The effect of mycobacterial mycolic acids on the cytokine profile of the immune response in murine tuberculosis

by

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Outline of the Dissertation

This dissertation consists of a list of abbreviations and a summary followed by three chapters:

The general introduction in chapter 1 involves a description of the type of cells involved in the immune response to infection with *Mycobacterium tuberculosis*. The immunological value of mycolic acids, *i.e.* long chain fatty acids found in the cell wall *of Mycobacterium tuberculosis* is also discussed.

In chapter 2 the possibility to quantify cytokines with semi-quantitative reverse transcriptase polymerase chain reaction is investigated by evaluating the linearity of densitometry and the PCR reaction.

In chapter 3, the role of various cytokines in the immune response to *Mycobacterium tuberculosis* is discussed. The potential of mycolic acids in preventing tuberculosis was addressed by determining the profile of the mRNA of these cytokines in mice pretreated with mycolic acids before infection with *Mycobacterium tuberculosis*. A survival study and an organ count of mycobacteria conclude this chapter.

The concluding discussion in chapter 4 is followed by a list of all the references used in this dissertation.

List of Abbreviations

β2m	β2-microglobulin
Ab	Antibody
AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
BCG	Bacille Calmette Guérin
CD	Cluster of differentiation
cDNA	Complementary DNA
CFU	Colony forming units
DC	Dendritic cell
DEPC	Diethyl pyrocarbonate
DN	Double negative
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotides
dT	Deoxythymidine
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FasL	Fas-Fas ligand
GM-CSF	Granulocyte macrophage colony stimulatory factor
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
i.n.	Intranasal
i.v.	Intravenous
IFN	Interferon
IL	Interleukin
iNOS	Inducible form of nitric oxide synthase
KO	Knock out
LAM	Lipoarabinomannan

LJ	Löwenstein-Jensen
M. bovis	*Mycobacterium bovis*
M. tuberculosis	*Mycobacterium tuberculosis*
MA	Mycolic acids
mM	Millimolar
TDM	Trehalose dimycolate
MDR	Multi-drug resistant
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NIH	National Institute of Health
NK	Natural killer
NKT	Natural killer T cell
NO	Nitrogen oxide
PCR	Polymerase chain reaction
PIM	Phosphatidylinositol mannoside
QCRT-PCR	Quantitative competitive RT-PCR
RNA	Ribonucleic acid
rRNA	Ribosome ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
ss	Single-stranded
TB	Tuberculosis
TBE	Tris-borate/EDTA
TCR	T cell receptor
TDM	Trehalose dimycolate
Th	T helper
TMM	Trehalose monomycolate
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
tRNA	Transfer RNA

V	Volts
WT	Wild type

Summary

Mycobacterium tuberculosis (*M. tuberculosis*), the etiological agent of tuberculosis, is an intracellular bacterium which persists within macrophages. Successful control of tuberculosis depends on T-cell-mediated immunity. Immune protection involves the development of a Th1 response characterised by the secretion of cytokines such as IL-12, IFN-γ and TNF-α. The progression towards disease in humans and mice is often associated with a Th2 response characterised by the secretion of cytokines such as IL-4 and IL-10.

Mycolic acids, the major cell wall lipid of *M. tuberculosis*, were previously shown to have a marginally protective effect on the development of disease in Balb/c mice when administered intravenously at an optimal dose of 25 µg one week before intravenous *M. tuberculosis* infection. Here it is shown that the protective effect is highly significant when infection is done intranasally. The protective effect of 25 µg mycolic acids against tuberculosis could not be explained by induction of a longer lasting Th1 response in Balb/c mice. This was determined by using semi-quantitative RT-PCR on the mRNA of cytokines characteristic of the different immune responses. It was observed that maximum sensitivity was obtained at the lowest possible PCR cycle and template concentrations for the samples.

Mycolic acids were the first non-protein antigens shown to induce an immune response after presentation on CD1 membrane proteins. Balb/c mice predominantly generate a Th1 response during the first 3 – 4 weeks of *M. tuberculosis* infection, whereas they generate a Th2 response in the following weeks. Even though the protective effect of 25 µg mycolic acids could not be associated with a prolonged Th1 immune response in infected mice, it did induce IL-12 and IL-10 mRNA in uninfected mice. These cytokines are primarily

macrophage secreted cytokines. Therefore, the protective effect of 25 µg mycolic acids may rather be associated with the innate immune response.

