and the volume of the homogenates adjusted to 1,5 ml. Serial dilutions were prepared in the diluting medium.

iv. In the course of these experiments the following dilutions of the lungs' and spleen's decontaminated homogenates were prepared: undiluted, 10^{-1}; 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}.

v. Duplicate or triplicate plates were prepared for each dilution by evenly spreading the introduced aliquots with the sterile glass rods. Each tube with a respective dilution was vortexed prior to plating.

vi. The inoculated plates were placed in an incubator at 37°C and regularly checked for growth and the presence of contaminations.

4.4.11 Decontamination of organ homogenates

Decontamination of spleen and lungs homogenates was carried out according to the method recommended by Kubica *et al.*, (1963, 1964) and was performed as follows:

Aliquots of the spleen or lungs homogenates were introduced into 10 ml centrifuge tubes. Into each tube an equal volume of N-acetyl-L-cysteine-sodium hydroxide (NACL-NaOH) was added. Caps on the test tubes were tightened securely and the content was mixed using a test tube vortex until completely liquefied, for approximately 10-20 seconds. The mixtures were allowed to stand for 15 minutes at room temperature.

The tubes were then filled up with sterile phosphate buffer pH 6,8 and centrifuged at 2000 g for 15 minutes using a Labofuge GL centrifuge. The supernatants were discarded and 1,5 ml of sterile phosphate buffer pH 6,8 was introduced into each tube. The sediments were resuspended and were considered to be undiluted, decontaminated sample homogenates.

4.4.12 Plating of decontaminated homogenates

Appropriate dilutions of the decontaminated homogenates were prepared in the diluting medium (0,01 % Tween 80 in saline) and aliquots of 100 μl of the respective dilutions were plated onto the Middlebrook H-10 agar plates.

Duplicate or triplicate plates were prepared for each dilution by evenly spreading the introduced aliquots with the sterile glass rods. Each tube with a respective dilution was vortexed prior to plating. The inoculated plates were placed in an incubator at 37°C and regularly checked for growth and the presence of contaminants.

4.4.13 Experimental set-up

The set up of the study is indicated in Table 4.2.

Table 4.2: Experimental setup of the the chemotherapy experiment groups.

Group No	Treatment and infection with *M. tuberculosis*	Administration of antibiotics	Number of mice per group
1	100 µl saline *i.n.*. No infection with *M. tuberculosis*	Yes	10
2	100 µl saline *i.n.*. No infection with *M. tuberculosis*	No	6
3	25 µg MA *i.v.* *M. tuberculosis i.n.*	Yes	12
4	25 µg MA *i.v.* *M. tuberculosis in*	No	12
5	100 µl serum *i.v.* *M. tuberculosis in*	Yes	12
6	100 µl serum *i.v.* *M. tuberculosis i.n.*	No	12
7	*M. tuberculosis i.n.*.	Yes	6
8	*M. tuberculosis i.n.*.	No	5

1) *i.v.* - intravenous inoculation and *i.n.* – intranasal inoculation

4.5 Results

4.5.1 Confirmation of doses of *M. tuberculosis* and mycolic acids

By determining the colony forming units (cfu) of *M. tuberculosis*, it was established that the sub-culture of *M. tuberculosis* used in this study was diluted to a concentration of 2,5 x 10^6 cfu/ml. The dose of *M. tuberculosis* introduced intranasally into the animals was therefore confirmed to be 2,5 x 10^5 cfu/mouse/60µl saline.

By analysing the mycolic acids sample used to treat the animals by means of HPLC, it was established that the mycolic acids/mouse serum conjugate used in study comprised 25 µg mycolic acids per animal.

4.5.2 Comparison of the masses of the removed organs

The animals in this experiment were randomly selected and sacrificed 4, 16, 37 and 54 weeks after the infection with *M. tuberculosis* H37Rv as indicated in fig. 4.2.

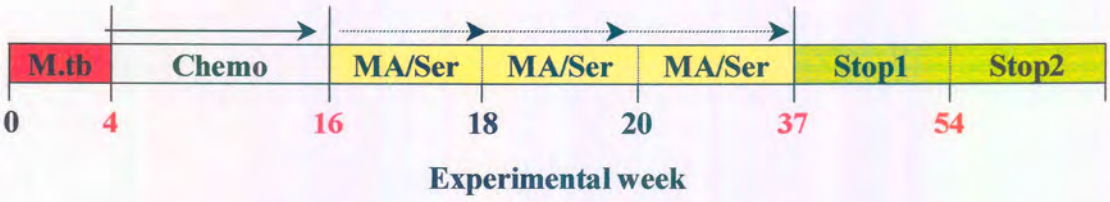


Figure 4.2: A time line display of the combined chemotherapy and MA pre-treatment experiment. The solid line indicates every day ad lib administration of antibiotics ending where the arrow ends and the dashed line indicates weekly administration of MA. The experimental weeks range from week 0 to 54 with weeks numbered in red depicting weeks of organ extraction. M.tb = intranasal *M. tuberculosis* infection; Chemo = administration of INH, PZA and RIF; MA/Ser = administration of mycolic acids or serum; Stop1 and Stop2 = organ bacterial counts to determine *M. tuberculosis* sterility.

The recorded masses of the removed organs were subsequently used for calculating the total number of bacteria present in each respective organ, assuming that the homogenates comprised a uniformly distributed suspension of mycobacteria present in the organs.

The organ masses were compared between various groups of mice. The masses of the spleens and lungs removed from the sacrificed *M. tuberculosis* infected and uninfected animals are presented as organ indices (Fig. 4.3). The masses and indices calculated of the spleens and particularly of the lungs of the infected and control mice at week 4 after *M. tuberculosis* infection appeared to be different. The mean value of the lungs' mass of the mice that had been infected with *M. tuberculosis* was found to be twice as high as that of the control group, that is: 0,4 g ± 0,1 compared to 0,2 g ± 0,1. The lung/body ratios were likewise affected with 1,77 ± 0,153 recorded for the infected group compared to 0,87 ± 0,5 for the control group. The results imply that intranasal infection was causing the expected disease. The viable mycobacterial cell counts from the infected and non-infected animals also confirmed this effect. The counts recorded for the mouse lungs (on average $5.2 \times 10^6 \pm 2{,}17 \times 10^5$) were two orders of magnitude higher than those obtained for the mouse spleens ($6{,}11 \times 10^4 \pm 4{,}71 \times 10^3$).

The organ mass determinations and concomitant viable mycobacterial cell counts at week 16 were done to establish whether the infection with mycobacteria was controlled by the antibiotics treatment and to what a degree the infection was spreading within the organs of mice. The organ indices of the spleens and lungs removed from the sacrificed *M. tuberculosis*-infected and uninfected animals at week 16 are presented in Fig. 4.3.

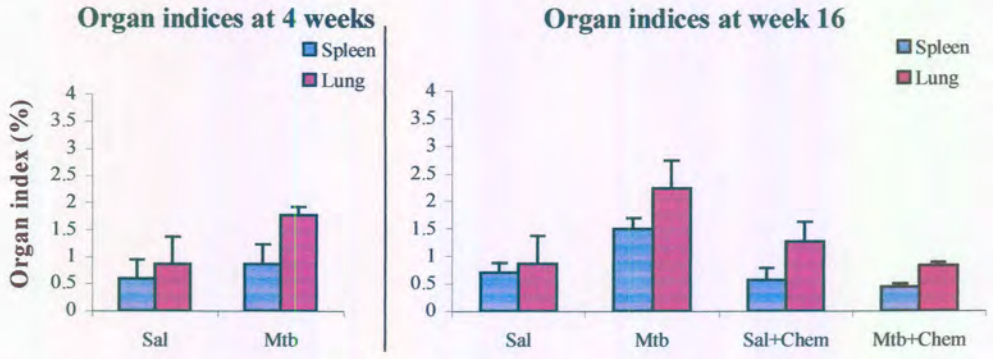


Figure 4.3: Measured organ indices of the spleens and lungs removed from Balb/c mice (4 weeks after infection and 16 weeks after infection). The organ index is expressed as a percentage of organ per body mass. The organs were removed four and sixteen weeks after infection with *M. tuberculosis* or saline treatment. The bars represent an average of three samples. "M.tb" on the x-axis of the graph represents infection with *M. tuberculosis*, "Sal" represents saline treatment, and Chem represents chemotherapy.

The mass of the spleens and the lungs isolated from the uninfected, control animals undergoing or not undergoing chemotherapy as well as from those comprising infected mice undergoing chemotherapy, were similar and considerably smaller than those isolated from the infected mice not undergoing chemotherapy. The spleens isolated from the animals of the first three groups (Sal+Chem, M.tb +Chem and Sal) were on average twice as small (0,133 g ± 0,05) as the corresponding organs isolated from infected and untreated animals (0,367 g ± 0,057). The lungs isolated from uninfected mice and those that received chemotherapy (Sal, Sal+Chem, and M.tb+Chem) were on average 2,5 times smaller than those isolated from the infected and untreated mice (M.tb), eg. Sal = 0,233 g ± 0,087 compared to M.tb = 0,533 g ± 0,115. The lung/body ratios accordingly registered 0,978 ± 0,363 for uninfected (Sal) compared to 2,23 ± 0,503 for the *M tuberculosis* infected (M.tb) mice.

The number of colonies (expressed as colony forming units, cfu) grown on agar plates, recovered from the organs of the control and experimental mice after a 16-week incubation period is presented in Table 4.3.

Table 4.3: Average number of colony forming units recovered from the suspensions of the lungs and of the spleens 16-weeks.after infection

Treatment Group	SPLEENS *M. tuberculosis* count (Mean cfu/organ +/- SD)	LUNGS *M. tuberculosis* count (Mean cfu/organ +/- SD)
Saline (in) and Chemotherapy		
Mouse 1	Neg	Neg
Mouse 2	Neg	Neg
Mouse 3	Neg	Neg
2 x 10^5 H37Rv (in) and Chemotherapy		
Mouse 1	Neg	Neg
Mouse 2	Neg	Neg
Mouse 3	Neg	Neg
Saline (in)		
Mouse 1	Neg	Neg
Mouse 2	Neg	Neg
Mouse 3	Neg	Neg
2 x 10^5 H37Rv (in)		
Mouse 1	2,4 x10^5 ± 6,7x10^4	1,4 x10^6 ± 1,1 x10^5
Mouse 2	1,1 x10^5 ±3,2x10^4	3,7 x10^6 ± 9,5 x10^5
Mouse 3	5,8 x10^5 ± 4,5 x10^3	Contamination

The results in Table 4.3 clearly illustrate the bactericidal properties of the antibiotics used for the treatment of the infected mice. The results recorded for the mice not undergoing chemotherapeutic treatment, might also indicate that the infection with *M. tuberculosis* H37 Rv in the lungs spread further in the spleens of the infected mice.

On analysing the results presented in Figures 4.4 the protective effect of the antibiotics is confirmed, showing that no macroscopic evidence for relapse was observed, 21 weeks after discontinuation of chemotherapy. In the remaining cases of infected animals not receiving chemotherapy it was evident that no protective effect was visible by mycolic acids treatment (Mtb+MA, compared to Mtb+Ser and Mtb alone)

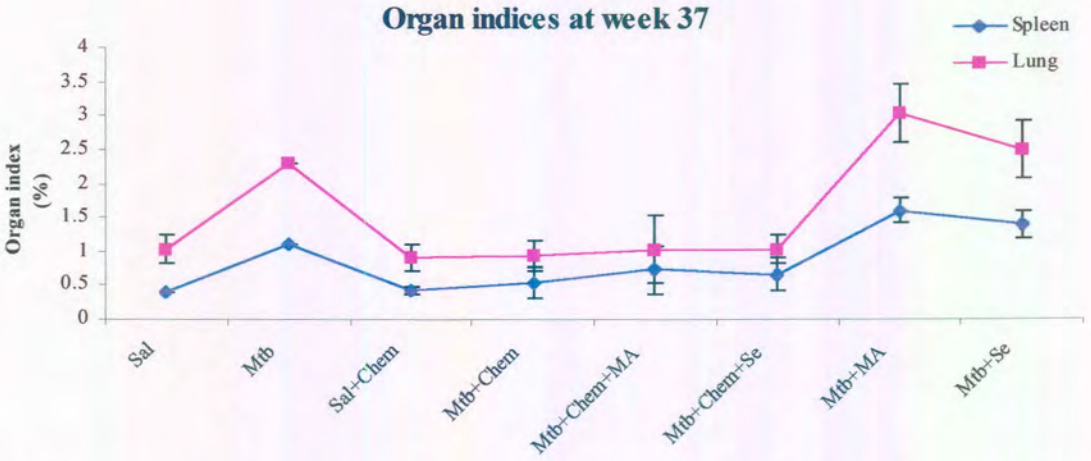


Figure 4.4: Organ indices of the spleens and lungs removed from Balb/c mice and expressed as a percentage of total body mass. The organs were removed 37 weeks after infection with *M. tuberculosis* or saline treatment. The bars represent an average of three samples. "Mtb" on the x-axis of the graph represents infection with *M. tuberculosis*, "Sal" saline treatment, "Chem" chemotherapy, "MA" treatment with mycolic acids conjugated to serum and "Ser" treatment with serum.

The mycobacterial counts from the lungs and spleens from the experimental mice sacrificed at week 37 confirmed the observations made by comparing the organ masses and indices (Table 4.4).

Table 4.4: Average number of colony forming units recovered from the suspension of the lungs and of the spleens 37 weeks after infection

Treatment groups	SPLEENS *M. tuberculosis* count (Mean cfu/organ +/- SD)	LUNGS *M. tuberculosis* count (Mean cfu/organ +/- SD)
Saline (in) and Chemotherapy		
Mouse 1	Neg	Neg
Mouse 2	Neg	Neg
Mouse 3	Neg	Neg
2×10^5 H37Rv (in) + Chemotherapy +25 μg MA		
Mouse 1	Neg	Neg
Mouse 2	Neg	Neg
Mouse 3	$1,2 \times 10^3 \pm 6,4 \times 10^2$	Neg
2×10^5 H37Rv (in) + Chemotherapy + Serum		
Mouse 1	Neg	Neg
Mouse 2	$2,4 \times 10^2 \pm 1,1 \times 10^2$	60
Mouse 3	Neg	$8,4 \times 10^3 \pm 3,9 \times 10^2$
2×10^5 H37Rv (in) + Chemotherapy		
Mouse 1	30 ± 10	$4,6 \times 10^4 \pm 2,2 \times 10^4$
Mouse 2	Neg	70 ± 14
Mouse 3	Neg	Neg
Saline (in)		
Mouse 1	Neg	Neg
Mouse 2	Neg	Neg
Mouse 3	Neg	Neg
2×10^5 H37Rv (in) + 25 μg MA		
Mouse 1	$1,3 \times 10^3 \pm 1, \times 10^3$	$5,9 \times 10^6 \pm 1,5 \times 10^6$
Mouse 2	$1,7 \times 10^3 \pm 4,2 \times 10^2$	$7,3 \times 10^5 \pm 6,4 \times 10^4$
Mouse 3	$1,2 \times 10^5 \pm 9,9 \times 10^2$	$6,7 \times 10^6 \pm 1,3 \times 10^6$
2×10^5 H37Rv (in) + Serum		
Mouse 1	$1,4 \times 10^4 \pm 4,1 \times 10^3$	$3,8 \times 10^5 \pm 1,1 \times 10^5$
Mouse 2	$1,1 \times 10^5 \pm 7,8 \times 10^4$	$2,2 \times 10^6 \pm 1,3 \times 10^6$
Mouse 3	$7,9 \times 10^5 \pm 1,9 \times 10^{24}$	$3,6 \times 10^5 \pm \times 10^2$
2×10^5 H37Rv (in)		
Mouse 1	$3,2 \times 10^4 \pm 5,6 \times 10^4$	$3,17 \times 10^2 \pm 4,2 \times 10^2$

No growth was found in the noninfected animals as expected. However, the cell counts show that organs were not sterilized from mycobacteria at week 37, with the exception of those that received combined chemotherapy and mycolic acids treatment. Table 4.5 summarizes the avarages of the groups that had a positive count.

Table 4.5: The calculated average colony forming unitsfrom groups with positive mycobacterial counts, 37 weeks after *M. tuberculosis* infection

Treatment group	**Spleens**	**Lungs**
Mtb + Chemotherapy + 25 μg MA	$1,2 \times 10^3 \pm 6,4 \times 10^2$ (1)*	Not detected
Mtb + 25 μg MA	$4,1 \times 10^4 \pm 6,8 \times 10^4$ (3)	$4,4 \times 10^6 \pm 3,2 \times 10^6$(3)
Mtb + Chemotherapy + Serum	$2,4 \times 10^2 \pm 1,1 \times 10^2$ (1)	$8,4 \times 10^3 \pm 3,9 \times 10^2$ (1)
Mtb + Serum	$3,05 \times 10^5 \pm 4,23 \times 10^5$ (3)	$9,8 \times 10^5 \pm 10,6 \times 10^5$ (3)
Mtb + Chemotherapy	30 ± 10 (1)	$3,3 \times 10^4 \pm 3,25 \times 10^4$ (2)
Mtb	$8,8 \times 10^4 \pm 3,5 \times 10^4$ (1)	$5,05 \times 10^5 \pm 3,6510^5$ (1)

Figures in brackets indicate the number of determinations

The significance of the sterilizing effect of MA and chemotherapy could not yet be determined, and was thus further investigated at week 54.

4.5.3 Mycobacterial cell counts at week 54

This was to establish the effectiveness of the antibiotic therapy 54 weeks since the start of the experiment and a potential effect of the simultaneous administration of mycolic acids on the number of mycobacteria detected in the spleens and lungs of the experimental animals. The results obtained using the organs isolated from 15 mice are presented in Table 4.6.

Table 4.6: Average number of colony forming units recovered from the suspension of the lungs and of the spleens 54 weeks after infection

Mouse No	SPLEENS *M. tuberculosis* count (Mean cfu/organ +/- SD)	LUNGS *M. tuberculosis* count (Mean cfu/organ +/- SD)
2 x 10^5 H37Rv (in) + Chemotherapy + 25 µg MA		
Mouse 1	Neg	Neg
Mouse 2	Neg	Neg
Mouse 3	Neg	Neg
Mouse 4	Neg	Neg
Mouse 5	Neg	Neg
Mouse 6	Not determined	$3{,}2x10^4 \pm 5{,}6\ x10^4$
2 x 10^5 H37Rv (in) + Chemotherapy + Serum		
Mouse 1	Neg	Neg
Mouse 2	Not determined	$3{,}17x10^2 \pm 4{,}2\ x10^2$
Mouse 3	Neg	Neg
Mouse 4	Neg	Neg
Mouse 5	Neg	Neg
Mouse 6	Neg	Neg
Mouse 7	Neg	Neg
Mouse 8	Neg	Neg
Mouse 9	Neg	Neg

Mycobacteria were not detected in any of the examined spleens and only in two out of 15 examined mouse lungs. This result confirms again that the antibiotic therapy appeared to be successful in controlling the infection with *M. tuberculosis* in Balb/c mice.

Mycobacteria were detected in the lungs of both the groups of mice that were placebo treated and treated with mycolic acids. This implied that no significance could be given to the observation at week 37, that mycolic acids appeared to exert additional protection over and above chemotherapy to combat progression of tuberculosis. It is also noteworthy that at week 37, the viable mycobacterial cell count of the spleens of mycolic acids plus

chemotherapy treated mice were higher than that obtained from infected mice receiving chemotherapy only.

The general observations made on these animals were that there was no cross-infection observed between the experimental animals assigned to various groups and maintained in the same isolator. Organs from groups of mice that were infected with *M. tuberculosis*, but did not received chemotherapy, showed high numbers of viable mycobacteria. The tested spleens showed approximately the same level of infection (approximately 1 x 10^4 cfu/plate) whereas the lungs appeared to harbour higher numbers of mycobacteria, ranging from 3,3 x 10^6cfu/plate (for the group treated with mycolic acids-mouse serum conjugate) to 1 x 10^6cfu/plate and 5 x 10^5cfu/plate (for the groups treated with the mouse serum only and not treated at all, respectively).

The results observed so far suggest that administration of mycolic acids had no effect in complementing chemotherapy by sterilizing infection where chemotherapy could not, as infection could be detected after week 54.

4.6 Discussion

This study envisaged emulating studies with DNA "vaccines" in combination with chemotherapy to effect clearance of the mycobacteria in a shorter chemotherapy. Mycolic acids were used to substitute for the immunotherapy to boost the immune system. This was motivated by previous observations that mycolic acids pre-treatment could slightly prolong survival of Balb/c mice that were intravenously infected with *M. tuberculosis* (Siko, 1999) and also induced the expression of IL-12 and IFN-γ in infected and uninfected Balb/c mice (Pretorius, 1999). As well as a later study (Chapter 2) where mycolic acids administration was shown to have a strong protective effect against intranasal infection with *M. tuberculosis*. The approach used in this study was to infect the mice with a minimal lethal concentration of *M. tuberculosis*, apply short treatment course with chemotherapeutic drugs to control the *M. tuberculosis* infection, later to be followed by mycolic acids treatment to boost the immune system.

In all the infected groups, more mycobacteria were detected in the lungs than in the spleen, primarily because in the intranasal infection route the lungs are the port of entry for the mycobacteria, simulating normal infection in humans. After 16 weeks of infection and 12 weeks of chemotherapy all the animals that received chemotherapy were found to be macroscopically clear of infection as compared to the *M. tuberculosis* infected animals that did not receive chemotherapeutic treatment. After termination of chemotherapy, mycolic acids were administered to animals three times from week 16 to week 20 and they were found to have no effect in containing the infection in either the lungs or the spleen after 37 weeks and 54 weeks of infection.

In the experiment performed here, no convincing evidence could be found of complementary benefit provided by mycolic acids administration in addition to the treatment of *M. tuberculosis* infection with chemotherapy. In previous studies (Siko, 1999; Pretorius, 1999) post-infection treatment of animals with mycolic acids were found to be ineffective in controlling tuberculosis. In contrast, pre-treatment with mycolic acids

was found to be effective to curtail subsequent *M. tuberculosis* infection (see Chapter 2). Pre-treatment of Balb/c mice with mycolic acids apparently primed the immune system by means of cytokine expression (Pretorius, 1999) to handle *M. tuberculosis* infection, whereas post-infection treatment with mycolic acids was ineffective against the spread of *M. tuberculosis* infection. In this study however, it was hoped that post-infection chemotherapy prior to mycolic acids administration would restore the responsiveness of the mice to mycolic acids. This appeared not to be the case.

In conclusion, mycolic acids were found to be inefficient as immunotherapeutic supplement to shorten the chemotherapy course to treat tuberculosis. Mycolic acids appeared to be efficient at priming the immune response to a protective mode before an infection, but found no application to be of benefit once the infection has already taken effect.

CHAPTER 5

Development of a biosensor antibody assay for *Mycobacterium tuberculosis* mycolic acids

5.1 Introduction

Scientists have discovered that the lipid rich mycobacterial cell wall (Minnikin and Goodfellow, 1980; Brennan and Nikano, 1995), contributes towards antigenicity of mycobacteria (Porcelli *et al.*, 1992; Sieling *et al.*, 1995). These observations led to the further discovery that mycolic acids, which are abundant mycobacterial cell wall lipids (Minnikin, 1982; Minnikin *et al.*, 1984), are specifically presented in an MHC independent mechanism on CD1 molecules (Beckman *et al.*, 1994; Sugita *et al.*, 1998).

Mycolic acids were found to induce an enhanced antibody response in Sprague Dawley rats that peaked at about 91 days (Siko, 1999). This evidence of the existence of antibodies against mycolic acids confirmed the observation in human tuberculosis patients of antibodies that recognise mycolic acids (Pretorius, 1999). Pan *et al.* (1999) independently corroborated these observations when they reported that the specificity of antibodies that were previously classified as anti-cord factor actually recognized a mycolic acids subclass.

5.1.1 Modern advances in immunoassays

The current rapid technology-driven advances in the genome and proteome research demanded an equal advancement in the techniques to analyse biomolecular interactions to keep pace. Since the antibody-antigen interactions involve reversible molecular

recognition, their interactions can be measured through association and dissociation in an immunoassay.

An immunoassay is a technique that measures the presence of a substance using an immunological reaction such as antibody-antigen interaction. In the 1950s, after the introduction of the technique of radiolabelling of proteins, antibody and antigen dissociation and association could be measured by using radiolabelled antigens or antibodies. The biggest limitation of radioimmunoassay (RIA) was the safety risk involved. Coupling of enzymes to proteins revolutionised immunoassays. The enzyme linked immunosorbent assay (ELISA) introduced in the late 60's (Avrameas, 1969; Avrameas, 1971) is today still extensively used to measure antibody-antigen interactions. The disadvantage of ELISA is that it detects only high affinity antibodies and the interactions are detected at the endpoint. Measurement of real-time binding kinetics of antibody-antigen interaction was possible already since the early 70's by means of techniques such as temperature dependent fluorescent polarization and fluorescent emission isotropy (Weltman and Davis, 1970; Portmann *et al.*, 1971; Dimitropoulos *et al.*, 1986) which required high purity of reagents and was limited by the unknown effect of covalent modification of the antibodies during labelling with fluorophores. The association and dissociation can now be quantified in impure samples of unlabelled antibodies by measuring the rate constants with optical biosensor technology (Badley *et al.*, 1987).

5.1.2 The Resonant mirror biosensor

Biosensors are analytical devices, which can convert a biomolecular interaction signal on a sensing surface into a quantifiable response signal without using any labels. There are two main configurations used for analysing a sample on the sensor surface: equillibrium analysis in the micro cuvette and steady state analysis in the micro flow cell (Fig. 5.1) (Freaney *et al.*, 1997). In both systems a wave of total internally reflected incident light is

generated at the interface of a substance with high refractive index and a substance with low refractive index.

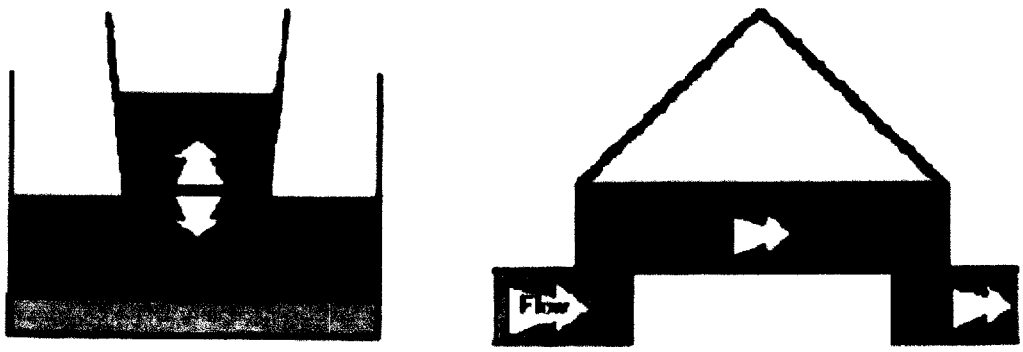


Figure 5.1: Target molecule delivery to the biosensor surface in either **A)** a micro cuvette or **B)** a micro flow cell.