Opsomming

Mycobacterium tuberculosis (*M. tuberculosis*), die etiologiese agent van tuberkulose, is 'n intrasellulêre parasiet wat in die makrofaag skuil. Suksesvolle beheer van tuberkulose is afhanklik van T-sel bemiddeling. Immuun beskerming word gekenmerk deur die ontwikkeling van 'n Th1 response wat gekarakteriseer word deur die sekresie van sitokiene soos IL-12, IFN-γ en TNF-α. Siekte word in mense en muise geassosieer met 'n Th2 respons wat gekarakteriseer word deur die sekresie van sitokiene soos IL-4 en IL-10.

Daar is voorheen aangetoon dat mikoolsure, die hoof selwandlipied van *M. tuberculosis*, 'n marginale beskermde effek gehad het op die ontwikkeling van siekte in Balb/c muise nadat dit intraveneus toegedien was teen 'n optimale dosis van 25 µg, een week voor intraveneuse *M. tuberculosis* infeksie. Hier word aangetoon dat die beskermende effek hoogs betekenisvol was na intranasale infeksie. Die beskermende effek van 25 µg mikoolsure teen tuberkulose kon nie verklaar word in terme van die induksie van 'n langer blywende Th1 respons in Balb/c muise nie. Dit was bepaal met behulp van semi-kwantitatiewe terugwaartse polimerase-ketting-reaksie op die mRNA van die karakteristieke sitokiene van die veskillende immuunresponse. Daar is opgemerk dat maksimum sensitiwiteit vekry word by die laagste moontlike polimerase-ketting-reaksie siklus en templaatkonsentrasies van die verskillende monsters.

Die eerste nie-proteïen antigene wat opgemerk is om 'n immuunrespons te induseer na presentering op CD1 membraanproteïene, was mikoolsure. Balb/c muise genereer hoofsaaklik 'n Th1 response gedurende die eerste 3 - 4 weke van *M. tuberculosis* infeksie met 'n Th2 response in die daaropvolgende weke. Alhoewel die beskermde effek van 25 µg mikoolsure nie geassosieer kon word met 'n verlengde Th1 immuunrespons nie, het mikoolsure IL-12 en IL-10 mRNA in ongeïnfekteerde muise geïnduseer. Diè sitokiene word hoofsaaklik deur die

makrofaag gesekreteer. Daarom kan die beskermde effek van 25 µg mikoolsure dalk eerder met die konstitutiewe immuunsisteem geassosieer word.

Chapter 1

Immune response to *Mycobacterium tuberculosis*

1.1. The disease tuberculosis

Tuberculosis (TB) is an ancient infection that has plagued humans throughout recorded and archaeological history. TB appears to be as old as humanity itself. Skeletal remains of prehistoric humans dating back to 8000 BC, found in Germany, show clear evidence of the disease. Egyptian skeletons dating from 2500 to 1000 BC have revealed spinal deformities typical of Pott's disease that is TB of the spine. Ancient Hindu and Chinese writings have documented the presence of the disease. From these descriptions, however, it is impossible to differentiate TB from diseases that produce similar pathology. Perhaps the best proof of prehistoric TB has come from an Inca mummy of an 8-year old boy who lived about 700 AD. The radiographic picture of the lumbar spine showed evidence of Pott's disease, and smears of the lesions revealed acid-fast bacilli, most likely *Mycobacterium bovis* (*M. bovis*) (Stead and Dutt, 1994).

TB is a chronic infectious disease caused by *M. tuberculosis*. *M. tuberculosis* is an intracellular bacterium which persists within macrophages (Kaufmann, 1993; McDonough *et al.*, 1993). In fact, if one injects *M. tuberculosis* intravenously into mice, the great majority of the bacteria will be within tissue macrophages after 24 hours (Bermudez and Sangari, 2001). Pulmonary TB is the infectious and common form of the disease, occurring in over 80% of cases. Extra-pulmonary TB is a result of the spread of TB via the blood stream or the lymphatic system to other organs. TB may affect any part of the body but is most commonly found in the pleura, lymph nodes, spine, joints, genito-urinary tract, nervous system and abdomen (Dannenberg, 1994).