The flow cell system is based on surface plasmon resonance (SPR). The SPR occurs when the sensing surface frees electrons in a film of metal, such as gold, oscillate and absorb energy from an incident light beam at a specific angle (SPR angle). The absorbance is then measured as a sharp decrease in the intensity as measured by a diode detector. In this system binding analysis is determined where one of the interacting partners is immobilised on a gold chip, which forms a wall of the flow cell (Fig. 5.2) (Myszka and Rich, 2000).

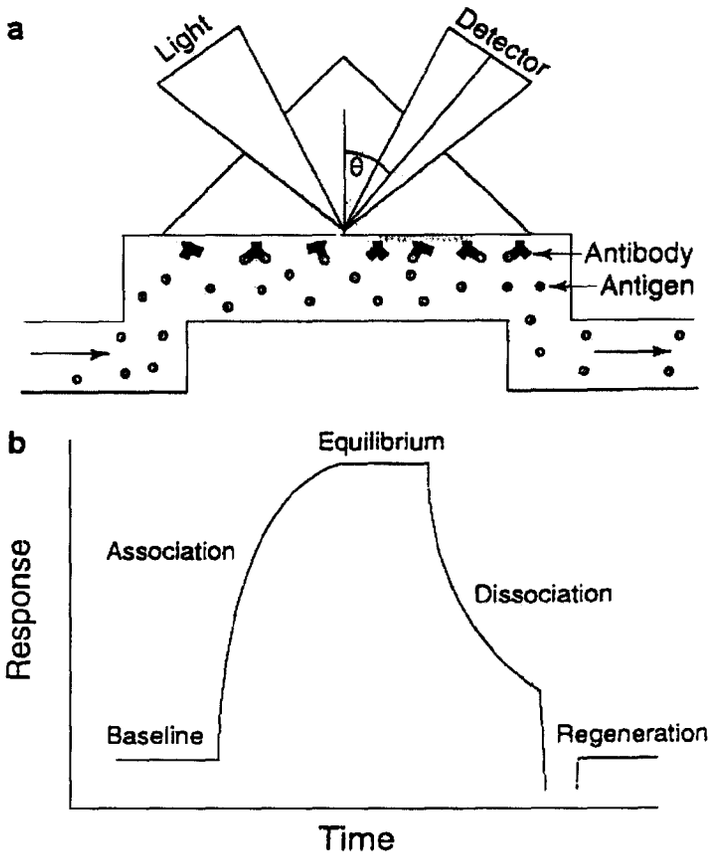


Figure 5.2: Flow system biosensor with the direction of the flow relative to the angle of the incident light depicted (a) and the resulting sensorgram (b) (Myszka and Rich, 2000).

Unlike the SPR flow system, the cuvette system employs a resonant mirror wave guiding technique. In this system, the resonant mirror internally reflects light beamed from one side of the cuvette through a prism to be measured the other side by a detector (Fig. 5.3). In this system, the changes in the evanescent field determines the angle that effects total reflection in the high refractive index layer that makes up the wave guide.

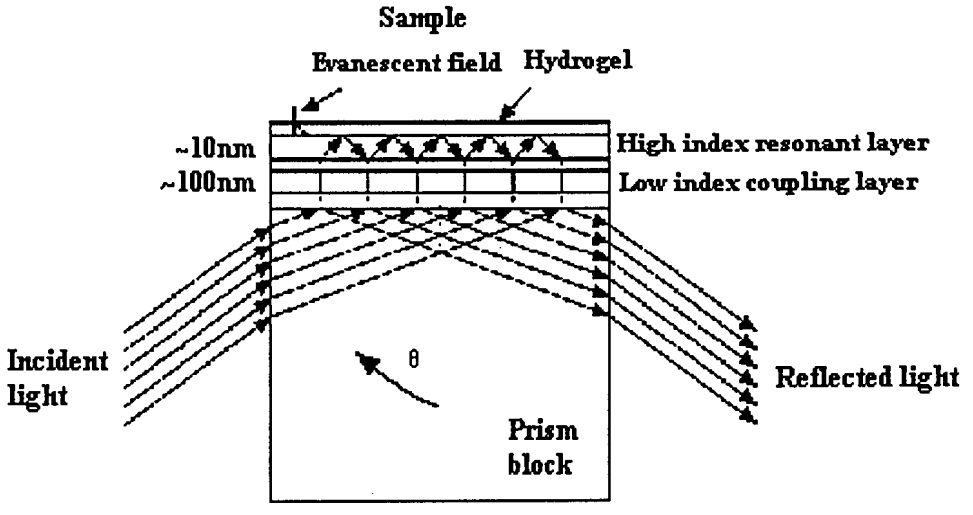


Figure 5.3: Representation of the IAsys resonant mirror-sensing device built in a cuvette (Pathak, 1995).

The region in the resonant layer where the path of the light is interrupted by the events that take place at the surface inside the cuvette is the evanescent field. The intensity decreases exponentially away from the resonant layer, which means that only reactions that occur close to this field can be monitored. When a ligand (such as an antibody or antigen) associates with the immobilised substance (antigen or antibody), the resonance angle changes and is registered with the instrument software in real-time. The change is measured in arc seconds units. An arc second is defined as 1/3600 of a degree with 3600 arc seconds making up a degree. Ligands immobilized onto the sensor surface by chemical means generate a sensorgram that can be characterised with available software (Fig. 5.2b).

In the equillibrium analysis configuration it is essential that the cuvette cell contents are stirred to ensure that mass-transfer is limited.

The IAsys affinity biosensor system uses a removable stirred cuvette system that is believed to have an advantage over the flow system in that the sample consumption is minimal, with no time constraints in terms of how long the sample is left in contact with its binding partner (Fig 5.4). This system is also available with two compartments (cells) suitable for measurements against a standard or direct comparison of sample binding properties.

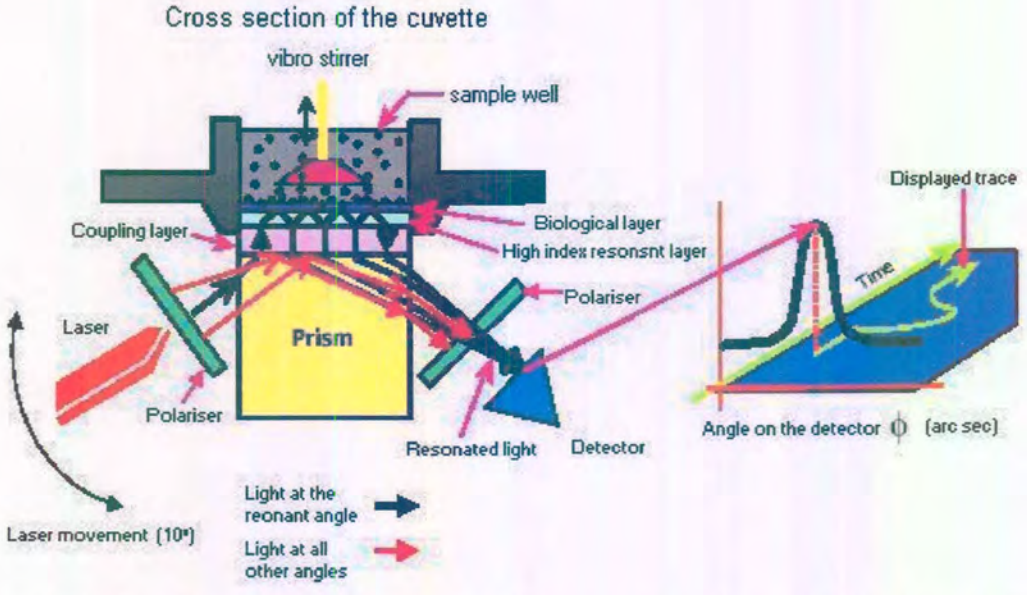


Figure 5.4: Schematic representation of the IAsys cuvette system with the vibro stirrers and oscillating laser light. (IAsys technical manual)

The sensor contains internal aspirators to remove solutions from the cells without removing the cuvette, making addition of other solutions fast and easy. The cuvettes are available with a choice of different derivatised sensor surfaces for different chemical immobilization reactions: carboxymethyl dextran for general protein immobilization, hydrophobic surfaces for creating lipid bilayers, carboxyl, and aminosilane surfaces for

specific immobilization of molecules, biotinylated surfaces for interacting with streptavidin labelled molecules and a non-derivatised surface. In this study the resonant mirror biosensor is used to develop a system by which anti-mycolic acids antibodies can be assesed to determine whether they can be regarded as surrogate markers for *M. tuberculosis* infection in sero-diagnosis.

5.2 Aims

This study aimed at developing a biosensor surface suitable for characterisation of the antibody-mycolic acids interaction using a resonant mirror biosensor.

5.3 Materials

5.3.1 Mycolic acids

Mycobacterial mycolic acids were isolated from a culture of *Mycobacterium tuberculosis* H37Rv (American Type Culture Collection 27294) as described by Goodrum, Siko *et al.* (2001).

5.3.2 Reagents

PBS-Azide EDTA buffer (PBS/AE): 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.05 g Na_2HPO_4 per 1 *l* ultrapure, distilled water with 1 mM EDTA and 0.025% (m/v) sodium azide, adjusted to pH 7.4

Guanidinium thiocyanate (GSCN, for synthesis; Merck, Munich, BRD; Cat No 820613) 3.5 mole per *l*

Cetylpyridinium chloride (CPC; Sigma, St. Louis, MO; Cat No C-9002) 0.02 mg per ml

Cholesterol (5-cholesten-3β-ol) (Sigma; Cat No C-8667): stock solution 100 mg per ml in chloroform (Merck, Darmstadt, BRD)

Phosphatidyl choline (pure) (PC-99; Sigma; Cat No P-3556): stock solution 100 mg per ml in chloroform

Phosphatidyl choline (from dried egg yolk) (PC-60; Sigma; Cat No P-5394): stock solution 100 mg per ml in chloroform

HCl 0.1 M

NaOH 50 mM

Ethanol 96% (v/v) in demineralized water

Saponin (Sigma; Cat No S-1252), 1 mg/ml in PBS-EDTA buffer

5.3.3 Resonant Mirror Biosensor apparatus

IAsys *plus* Resonant Mirror Biosensor (IAsys Affinity Sensors, Saxon Way, Bar Hill, Cambridge, UK)

5.3.4 Human sera

Human sera from three different sources were tested:

- Negative control sera from people who had never suffered from tuberculosis were obtained from healthy students and staff of the Department of Biochemistry, University of Pretoria, Pretoria, RSA and from healthy students from the Department of Medicine, University of Witwatersrand, Johannesburg, RSA.
- Tuberculosis patient sera obtained in the year 1994 at the King George V Hospital, Domerton, RSA, were a gift from the Medical Research Council National Tuberculosis Institute in Pretoria, RSA.
- Patient sera obtained in the year 2000 were kindly provided by Dr. A.C. Stoltz, Pretoria Academic Hospital, Pretoria, RSA, and Dr. G. Schleicher, Helen Joseph Hospital, Auckland Park, RSA.

5.3.5 Liposomes

- Phosphatidyl choline (PC) – 100mg/ml in chloroform
- Mycolic acids (MA) – 1mg
- Cholesterol (Chol) – 100mg/ml in chloroform
- PBS Azide EDTA (PBS/AE)
- Saline

5.3.6 ELISA

Goat anti-human IgG (H + L chains) antibody conjugated to peroxidase was obtained from Sigma (St Louis, MO, USA)

o-Phenylenediamine (Sigma, St Louis, MO, USA)

Hydrogen peroxide (Merck, Darmstadt, BRD).

Sterile, disposable 50 ml centrifuge tubes (Bibby Sterilin Ltd, Stone, UK)

Disposable pipettes (Bibby Sterilin Ltd, Stone, UK)

Disposable pipette tips (Bibby Sterilin, Serowell; Bibby Sterilin Ltd, Stone, UK)

Serowell ELISA plates: flat-bottom 96-well plates (Bibby Sterilin Ltd, Stone, UK)

Buffers

- **Acidification buffer:** Glycine HCL (0,2 M, pH 2.8)
- **Neutralisation buffer:** K_2HPO_4 (1 M in dddH_2O). The pH was adjusted to 9.0 with H_2KPO_4 (1 M) if necessary.
- **PBS buffer**: 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 (anhydrous) and 1.05 g Na_2HPO_4 (anhydrous) per 1 *l* distilled water, adjusted to pH 7.4.
- **Diluting buffer:** 0.5% (m/v) carbohydrate- and fatty acid free casein (Calbiochem, La Jolla, CA) in PBS buffer adjusted to pH 7.4 was used for diluting of the sera and the immunoreagents.

5.4 Methods

5.4.1 Mycobacterial culture

Mycobacterium tuberculosis H37Rv (ATCC 27294) was cultured on Löwenstein-Jensen (LJ)-slants at 37°C for 3 to 6 weeks. Harvested cells with a total count varying between 10^4 and 10^6 per ml were suspended in 0.89 % sterile saline, washed twice by centrifugation (2000 x g for 15 min) and re-suspended in 0.89 % saline. Bacterial titres (viable counts) of samples were determined by counting colony forming units (CFU) in 1:10 to 1:1 000 000 dilutions on Middlebrook 7H10 agar after incubation at 37 °C for 4 to 6 weeks.

Total count was determined by means of a direct count using a Neubauer counting chamber and various dilutions of bacterial suspensions. Staff from the National Tuberculosis Institute of the Medical Research Council of South Africa, Pretoria, prepared the media. The sterility of all the media was confirmed before use in the experiments by incubating them at 37°C for 24 h to detect any turbidity.

The harvested bacteria were washed with sterile 0,9% m/v NaCl (Saarchem, Chemically Pure, RSA). Medium used for the preparation of serial dilutions, preceding the determination of viable counts of *M. tuberculosis* was prepared by dissolving Tween 80 (Merck, Chemically Pure) in 0,9% m/v NaCl (Saarchem, Chemically Pure) to a concentration of 0,01% v/v and distributing it in 9,0 ml aliquots into test-tubes. The autoclaved media were stored at 4°C.

5.4.2 Mycolic acids preparation

Mycolic acids were prepared from *M. tuberculosis* following a method described in Chapter 2.

5.4.3 Liposomes

For the preparation of mycolic acids only-containing liposomes, 90 µl of the phosphatidyl choline (PC-99) stock solution was added to an amber glass vial containing 1 mg of mycolic acids, mixed well to dissolve the mycolic acids, dried at 80°C under a stream of N_2, and then sonified in 2 ml saline for 5 min at room temperature. The "empty" phosphatidyl choline liposomes were made in the same manner with omission of the mycolic acids. For the cholesterol-containing liposomes, 60 µl of the phospatidyl choline (PC-60 or PC-99 as applicable) stock and 30 µl of the cholesterol stock were added to an amber glass vial with or without mycolic acids, mixed well, and then dried, suspended in saline and sonified as above.

Liposomes were divided into 200 µl aliquots, freeze-dried and stored at -20°C until use. Before use the liposomes were reconstituted with 2 ml PBS/AE buffer (PBS-EDTA buffer: 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.05 g Na_2HPO_4 per 1*l* ultrapure, distilled water with 1 mM EDTA and 0.025% (m/v) sodium azide, adjusted to pH 7.4), heated at 80°C for 15 min and then sonified as above. The final liposome concentration therefore came to 50 µg/ml.

5.4.4 ELISA

Mycolic acids originating from *Mycobacterium tuberculosis*, isolated as described above, and cholesterol (Sigma, St. Louis, MO; Cat No C-8667) were used at final concentrations of 60 µg/ml and 75 µg/ml, respectively. To prepare the coating solutions, the antigens were heated in PBS buffer for 20 min at 85°C. The hot solutions were sonicated at 20% duty cycles and optimal output level for 1 min. The solutions were kept at 85°C and loaded into the ELISA plates. The respective antigens were dissolved in hot PBS and then sonicated, as described above. The wells were coated overnight at 4°C with 50 µl/well of antigen solution. The final antigen load was approximately 3 µg/well for mycolic acids or approximately 3.75 µg/well for the cholesterol.

The coating solution was flicked out of the plates and replaced with 400 µl blocking buffer per well. Blocking was carried out for 2 hours at room temperature.

To undiluted serum (100 µl) in an Eppendorf tube, an equal volume of PEG 8000 in 0.01M PBS (pH = 7.4) was added, mixed and left overnight at 4°C. The precipitate was collected by centrifugation at 4°C for 30 min. The supernatant was discarded and the pellet washed two times with 4% PEG. After the pellet was washed, it was dissolved in 100 µl 0.154M PBS. Acidification buffer (50 µl) was added to the sample on ice to release the antibodies from the immune complexes. After standing for 15 minutes, neutralisation buffer (25 µl) was added. Double distilled water (25 µl) and ELISA diluting buffer (1800 µl) was added to the samples to obtain a final volume of 2 ml, representing a 1:20 dilution of serum.

The blocking solution was aspirated from the wells before loading of the serum or serum precipitate samples. Sera were diluted 20 times in diluting buffer. Aliquots of 50 µl were introduced into wells in quadruplicate. The plates were incubated at room temperature for 1 hour. The serum samples were removed from the wells, the wells washed three times with washing buffer using an Anthos Autowash automatic ELISA plate washer (Labsystems, Finland)and then emptied by aspiration.

Peroxidase-conjugated anti-human IgG diluted 1:1000 in diluting buffer was introduced in aliquots of 50 µl per well and the plates were incubated for 30 min at room temperature. After removal of the conjugate, the wells were washed three times with the washing buffer and then emptied by aspiration.

The substrate solution comprising 10.0 mg *o*-phenylenediamine and 8.0 mg hydrogen peroxide in 10 ml of 0.1 M citrate buffer pH 4.5, was prepared immediately before use and introduced in 50 µl aliquots per well. The plates were incubated at room temperature

and the colour development was monitored at 5, 30 and 60 min after addition of the substrate using an SLT 340 ATC photometer (Thermo-Labsystems, Finland) at a wavelength of 450 nm.

5.4.5 Coating of biosensor cuvettes

Each cuvette was first allowed to equilibrate to room temperature before insertion in the IAsys apparatus. Binding of each component was monitored in real-time.

5.4.5.1 Coating of the hydrophobic cuvette

The following steps were followed for coating a hydrophobic cuvette:

- The inserted cuvette was washed 5 times with 50 µl 2-propanol followed by data collection for 1 minute and then washing 7 times with 60 µl PBS/AE with 10 minutes data collection.
- This was then followed by washing 7 times with 50 µl 2-propanol and 1 minute data collection after that. With 50µl remaining in the cuvette, 22µl of MA-cholesterol mixture dissolved in chloroform was added in one cell and in the reference cell, 20 µl of the control solution was added and coating data was collected for 1 minute followed by washing 7 times with 60µl PBS/AE and 5 minutes data collection.
- The cuvette cells were then washed 5 times with 50µl HCl and data collected for 1 minute, and then washed 7 times with 60µl PBS/AE with 5 minutes data collection. This was followed by 5 washes with 50µl NaOH for 1 minute and the cells were washed 7 times with 60µl PBS/AE followed by data collection for 5 minutes.
- Blocking was performed by adding negative sera to a final concentration of 10%, washed 5 times with 60µl PBS/AE and followed by 5 minutes data collection.

5.4.5.2 Coating of underivatised cuvettes with liposomes

The following steps were followed for liposome coating of the underivatised cuvette:

- The cuvette was washed 10 times with 60 µl PBS/AE. The buffer was replaced with 50 µl cetylpyridinium chloride solution. After 10 min the cuvette was washed 5 times with 60 µl PBS/AE.
- After the final wash, 25 µl of PBS/AE was added to the cuvette, allowing 5 min for the baseline to be established. Then 25 µl of liposomes (containing either phosphatidyl choline plus mycolic acids, phosphatidyl choline plus cholesterol, or phosphatidyl choline only) were added and data collected for 20 min.
- The cuvette was again washed 5 times with 60 µl PBS/AE and then aspirated. In order to stabilize the lipid complex on the cuvette surface and to reduce non-specific binding, 50 µl of saponin solution was added to the cuvette. After 20 min of data collection the cuvette was washed 5 times with 60 µl PBS/AE.
- After 10 min of data collection base line was established by aspirating and replacing with 25 µl of PBS/AE. At least 5 min was allowed for a stable baseline to be established. In the first experiment, saponin (1 mg/ml) was included in the final washing and base-line solutions.

5.4.6 Binding of human antibodies in the biosensor

Serum dilutions were prepared in PBS/AE, except for the first experiment where saponin (1 mg/ml in PBS/AE) was used. An appropriate amount of serum dilution was introduced into the cuvette to give a final dilution as indicated in the result graphs. Data for the antibody binding were accumulated for 10 min after each serum addition. Washing the cuvette 3 times with 60 µl PBS/AE, after the final serum addition, effected the dissociation. Dissociation data were accumulated for 5 min.

5.4.7 Inhibition of antibody binding by pre-incubation with liposomes

Serum was diluted in the appropriate liposome suspension (made with PC-99) and incubated for 20 min at room temperature. A 10 µl aliquot of the mixture was then added to the cuvette and binding data were accumulated for 10 min. Dissociation was effected by washing the cuvette 5 times with 60 µl PBS/AE. Data on the dissociation were accumulated for 5 min.

5.4.8 Washing and regeneration of biosensor cuvettes

In order to regenerate the cuvette surface, the cuvette was washed five times with 50 µl 0.1 M HCl, seven times with 60 µl PBS/AE, five times with 50 µl 100 mM NaOH, seven times with 60 µl PBS/AE, three times with 50 µl 96% ethanol and finally 7 times with 60 µl PBS/AE. In the first experiment, the cuvette was regenerated after the dissociation event by washing 5 times with guanidinium thiocyanate (3.5 M), followed by extensive washing with saponin (1 mg/ml) in PBS/AE, before the second incubation with antibody at a higher concentration was introduced.

5.5 Results

IAsys biosensor cuvettes are supplied in either underivatised, or in various pre-derivatised forms. Of these, only the hydrophobic and underivatised cuvettes appeared to be suitable for coating with mycolic acids.

5.5.1 Anti-mycolic acids antibodies on a hydrophobic cuvette

The hydrophobic cuvette was considered to be suitable over the other types, based on the applicable IAsys protocol provided. The protocol supplied by the instrument manufacturer was first adapted for the purpose of this study. In order to determine the feasibility of testing anti-mycolic acids antibodies on the biosensor, the ability to differentiate between anti-mycolic acids antibodies in a tuberculosis patient serum and a negative control serum was tested.

The hydrophobic cuvette was coated as described in 5.4.5.1. The binding of antibodies to a mycolic acids-cholesterol phosphatidyl choline-60 (MA-Chol-PC60) coated surface differed between tuberculosis positive patient and tuberculosis negative control sera in accordance with their respective ELISA signal (Figure 5.5). ELISA signal to background values of patient 1799 was 6 times (with an absorbance reading of 1577) compared to 1.5 times (with an absorbance reading of 679) for the negative control serum GS8. The ELISA signal was obtained from mycolic acids coated plate and the background signal from an un coated plate.

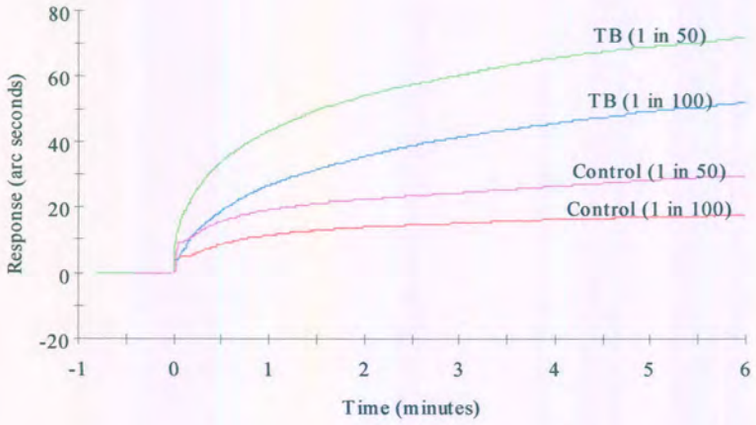


Figure 5.5: Binding profile of tuberculosis positive patient serum (TB) and negative control serum (Control) on a hydrophobic cuvette coated with mycolic acids-cholesterol phosphatidyl choline. Sera analysed were diluted 1in 50 and 1 in 100 in PBS/AE.

In spite of the difference between tuberculosis patient and negative control serum binding, it remained important to indicate the specificity of the interaction with the mycolic acids surface. This was attained through comparing the anti-mycolic acids antibody binding profiles of patient serum on a MA-Chol-PC60 and Chol-PC60 coated surfaces.

The results are indicated in Fig. 5.6. There were no obvious differences in binding of antibodies from the tuberculosis positive patient (diluted 1 in 800 PBS/AE) on either mycolic acids-cholesterol (MA-Chol-PC60), or cholesterol phosphatidyl choline 60 (Chol-PC60). There are two possible explanations for this observation: The patient serum binds non-specifically to the coated biosensor surface, or there is a cross-reactivity of binding of antibodies or serum components to cholesterol and mycolic acids.

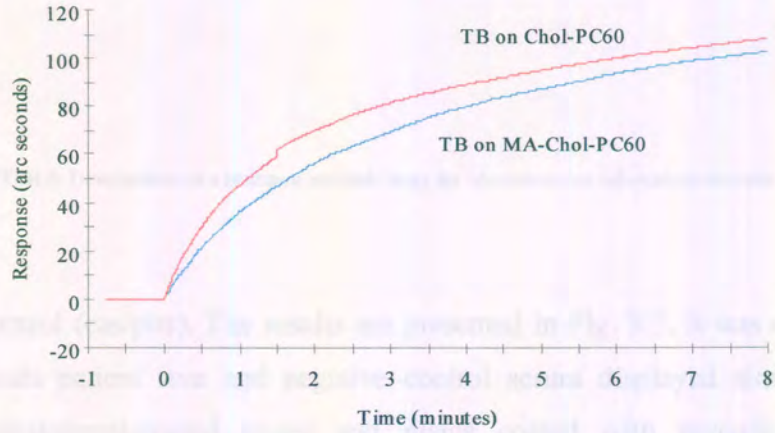


Figure 5.6: Binding profile of tuberculosis patient 1799 serum (TB) on a hydrophobic cuvette coated with mycolic acids-cholesterol phosphatidyl choline 60 (MA-Chol-PC60) or cholesterol phosphatidyl choline 60 (Chol-PC60). Sera analysed were diluted 1 in 800 PBS/AE.

5.5.2 ELISA confirmation of the cholesterol and mycolic acids cross-reactivity

Enzyme-linked immunosorbent assay (ELISA) was used to investigate a possible cross-reactivity of antibody binding between cholesterol and mycolic acids. Whereas the biosensor cannot distinguish between binding of antibodies or of other serum components, ELISA specifically reports the binding of antibodies. As patients who suffer from tuberculosis produce antibodies directed against the mycolic acids in the cell wall of *M. tuberculosis*, ELISA can detect these antibodies, as well as antibodies directed against cholesterol. The response in antigen-coated wells was compared to the response signal in control wells that had not been coated with antigen.