The primary mode of transmission is through airborne droplets expelled (usually when coughing) by an individual who has active TB. Active TB develops in three ways: primary progression of infection, endogenous reactivation of latent bacilli from previous infection, and exogenous re-infection (*i.e.* aerogenic) following resolution of a previous infection. The first form is known as primary TB, and the last two are known as post-primary TB. The clinical manifestation of post-primary TB is different (in general, milder) than that of primary TB (McKinney *et al.*, 1998). Post-primary pulmonary TB or reactivation TB is the most prevalent form of TB and develops despite the existing immunity developed during the primary exposure (Fenton and Vermeulen, 1996). The vast majority of healthy individuals infected with *M. tuberculosis* do not go on to develop progressive primary or reactivation TB. Instead, innate and acquired immune responses become activated and successfully control the focus of infection, which is usually in the lung parenchyma and draining lymph nodes (Dannenberg, 1989). Active TB develops eventually in 10% of infected subjects; 5% within 5 years of exposure and the other 5% later in life as their immune system weakens (Stead and Dutt, 1994). Thus, the immune response is adequate to control the infection in most people but apparently is not generally effective at destroying the organism. Active TB usually occurs concomitantly with a period of excessive environmental stress, malnutrition or lowering of immune competence of the body by diseases such as acquired immune deficiency syndrome (AIDS).

M. tuberculosis is one of the most successful bacterial parasites of humans. Epidemiologists have estimated, based on tuberculin skin test reactivity, that one-third of the world's population, *i.e.* 1.7 billion people, is infected with *M. tuberculosis* (Enarson and Murray, 1996). Although *M. tuberculosis* resides in large proportion of the world population in a clinically unapparent form, seven to eight million people develop the disease every year. About 1.8 million people died of this disease in 1998 (World Health Report, 1999). *M. tuberculosis* is responsible for more human deaths than any other single infectious agent – 26% of all preventable deaths or 7% of all deaths can be attributed to TB (Enarson

and Murray, 1996). The emerging health problem caused by *M. tuberculosis* not only affects the developing countries, where a persistently high incidence rate yields a mortality rate of 20% of all deaths in adults. Since the turn of the century, when TB was the number one killer among all infectious agents in Western Europe and the United States, mortality due to TB has drastically declined in these regions (Smith and Moss, 1994). In the mid-eighties it gained a renewed significance in the western world when, after decades of decline, the number of TB cases increased drastically, especially in the United States (Bloom and Murray, 1992). Reasons for this development may be found in the worsened economic and social infrastructure of part of the population in industrial countries as well as in the spread of the human immunodeficiency virus (HIV) epidemic. HIV infection is the greatest known risk factor for progression from latent *M. tuberculosis* infection to active TB (Narain *et al.*, 1992). It is estimated that TB is responsible for 32% of deaths among HIV-positive people globally, compared to 11% due to septicaemia, 10% to cerebral toxoplasmosis, 8% to pneumonia, 6% to malignancies, 5% to meningitis and 10% due to other infections. Approximately 8% of the TB patients and one quarter of those who died of TB in 1998 were already co-infected with HIV, and it is likely that this proportion will increase in future (Kaufmann and Hess, 2000).

Although Robert Koch already identified *M. tuberculosis* as the causal agent of TB in 1882 (Barnes, 2000), prevention and therapy of TB is still not satisfactory. In the 1906s, Albert Calmette and Camille Guérin developed a vaccine for human TB from a weakened strain of virulent cow TB (*M. bovis*) called Bacille Calmette Guérin (BCG) (Calmette *et al.*, 1927). This vaccine has been used extensively since 1928. Although BCG is the most widely used vaccine world-wide, with 3 billion doses of BCG given to people around the world from 1921 to 1990, its application has been controversial with respect to its efficacy and safety (Colditz *et al.*, 1994). The results of trials in the past ranged from possibly detrimental to an 80% protective effect. Meta-analysis of all of the published prospective trials and case-control studies indicates approximately 50% efficacy against all forms

of TB (Colditz *et al.*, 1994). General agreement exists that BCG can protect against or at least ameliorate severe forms of systemic TB in children, particularly meningitis (Huebner, 1996), yet it seems to be of low or no protective value in adults. The most prevalent form of TB, namely reactivation of dormant TB in adults, cannot be prevented by this vaccine in a satisfactory way (Huebner, 1996).