In this experiment, the antibody recognition of cholesterol was compared with that of mycobacterial mycolic acids within the same ELISA plate, using tuberculosis patient serum (PC se), negative control (NC se), high cholesterol patient serum (+CP se) and

casein-PBS control (cas/pbs). The results are presented in Fig. 5.7. It was observed that both tuberculosis patient sera and negative control serum displayed similar antibody binding on cholesterol-coated plates and plates coated with mycolic acids. The tuberculosis patient serum (patient 1799) was the strongest antibody signal generating serum from a group of 214 tuberculosis patients tested on ELISA plate coated with mycolic acids. This strong signal was again observed on the cholesterol-coated plates but was not observed with serum from a patient suffering from high blood cholesterol levels. This confirmed the observations made on the biosensor that there could be cross-reactivity of binding of tuberculosis patient antibodies to cholesterol and mycolic acids.

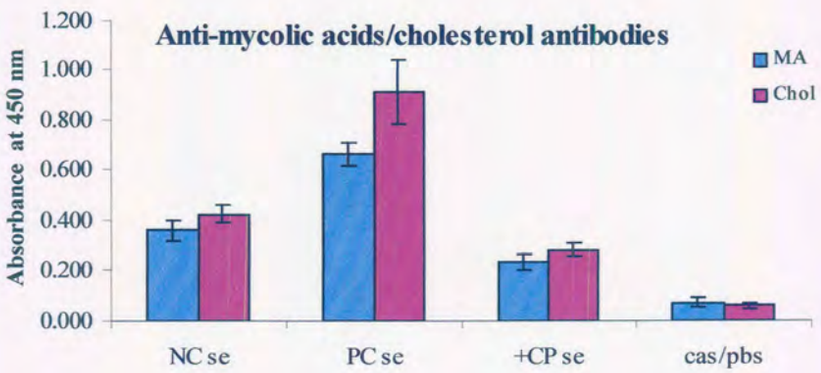


Figure 5.7: Antibodies binding profile on ELISA plates coated with either cholesterol (Chol) or mycolic acids (MA). Sera from different samples were analysed. NC se = healthy control serum, PC se = tuberculosis patient 1799 serum, CP se = cholesterol patient serum, cas/pbs = casein-PBS.

In this study, where mycolic acids were used together with cholesterol as antigens in the ELISA using tuberculosis patient as test serum, almost equal signals were produced in wells coated with cholesterol or mycolic acids alone. It was thus appropriate to look into possible ways in which mycolic acids could theoretically mimic the chemical structure of cholesterol.

5.5.3 Modeling of the possible molecular structural mimicry between cholesterol and mycobacterial mycolic acids

Based on the observations made on the biosensor and ELISA on the possible existence of molecular mimicry between mycolic acids and cholesterol, different mycolic acids folding structures were investigated. This generated a possible folded structure of the two oxygenated mycolic acids species (i.e. keto mycolic acids and methoxy mycolic acids, Figure 5.8). Only oxygenated methoxy-mycolic acids could assume a folded structure in which mimicry of cholesterol could be envisaged, with the methoxy-group of mycolic acids corresponding to the hydroxyl position of cholesterol. In its folded structure with all the oxygenated groups clustered on one side of the molecule and a hairpin bend induced by the cyclo-propane moiety of the long hydrocarbon chain of mycolic acids, a mimicry to the structure of cholesterol certainly appeared feasible. Keto mycolic acids do not mimic cholesterol as the double bonds between oxygen and carbon (red circle on Fig 5.8) prevent formation of such a structure.

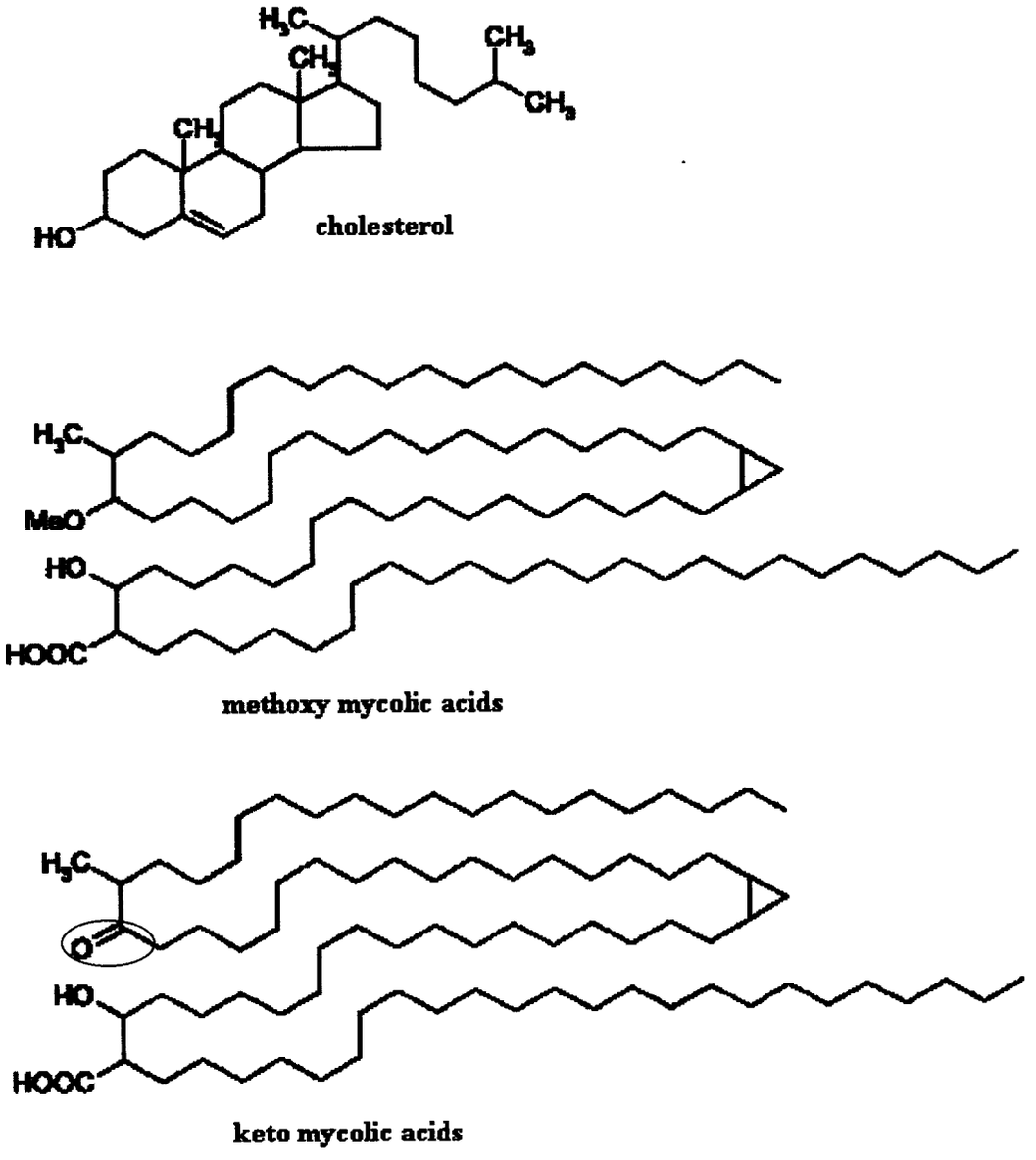


Figure 5.8: The structures representing the proposed possible molecular mimicry between the methoxy- mycolic acids and keto-mycolic acids and cholesterol

With evidence gathered so far, it became important that this cross-reactivity between mycolic acids and cholesterol be further characterised on the biosensor. The limiting factor in this regard was the instability of the available hydrophobic cuvette. The hydrophobic cuvette proved to be difficult to work with, as experiments could not be reproduced on the same cuvette after regeneration steps. Experiments could only be reproduced on new cuvettes, which are very expensive.

5.5.4 Coating the underivatised cuvettes with mycolic acids

A new approach was needed to be implemented, that would allow the repeated use of non-derivatised cuvettes coated with mycolic acids or cholesterol. The problem was solved by using a novel approach that involved the activation of the surface with a cationic detergent. The hydrophilic surface of the non-derivatised cuvette was made hydrophobic by activation with cetylpyridinium chloride (CPC), and could subsequently be coated stably with mycolic acids or cholesterol containing liposomes.

The optimum concentration of CPC was found to be around 0.02 mg/ml. The results indicated that addition of MA-liposomes before CPC addition produced inadequate binding, while addition of mycolic acids-liposomes after CPC activation showed an increase in binding of about 500% (Fig 5.9).

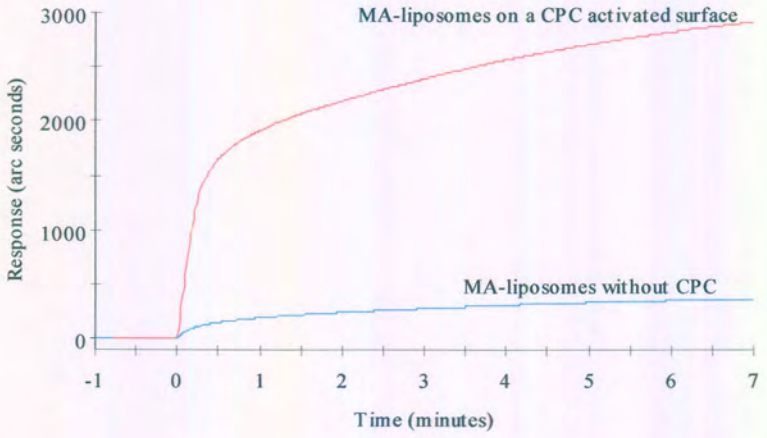


Figure 5.9: Mycolic acids-cholesterol liposomes (PC 60) coated on a non-derivatised cuvette activated with CPC (red line) or left in PBS/AE (blue line).

With the liposomes immobilised on the surface, the next step was to find the optimum concentration of a neutral surfactant (saponin) to be used to stabilise the surface and also to block. Saponin was used as it also binds cholesterol. The results indicate that titration with aliquots of a 1 mg/ml saponin solution produced saturation at around 2000 arc seconds. Titration with a higher (10 mg/ml) concentration of saponin did not have any further beneficial effects (Fig. 5.10). From this data it was concluded that an effective amount of saponin was 25 µl of a 1mg/ml saponin solution to effect optimal blocking of cholesterol containing liposome coats in underivatised cuvettes. It is expected that this optimisation needs to be done for every batch of saponin that is acquired for biosensor applications.

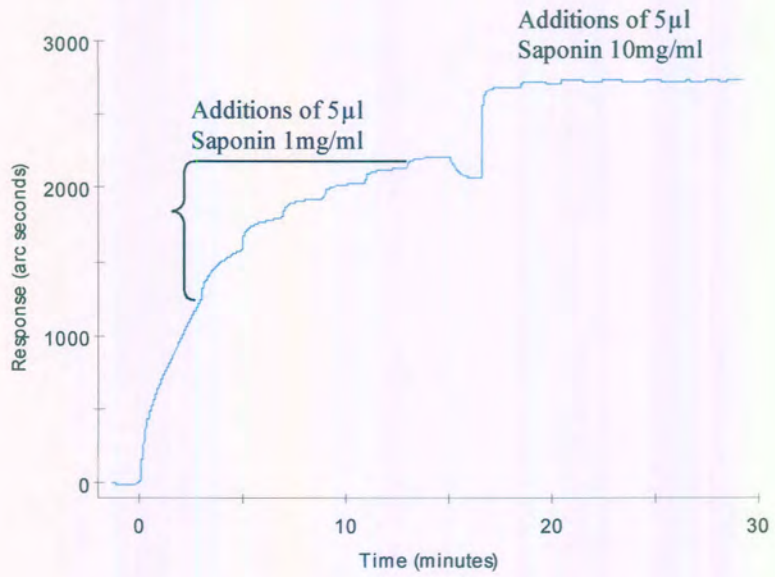


Figure 5.10: Titration of saponin on a non-equilibrated mycolic acids-cholesterol PC60 liposome surface.

With the non-derivatised cuvette coated with MA-chol liposomes, it was necessary to determine if this surface will be stable towards repeated binding cycles. The results indicated that the baseline remained stable and unchanged after repeated cycles, but repeated loading of tuberculosis patient serum at 1:200 dilution brought about decreased binding with every cycle without inflicting a drop in the baseline. Fig. 5.11 shows the decline in signal of the same serum concentrations after each regeneration step that removed the bound antibodies. It was as if repeated binding of tuberculosis patient serum of the same concentration caused the gradual depletion of mycolic acids from the surface.

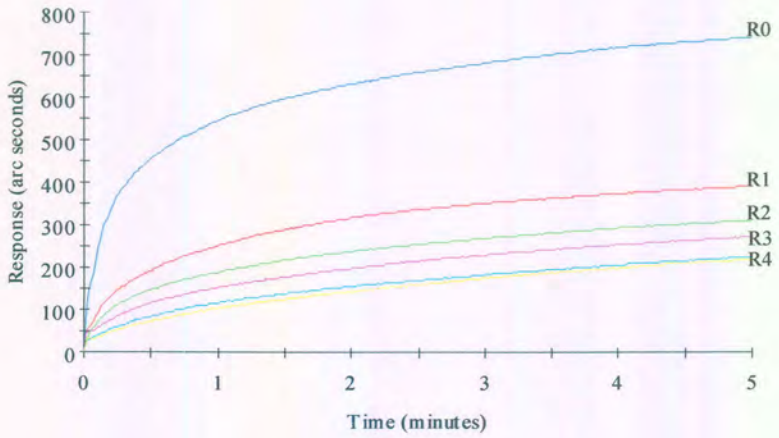


Figure 5.11: Apparent depletion of mycolic acids on the liposome-coated surface due to continued regeneration. After the first binding event (R0) depletion was measured by the declining binding signal after each acid-base regeneration cycle (R0 to R4).

The decrease in signal suggested that the affinity of the antibodies towards mycolic acids may be strong enough to effect removal of mycolic acids from the liposome coat during antibody removal at each regeneration. From these results it appeared to be essential that the surface be totally stripped after each binding and dissociation cycle and then recoated to be able to measure different samples.

Stripping of a cuvette surfaces was achieved by using washing steps that contained higher concentrations of acid (HCL 0.1M), base (NaOH 50mM), ethanol (96%, capable of removing surfactants from glass surfaces) and guanidinium thiocynate (GSCN, 3.5M) to restore the evanescent layer. It was then imperative to once again compare tuberculosis patient serum with negative control serum in this new approach towards coating the biosensor with mycolic acids containing liposomes. In these experiments the cuvette was coated with liposomes comprising phosphatidyl choline (PC-60), cholesterol and mycobacterial mycolic acids. Dilutions of tuberculosis patient 1799 (TB 1:200 and

1:800) serum displayed a stronger binding curve on this surface than similar dilutions of healthy control serum (Control 1:200 and 1:800) as shown in Fig. 5.12. This result was comparable to what was found with the hydrophobic cuvette coated with phosphatidyl choline (PC-60), cholesterol and mycobacterial mycolic acids, although the signal was here enhanced. These results were also comparable to those obtained with ELISA previously, implying that the binding to the coated biosensor surface was probably due to the binding of antibodies from the serum samples.

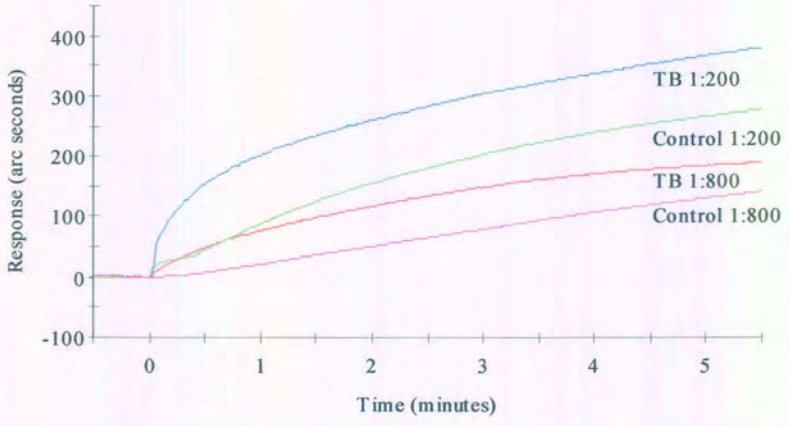


Figure 5.12: Resonant mirror biosensor binding profiles of tuberculosis patient (TB) and healthy control (Control) sera on underivatised CPC activated cuvettes coated with liposomes consisting of PC-60, cholesterol and mycolic acids. Serum dilutions are indicated on each curve.

5.5.5 Inhibition tests

Up to this stage the liposomes that were used contained cholesterol for stability. With the observations made on the apparent cross-reactivity between cholesterol and mycolic acids, the question remained as to whether anti-cholesterol antibodies can also bind to mycolic acids, i.e. whether the anti-cholesterol antibodies could be the same as those directed against mycolic acids. Inhibition tests were employed to assess the degree of cross-reactivity between cholesterol and mycolic acids. This required the use of lipomes that were free of cholesterol and that comprised of high purity of phosphatidyl choline (PC99). In order to determine the specificity of binding of antibodies to the mycolic acids coated biosensor surface, cholesterol-free liposomes were constructed for coating. These contained phosphatidyl choline (PC-99) and mycolic acids only. Tuberculosis patient 1799 serum was added in both cells of the mycolic acids coated cuvette in 1:1000 dilution to ensure that the response of the two cells were identical. Subsequently 1:100 dilution of the tuberculosis patient serum was added in both cells, the first pre-incubated with PC-99 liposomes and the second with PC-99/mycolic acids liposomes.

Pre-incubation of the patient serum with PC-99/mycolic acids liposomes resulted in an inhibition of binding to the surface, as shown in Fig. 5.13. No inhibition was seen when pre-incubation was done with PC-99 liposomes. These results provide evidence that the sera of tuberculosis patients contain antibodies that specifically recognize mycolic acids. The results also show that pre-incubation of tuberculosis patient serum with PC-99/cholesterol liposomes could not inhibit the antibody binding response on a mycolic acids-containing biosensor surface. This suggested that even though the tuberculosis patient may have produced antibodies that recognised cholesterol they have an even higher affinity for mycolic acids. Alternatively, these results may imply that the antibodies to mycolic acids are not identical to those against cholesterol.

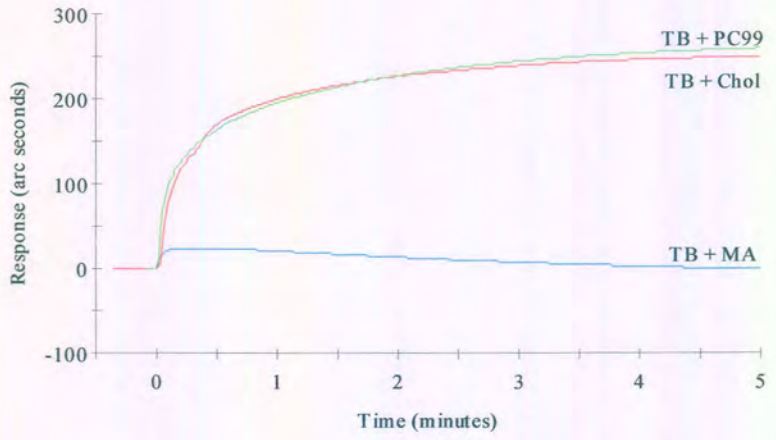


Figure 5.13: Inhibition assay of TB patient sera with mycolic acids on a mycolic acids coated surface. TB patient serum of the highest titre on ELISA was compared to the inhibitory effects of cholesterol and empty liposomes (PC99). TB + MA = TB patient serum pre-incubated with mycolic acids liposomes/PC99, TB + Chol = TB patient serum pre-incubated with cholesterol liposomes/PC99, and TB + PC99 = TB patient serum pre-incubated with empty liposomes/PC99

The specificity of the antibody-antigen interaction was tested with tuberculosis negative sera as negative control, to establish that the mycolic acids inhibition that was observed was only due to the presence of antibodies to mycolic acids in tuberculosis patients. The results indicated that when the cuvette cell was coated with mycolic acids and the negative serum was also incubated with mycolic acids, there appeared to be no inhibition (Fig.5.14). The strong binding of negative control serum to the mycolic acids coated surface can possibly be due to antibodies against cholesterol that also bind mycolic acids. The interaction between these antibodies and mycolic acids could not be inhibited by

cholesterol, arguing against such reasoning. The binding of negative control serum to mycolic acids therefore could simply be due to non-specific binding

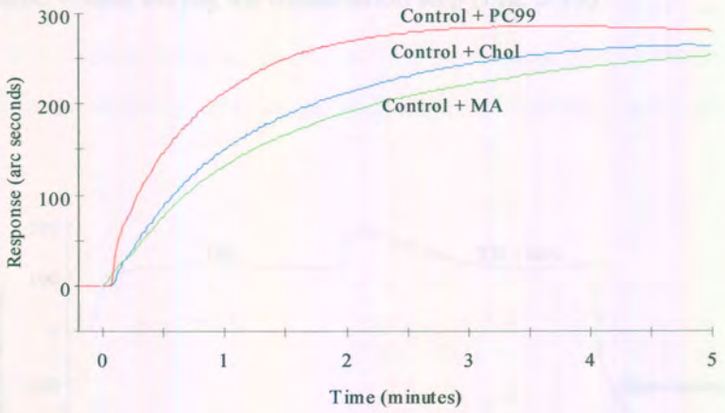


Figure 5.14: Inhibition assay of negative control sera with mycolic acids on a mycolic acids coated non-derivatised cuvette surface. The negative control serum was also pre-incubated with cholesterol and empty liposomes (PC99). Control + MA = control serum pre-incubated with mycolic acids PC99 liposomes, control + Chol = control serum pre-incubated with cholesterol PC99 liposomes, and control + PC99 = control serum pre-incubated with empty PC99 liposomes.

As the results from ELISA suggested that antibodies that recognise mycolic acids may also recognise cholesterol, the cuvette cells were also coated with cholesterol and the inhibition experiments repeated as it was done with mycolic acids coated cells. Immobilisation of cholesterol/PC99 liposomes followed by binding of tuberculosis patient sera that had been pre-incubated with mycolic acids, resulted in the inhibition of the binding. This however, did not only result in the inhibition of the binding, but also

resulted in the mycolic acids damaging the coat surface. This made it especially difficult to establish if the inhibition was specifically due to the interaction of the mycolic acids with the antibody or whether it also involved the destabilisation of the cholesterol coated surface that became visible during the dissociation step (Fig. 5.15).

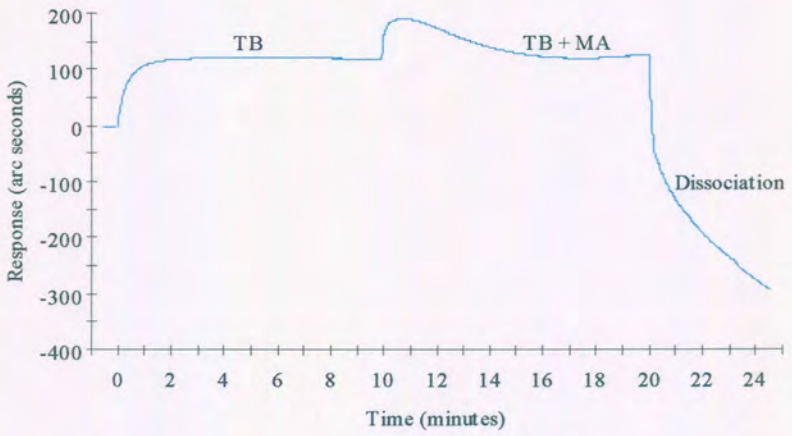


Figure 5.15: Inhibition of binding assay of tuberculosis patient 1799 serum with mycolic acids/PC99 liposomes on a cholesterol coated cuvette surface. TB + MA = TB patient serum pre-incubated with mycolic acids liposomes/PC99 and Dissociation = three times PBS/AE wash. Note the decay of the cholesterol coat by the response falling below the base-line during dissociation.

The effect that mycolic acids had on the cholesterol surface suggested that even though there was inhibition of binding, it would be difficult to interpret the result. It thus became important to investigate the possibilities of mycolic acids interacting with the liposomes bound on the biosensor surface.

5.5.6 Cholesterol-mycolic acids interactions

Addition of soluble mycolic acids/PC99 to a cholesterol/PC99 coated surface in the biosensor, revealed that there might be strong interactions between mycolic acids and cholesterol on the surface (Fig. 5.16). Addition of soluble PC99 liposomes had no such effect. The mycolic acids may have folded into structures that resemble the stacked cholesterol molecules, thereby interacting with coated cholesterol and removing it from the surface.

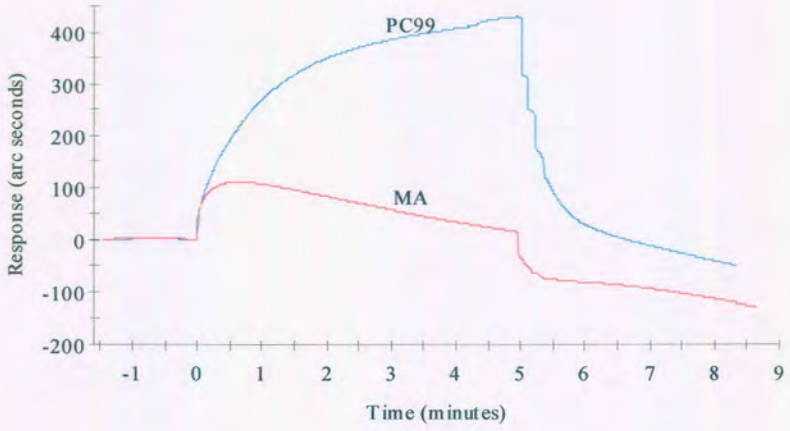


Figure 5.16: Binding assay of mycolic acids on a cholesterol-coated surface. MA= mycolic acids / phosaphatidyl choline (99 % pure) liposomes and PC99 = phosaphatidyl choline (99 % pure) liposomes.

An experiment was done to investigate if cholesterol will be able to bind to a mycolic acids surface. The results in Fig. 5.16 indicated that cholesterol goes after the mycolic acids, thereby escaping from the cholesterol coat when incubated with soluble mycolic acids, but accumulating into the mycolic acids coated surface when incubated with soluble cholesterol (Fig.5.17). This implies that pre-incubation of patient serum with

cholesterol/PC99 liposomes that may be expected to show inhibition of binding to a mycolic acids surface, if cross-reactivity between cholesterol and mycolic acids binding to the antibodies existed, may result in an artefactual positive binding curve due to cholesterol accumulation into the mycolic acids layer. The results taken together therefore argue in favour of a direct cross-reactivity of binding of antibodies to cholesterol and mycolic acids.

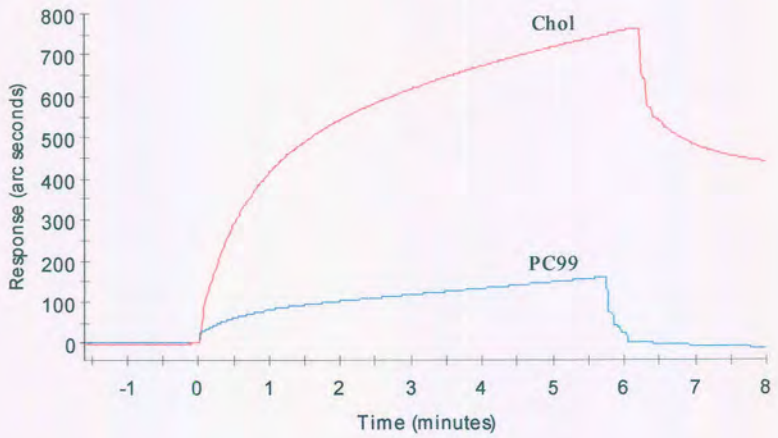


Figure 5.17: Biosensor binding profile of cholesterol on a mycolic acids -coated surface. Chol = binding profile of after addition of cholesterol/PC99 liposomes; PC99 = binding profile after adding PC99 liposomes

Data presented so far indicated that a tuberculosis patient serum that gave a high antibody binding signal to mycolic acids on ELISA could be inhibited by pre-incubation with mycolic acids. It was also indicated that there might be cross-reactivity between antibodies binding to cholesterol and mycolic acids. The question that arose from these observations was whether this could be extended to other tuberculosis patient sera that had low signal on ELISA. The results indicated that the binding of the antibodies could

still be inhibited with mycolic acids although it was not as pronounced as displayed by that of the high titre patient serum (Fig. 5.18).

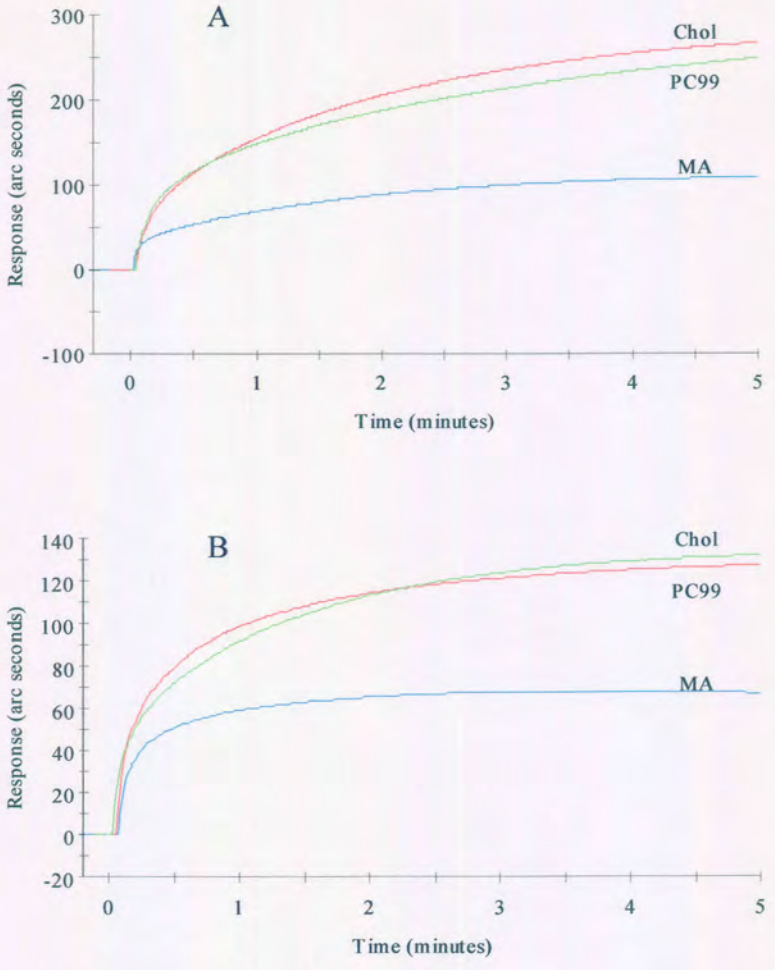


Figure 5.18: Biosensor binding profile of an inhibition assay of TB and HIV positive patient sera with mycolic acids on a mycolic acids coated surface. TB patient sera of intermediate titre on ELISA was compared with the inhibitory effects of cholesterol and empty liposomes (PC99). MA = TB patient serum pre-incubated with mycolic acids PC99 liposomes, Chol = TB patient serum pre-incubated with cholesterol PC99 liposomes, and PC99 = TB patient serum pre-incubated with empty PC99 liposomes. A= Medium titre tuberculosis patient P50 serum (relative ELISA signal = 1.23), B= lower titre tuberculosis patient P48 serum (relative ELISA signal = 0.56).

5.6 Discussion

Tuberculosis patients produce antibodies directed against *Mycobacterium tuberculosis* mycolic acids (Pretorius, 1999; Pan *et al.*, 1999). Mycolic acids constitute the most abundant lipids in the cell wall of *Mycobacterium tuberculosis*. These antibodies were detected by an enzyme-linked immunosorbent assay (ELISA). The response in antigen-coated wells was compared to the response signal in control wells that had not been coated with antigen. The ability to detect antibodies directed against mycolic acids using ELISA prompted further characterization of the binding of these antibodies. Because the biochemical reactions between antigen and antibody are normally reversible, the association and dissociation could then be determined in real-time using a resonant mirror biosensor. Because of the hydrophobic nature of the antigen, the first challenge was to find and develop a suitable surface on the biosensor cuvette. After several attempts on the hydrophobic biosensor surface using standard protocols, the underivatised surface was found to be suitable for innovating a homogenous mycolic acids coat after activating the surface with a cationic detergent and coating with mycolic acids containing liposomes. Both cholesterol/PC60 and mycolic acids/cholesterol/PC60 containing liposomes coated cuvette surfaces produced similar binding profiles with tuberculosis patient serum. This corroborated the results found with ELISA that mycolic acids specific antibodies were present in tuberculosis patients.

In this study, it was discovered by serendipity that there could be a relationship between mycolic acid and cholesterol structure, as antibodies binding to mycolic acids were found to also bind cholesterol. ELISA determination confirmed that these binding results wer due to antibodies specifically binding to mycolic acids and cholesterol thereby pointing to a possible molecular mimicry between mycolic acids and cholesterol. Theoretical folding of mycolic acid structure indicated that methoxy mycolic acids could take on a form that resembled cholesterol. This agrees with the observations made by Pan *et al.* (1999) that tuberculosis patients have antibodies that specifically have affinity for methoxy-, but not for keto- oxygenated mycolic acids, nor non-oxygenated α-mycolic acids.

On the basis of these results it is proposed that mycobacterial mycolic acids may show a structural mimicry with cholesterol, such that antibodies directed against mycolic acids can also recognize cholesterol. In support of this, immobilized mycolic acids were found to capture cholesterol from dissolved cholesterol liposomes in the biosensor, while cholesterol was withdrawn from the cholesterol coated surface when exposed to dissolved mycolic acids liposomes thereby demonstrating the attraction of cholesterol to bind to mycolic acids. This opens the possibility that mycobacteria may accumulate cholesterol from the host body fluids and that the mycolic acids cholesterol sandwich may present itself for binding to cell-receptors and antibodies. Interestingly, although cholesterol could not inhibit the binding of tuberculosis patient antibodies to a mycolic acids coated biosensor surface, the reaction could actually have remained invisible due to the concomitant accumulation of cholesterol into the mycolic acids-liposome coated cuvette surface.

Altogether, the results obtained with the biosensor experiments come up in support of a structural mimicry between mycolic acids and cholesterol. This conclusion relies on the observation that cholesterol serves well as an immobilised binding ligand for anti-MA antibodies. The binding of antibodies to mycolic acids can be prevented by pre-incubation of the patient serum with mycolic acids-liposomes, confirming the specificity of the antibodies. Additional experiments may be required to provide absolute proof of specificity of binding of antibodies to mycolic acids. Lipoproteins complexed with antibodies may hydrophobically associate with mycolic acids or cholesterol coat, thereby giving only apparent specificity of binding. Although unlikely, this possibility cannot yet be totally excluded.

The results obtained in this study open up new insight into a possible way of infection by *M. tuberculosis*. A number of recent studies have indicated that cholesterol is important for mycobacterial entry into the macrophages (Av-Gay and Sobouti, 2000; Gatfield and Pieters, 2000; Pieters and Gatfield, 2002). Pathogenic *M. tuberculosis* was found to

require cholesterol not only as a carbon source but also for mycobacteria to be able to infect cells. In this study cholesterol has been shown to interact with mycolic acids, suggesting a possible mechanism involved in mycobactrial entry into macrophages.

Based on these data we hypothesise that mycobacteria accumulate cholesterol by a molecular attraction between mycobacterial mycolic acids and cholesterol, thereby mimicking the structure of oxidised LDL. In this way, *M. tuberculosis* may facilitate their uptake into the host macrophage via attachment to type A scavenger receptors. The proposed molecular attraction between the mycobacterial mycolic acids and the cholesterol in the host cell membrane may aid in the engulfment of the mycobacterium via a "lipid zipper"-like mode of action (Gatfield and Pieters, 2000). The molecular association between mycobacterial mycolic acids and membrane cholesterol may also explain why the membrane of the phagosome appears to be tightly associated with the engulfed mycobacterium (de Chastellier *et al.*, 1995; de Chastellier and Thilo, 1997). Because the mechanism of cholesterol involvement has not been fully elucidated, further investigations into the interactions between cholesterol and mycolic acids may shed new light in the development of new anti-tuberculosis drugs.

CHAPTER 6

General Discussion

Tuberculosis has been the scourge of mankind since the beginning of human history. This is mainly because *M. tuberculosis* can persist inside the human body without causing disease. The major reasons for the unabated spread of tuberculosis can be identified as insufficient protection offered by the world-wide vaccination programme based on the use of BCG, problems associated with the detection of tuberculosis, problems associated with treatment of tuberculosis and the occurrence of multi-drug resistant strains of *M. tuberculosis*, interaction with HIV infection, and socio-economic aspects. These problems call for a fresh approach to control the tuberculosis problem.

Raffel *et al.*, had in 1949 for the first time showed that the immunogenicity of mycobacterial cell wall can be extended to mycobacterial lipids. Most mycobacteria are endowed with specific, highly antigenic glycolipids that are immunogenic like the phenolic lipids of *M. leprae* and *M. bovis*, the peptidolipids of the *M. avium*, and the acylated trehalose-containing lipooligosaccharides of species such as *M. kansasii*, *M. szulgai*, and *M. malmoense*. A search for analogous structures in *M. tuberculosis* has revealed antigenicity in diglycosyl-diacylglycerol and the immunogenic phosphomannoinositides. The dominant carbohydrate-containing antigen of *M. tuberculosis* (responsible for the high-titer anti-arabinofuranosyl activity in tuberculous sera) is lipoarabinomannan, which has been purified in the native state from *M. tuberculosis* and shown to contain both phosphatidylinositol and phosphoinositol side-branches (Brennan, 1989; Barry *et al.*, 1998; Barry, 2001). Trehalose dimycolate (cord factor from *M. tuberculosis* cell wall) was found to produce extensive granuloma formation in mice (Bekierkunst *et al.*, 1969). Tuberculosis patients were also later found to produce antibodies to cord factor (Kato, 1972). This confirmed that the complex *M. tuberculosis* cell wall lipids contribute to the outcome of the dialogue between the pathogen and the host.

The cell wall of *Mycobacterium* is a very complex structure that accounts for the spore-like properties of the bacterium (Brennan and Nikaido, 1995; Daffe and Draper, 1998; Barry, 2001). In particular mycolic acids exist as the most abundant cell wall lipid in *M. tuberculosis* with many biological functioning other than simply providing a protective layer. In 1994, the group of Michael Brenner in Boston (USA), found that mycolic acids evoked an immune response through presentation on human CD1b (Beckman *et al.*, 1994). Since then the group in Boston has focussed their attention on the further characterisation of antigen presentation on CD1 molecules.

In this laboratory the focus fell on various applications of mycolic acids in fields including immunotherapy and tuberculosis diagnosis. The first challenge was to obtain purified mycolic acids in large quantities. This hurdle was crossed using a liquid-liquid extraction process on a counter current distribution system (Siko, 1999; Goodrum *et al.*, 2001). Experiments preformed on cell cultures, mice, rats, and tuberculosis patient serum suggested that:

- Mycolic acids pre-treatment of *M. tuberculosis* infected mice enhanced survival (Lombard, 2002)
- Mycolic acids pre-treatment of *M. tuberculosis* infected mice induced expression of IL-12 and IFN-γ in the lungs (Pretorius, 1999)
- Administration of mycolic acids to uninfected mice induced expression of IL-12 and IFN-γ in the lungs (Pretorius, 1999)
- Administration of mycolic acids to Sprague Dawley rats elicited anti-mycolic antibodies (Siko, 1999; Ten Bokum *et al.*, 2002 submitted)
- Mycolic acids pre-treatment prevented the onset of heat-killed *M. tuberculosis* induced adjuvant arthritis in Lewis rats (Siko, 1999; Ten Bokum *et al.*, 2002 submitted)
- Treatment with mycolic acids induced proliferation of CD4/CD8 double negative T cells (Goodrum *et al.*, 2001)

- Tuberculosis patients express antibodies against mycolic acids (Pretorius, 1999; Schleicher *et al.*, 2002)

With such overwhelming evidence on the potential of mycolic acids to be of benefit in the medical field and to elicit immune responses, the question remained about the mechanism of mycolic acids action.

6.1 Mycolic acids in murine tuberculosis

Previous studies indicated that protection against tuberculosis provided by mycolic acids could be due to production of pro-inflammatory cytokines such as IFN-γ and IL-12 in the lungs and not in the spleen (Pretorius, 1999). Induction of these cytokines is normally associated with a shift of the immune system towards a Th1 mode (Mossmann and Coffman, 1989). In these studies, an unequivocal shift in the Th1/Th2 balance was not detected in either the spleen or the lungs (Siko, 1999). The animals for these studies were infected intravenously, leading to systemic infection and cytokines were determined by means of end-point PCR. Because a response to mycolic acids administration was prominent in the lungs, the experiment was repeated, but this time expression of cytokines were analysed after the intranasal route of infection to introduce *M. tuberculosis* directly into the lungs of mycolic acids pre-treated animals. In addition, real-time quantitative PCR was compared with semi-quantitative endpoint PCR. The results indicated that expression of pro-inflammatory cytokines IL-12, IFN-γ, and TNF-α in mice that were pre-treated with a sub-optimal dosage of mycolic acids (5μg) was inhibited. This was not directly in agreement with the survival data observed. The optimum dose of mycolic acids (25μg/mouse) maintained the expression of pro-inflammatory cytokines to the same level as in *M. tuberculosis* infected mice that did not receive mycolic acids, even though these levels were generally higher than those of the animals receiving 5 μg/mouse. Compared to other studies where systemic *M. tuberculosis* infection was established via intravenous infection, mycolic acids pre-treatment did not enhance IL-12 or IFN-γ expression.

The pre-treatment of mice with 25 μg mycolic acids/mouse correlated with an enhanced (90%) protection of mice against subsequent disease progression over the 40 weeks following *M. tuberculosis* infection. Although, a significant expression of IL-12 in mycolic acids treated, uninfected mice were detected compared to untreated-uninfected mice the IL-4/IL-12 and IL-4/IFN-γ ratios clearly excluded any shift towards Th1. This indicates that mycolic acids may contribute to the expression of pro-inflammatory cytokines, but the protective mechanism involved is not simply a shift towards Th1 orientation. Flynn *et al.* (1995) indicated that with IL-12 administration, enhanced survival could be attributed to a shift towards a Th1 mode in the lungs. Although mycolic acids were also able to elicit an IL-12 expression response, the mechanism of induced protection was clearly different and did not rely on subsequent IFN-γ production in the lungs. Rather, an effect of mycolic acids on macrophages appeared to contribute to protection. Stoltz (2002) explored the possible mechanism of mycolic acids action and found that in the peritoneal macrophages, mycolic acids appeared to have interesting effects. The observations made were that peritoneal macrophages were not activated according to the classical pathway, which only involved the Th1 cytokines but rather were activated via the alternative pathway which also involved Th2 cytokines. In these studies mycolic acids were shown to induce IL-10. An alternative macrophage activation response to mycolic acids in the peritoneum was in contrast with what was previously observed in the lungs (Pretorius, 1999) and requires further investigation with alveolar macrophages.

6.2 Mycolic acids in immunocompromised mice

C57Bl/6 mice are more resistant to *M. tuberculosis* infection than Balb/c mice. Previous studies indicated that mycolic acids pre-treatment of Balb/c mice intravenously infected with *M. tuberculosis* brings them to the level anti-tuberculosis resistance of C57Bl/6 mice (Siko, 1999). Mycolic acids administration were however shown to have no significance effect on the survival of C57Bl/6 mice. C57Bl/6 mice were shown to have a high base

level of IL-12 in the lungs (Pretorius, 1999). The observation that mycolic acids brought the resistance of Balb/c mice to that of the level of C57Bl/6 mice, inferred the possibility that if C57Bl/6 mice could become susceptibile to *M. tuberculosis* infection then mycolic acid pre-treatment may restore resistance. This aims at the possibility of enhancing the innate immune response to control tuberculosis in HIV co-infected individuals. Like C57Bl/6 mice, humans are significantly resistant to *M. tuberculosis* infection. Of persons infected with *M. tuberculosis*, only 10% develop tuberculosis. Infection with HIV renders them much more susceptible to disease progression upon infection with *M. tuberculosis.*

Infection of C57Bl/6 mice with LP-BM5 murine leukaemia virus renders them immunodeficient, with symptoms similar to those observed in humans that have developed AIDS. For this reason this model of C57Bl/6 mice infected with LP-BM5 MuLV has been termed murine AIDS (MAIDS) and has been extensively used in anti-retroviral drug research. Ian Orme and co-workers (1992) indicated that *M. avium* infection is increased when MAIDS developed in C57Bl/6 mice. The MAIDS model was therefore chosen to investigate the potential benefits of mycolic acids pre-treatment in LP-BM5 infected C57Bl/6 mice before infection with *M. tuberculosis.*

In this study, co-infection of C57Bl/6 mice with LP-BM5 MuLV and *M. tuberculosis* manifested itself as a realistic model as there was increased susceptibility to tuberculosis disease progression compared to animals that were mono-infected. This suggested that the model could be useful as a valuable tool in studying tuberculosis in an AIDS environment, aiming at a better control of tuberculosis in sub-Saharan Africa.

This study provided an unexpected outcome: Unlike with Balb/c mice, the effects of mycolic acids administration in C57Bl/6 mice were not observed in the lungs but were now observed in the spleens. Mycolic acids induced expression of IL-12 in the spleens of mice that were co-infected with LP-BM5 MuLV and *M. tuberculosis*. This induction was however maintained to the level of uninfected mice. There was no observable effect on the level of IFN-γ expression. There was also a down-regulation of IL-10 expression in these animals suggesting that mycolic acids induced a shift towards pro-inflammatory

cytokine expression as opposed to anti-inflammatory cytokine expression. Anti-inflammatory/pro-inflammatory cytokine ratios emphasised that mycolic acids did enhance the expression of pro-inflammatory cytokines. This trend was apparent in both IL-10/IL-12 and IL-10/IFNγ ratios in the spleens and suggested that mycolic acids induce a pro-inflammatory cytokine response in C57Bl/6 mice, which was, however, not enough to uphold innate protection in immunocompromised animals.

6.3 Immunotherapy with mycolic acids

The major problem with tuberculosis chemotherapy is that it should be maintained without interruption for a period of 6 to 9 months. Lack of compliance results in multi-drug resistant strains. Studies have focused on combining chemotherapy with immunotherapy to combat this problem. Because mycolic acids have been shown to induce expression of IL-12, this study focused on applying this property of mycolic acids as a potential immunotherapeutic support treatment in combination with chemotherapy. Studies have indicated that administration of IL-12 alone is not sufficient to control *M. tuberculosis* infection, but IL-12 in combination with chemotherapy was successful (Flynn *et al.*, 1995; Lowrie *et al.*, 1999). Similarly, mycolic acids as such could not be used independently to cure tuberculosis. In Balb/c and C57Bl/6 mice already infected with *M. tuberculosis*, treatment with mycolic acids had no observable effects (Siko, 1999).

When Balb/c mice were infected with *M. tuberculosis* and the animals were subsequently treated with chemotherapeutic drugs as well as mycolic acids, no improved sterilisation of the lungs in terms of growth of *M.tuberculosis* could be achieved, in comparison to mice that only received chemotherapy with isoniazid, pyrazinamide and rifampicin. There could be any, or a combination of several explanations for the negative outcome of this experiment:

- Mycolic acids may not have induced IL-12 at sufficient levels of expression to effect the same support for chemotherapy as was obtained with the IL-12 gene therapy repoted by Lowrie *et al.* (1999).
- Mycolic acids administration may have induced biological effects in addition to IL-12 expression that could have antagonised the effect of IL-12, such as IL-10 secretion or the activation of a type of macrophage that could antagonise the effect of IL-12 (Stoltz, 2002).

Taken together, the potential of mycolic acids to be applied as an immunotherapeutic support for chemotherapy against tuberculosis found no support in the mouse experiment performed here.

6.4 Mycolic acids in a biosensor based serodiagnostic assay for tuberculosis

The tuberculosis epidemic is aggravated by lack of fast and reliable methods for diagnosing *M. tuberculosis* infection. In 1999, Pan *et al.* indicated that oxygenated mycolic acids are recognised by antibodies in tuberculosis patients in an ELISA assay, and that such antibodies may act as surrogate markers for infection with *M. tuberculosis*. Schleicher *et al.* (2002) from our group, reported however that the predictive value of such an assay was poor, when tested among South African patients. It thus became important to understand the mechanisms of interactions between specific patient antibodies and mycolic acids. The light evanescent biosensor appeared well suited to offer fast and reliable information on this as it measures the interaction of molecules in real time. Mycolic acids are highly insoluble in a number of solvents and this provided a challenge in first finding a suitable surface for analysing the interaction of mycolic acids and their antibodies. This study envisaged developing such a surface to measure interactions between antibodies and the mycolic acids immobilised in the cells of a biosensor cuvette.

A non-derivatised biosensor cuvette was selected to immobilise mycolic acids, after activation of the surface with a cationic surfactant. Mycolic acids were deposited on the

surface using cholesterol-containing liposomes. The coat was blocked with a neutral surfactant. A binding profile was established from a tuberculosis patient serum that differed from negative control serum on a mycolic acids coated surface. The anti-mycolic acids antibodies could be inhibited by pre-incubation with mycolic acids indicating specificity of these antibodies. Interestingly a similar binding profile was established on a control surface coated with cholesterol liposomes that contained no mycolic acids. Binding of patient antibodies to cholesterol was confirmed with ELISA to be an antibody antigen interaction. This suggested that both mycolic acids and cholesterol may act as ligands for antibodies in patient and control sera, and that there may even be a degree of cross-reactivity between mycolic acids and cholesterol towards antibody recognition.

Cross-reactivity between cholesterol and mycolic acids appeared not to be far-fetched according to theoretical modelling of folded mycolic acids and cholesterol structures. The insight obtained by modelling could even explain why Pan *et al.* (1999) had found oxygenated mycolic acids to be the specific ligand for anti-mycolic acids antibodies. The cross-reactivity between cholesterol and mycolic acids as ligands for anti-mycolic acids antibodies require more rigorous testing with antibody binding assays other than ELISA and the biosensor, which rely on ligand-coated surfaces of a hydrophobic nature. Final proof of specificity and cross-reactivity will require determination of the affinity of these ligands with purified immunoglobulins from patient sera. This will be addressed by other researchers on this project.

6.5 Conclusion

The cholesterol-mycolic acids mimicry observed with biosensor analysis provides some tantalizing possibilities and explanations of recent observations: During the year 2000 a number of reports appeared in the scientific literature implicating cholesterol in the survival of pathogenic *M. tuberculosis* in macrophages *in vitro*. Av-Gay and Sobouti (2000) in their observations suggested that cholesterol might have a role in mycobacterial infection other than as a carbon source. Gatfield and Pieters (2000) reported that

cholesterol depletion of macrophages blocked mycobacterial entry. These observations suggested a crucial role played by cholesterol during infection.

In previous studies it was shown that mycolic acids could not protect mice against tuberculosis if administered after infection but was only effective when administered as a pre-treatment before *M. tuberculosis* infection (Siko, 1999). Protection rendered by mycolic acids could be due to their blocking of the scavenger receptor (SR). Macrophages express SRs that are able to bind oxidised low-density lipoproteins (LDL). Low-density lipoproteins are rich in cholesterol. Scavenger receptors derive their names from the fact that they bind a variety of unrelated ligands. The SRs were reported to be the most important cell surface receptors on the macrophage involved in the binding and subsequent entry of *M. tuberculosis* (Zimmerli *et al.*, 1996). The possible role played by mycolic acids in the protection against tuberculosis could be through binding the SRs and thereby preventing the adherence of the bacterium. Mycolic acids may also have biological effects that may explain why there is a narrow window of dosage of mycolic acids where they are protective. Concentrations of mycolic acids higher or lower than 25μg were previously observed to have no protective effect (Siko, 1999).

A different perspective on the possible mechanism of mycolic acids protection is provided by the observations that mycolic acids pre-treatment induced pro-inflammatory cytokine expression in the lungs of *M. tuberculosis* mice, without changing the T-heper bias. This protection could be through activation of macrophages. Lower concentrations of mycolic acids resulted in suppression of these cytokines and rendered the animals more susceptible to disease progression than untreated *M. tuberculosis* infected mice. One possible explanation for the narrow window of dosage affectivity was provided by Stolz (2002): He found that mycolic acids may render the macrophages super-targets by inducing a cholesterol hunger that prepared the macrophages for *M. tuberculosis* infection. Higher doses were however enough to evoke protective cytokine responses and thus induce the bactericidal activity of macrophages, while overdoses may block the bactericidal activity similar to high dose antigen tolerance in activation of B-cells.

Another view could be that mycolic acids are involved in determining the critical macrophage membrane lipid structural transition by association with cholesterol. In this case the lower dose of mycolic acids may render the macrophage super-targets of *M. tuberculosis*, the optimal dose of mycolic acids could then be protective, and a higher dose could induce a stage where the macrophage becomes paralysed to respond to infection.

In more practical terms, a molecular mimicry between mycolic acids and cholesterol as derived from the results with the biosensor may create the possibility of synthesising a more effective drug that targets the cholesterol, by binding to the mycolic acids surrounding the invading bacterium. This study opens the possibility of designing a compound comprising known organic compounds that bind to cholesterol, cholesterol-containing molecular complexes or chemical entities with cholesterol structural mimicry, linked to organic compounds with anti-mycobacterial or anti-inflammatory or anticoagulant properties via a weak covalent bond or a strong non-covalent bond, which should slowly dissociate in an aqueous environment. The cholesterol-binding organic compound can also be linked to a reporter molecule for the purpose of selectively binding to the infectious agent, thereby allowing its detection. These options may provide a worthwhile approach to future research that may contribute to controlling tuberculosis in the world and specifically in sub-Saharan Africa, where the disease is so dramatically aggravated by AIDS.

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