It was not until 1943 that an effective treatment for TB was found; in that year, soil biologist Selman Waksman's laboratory at Rutgers University in New Jersey developed streptomycin, the first of a series of antibiotics that proved effective against TB (Barnes, 2000). TB therapy requires at least six months of treatment with three or more drugs. Furthermore, after a few weeks, the symptoms of TB as well as patient compliance wane, preventing completion of adequate therapy and thus favouring the emergence of drug-resistant strains (Bloom and Murray, 1992). Multi-drug resistant (MDR) TB refers to TB, which is resistant to at least isoniazid and rifampicin. MDR strains of *M. tuberculosis* have an associated fatality rate of 40 to 60%, equivalent to untreated TB (Barnes *et al.*, 1991; Bloch *et al.*, 1994; Heym *et al.*, 1994). The figures reported for initial drug resistance in China, Pakistan and the Philippines are about 20-30% (Kim and Hong, 1992). At the United States-Japan TB-Leprosy Panel meeting in Nagasaki in July 1996, Dr. Abe reported overall rates of resistance to one or more drugs among isolates from Yemen, Thailand and Myanmar in the range of 40-60% (Hirano *et al.*, 1996). Prevalence rates for drug resistance in Japan were reported at 10.6% in 1992. For the United States the prevalence rates were 14.2% in 1991 and only 7.6% in 1995. In a recent study initiated by the Global Tuberculosis Program of the World Health Organisation, multi-drug resistance was found to be highest in Latvia (22.1%) followed by India (New Delhi region) (13.3%), Estonia (11.7%), the Dominican Republic (8.6%) and Argentina (8.0%). By contrast, low rates were found in most countries in Western Europe, in Africa and in the US (Chan-Tack, 1998). Currently the cure rate of MDR TB patients is less than 50%. This situation reaffirms the need to develop better prophylactic and therapeutic

strategies by applying novel approaches recently made possible by advances in basic research on the immunology of TB.

1.2. Immunological control of mycobacterial infection

Immunity to *M. tuberculosis* infection is based on a complex interaction between macrophages and several T cell subsets. Macrophages present mycobacterial antigens to T lymphocytes. The T lymphocytes participate by means of two major functions.

Firstly, they are activated to secrete cytokines such as interferon gamma (IFN-γ) after recognising the mycobacterial antigens through highly specific receptors (Orme *et al.*, 1993b; Cooper *et al.*, 1993). IFN-γ also stimulates release of the cytokine tumour necrosis factor alpha (TNF-α) by the macrophage. IFN-γ and TNF-α increase anti-mycobacterial activity of both human (Denis, 1991; Hirsch *et. al.*, 1994) and murine (Bermudez and Young, 1988) macrophages by eliciting the production of toxic radicals derived from oxygen and nitrogen, which lead to the destruction of the microbe. Blocking with anti-TNF-α mouse antibody (Ab) increased the proliferation of *M. tuberculosis* in the spleens and livers, but not the lungs, of infected animals. Further addition of anti-IFN-γ mouse Ab was required in order to enhance *M. tuberculosis* growth within the lungs (Appelberg *et al.*, 1995). Moreover, it was reported that IFN-γ knock out (KO)-mice do not produce reactive nitrogen intermediates and, consequently, were unable to restrict the growth of *M. tuberculosis* (Flynn *et al.*, 1993). In mice with a latent *M. tuberculosis* infection, treatment with aminoguanidine, an inhibitor of the inducible form of nitric oxide synthase (iNOS), reactivates disease, providing further evidence for the role of iNOS and nitrogen oxide (NO) in disease control (Murray, 1999). Both TNF-α and IFN-γ are also instrumental in development of granulomas by stimulating the local production of chemokines (Rhoades *et al.*, 1995), a family of chemoattractant molecules that will accelerate the influx of

monocytes from the blood. The granuloma consists of activated macrophages often fused into multinucleated giant cells surrounded by T cells. The micro-organisms become sequestered in the granulomas and cause no clinical disease in 90% of infected individuals (American Thoracic Society and Centres of Disease Control, 1990). It is not known whether among the 90% of infected individuals who do not develop the active disease the TB bacilli are sterilised or merely remain in a latent state. Mice deficient for IFN-γ and TNF-α develop granulomas only in a delayed fashion (Flynn *et al.*, 1995a; Cooper *et al.*, 1993).

Secondly these cells also express cytolytic activities, *i.e.* they lyse infected target cells (Kaufmann, 1999). Lysis of infected macrophages, themselves insufficiently equipped for mycobacterial killing, could allow release of micro-organisms that are then attacked by more proficient monocytes. Some T cells also possess granulysin, which can directly attack and kill mycobacteria.

If macrophages do not become activated within the granuloma, bacilli escaping from the edge of the granuloma can multiply in these nonactivated (and partly activated) macrophages present perifocally. The patient starts to display the clinical symptoms of TB such as loss of weight, night sweats, persistent cough, loss of appetite and fatigue, as caseation necrosis develops and spreads. The host can eventually die due to the excessive tissue destruction (Dannenberg, 1994, 1999).

1.3. T cells involved in the immune response against tuberculosis

Peripheral T lymphocytes express on their surface an antigen receptor noncovalently linked to the cluster of differentiation (CD)3 complex, which is responsible for the transduction of the antigenic signal to the cytoplasm (MacDonald and Nabholz, 1986). The T cell receptor (TCR) is a disulphide-linked heterodimer, either composed of α and β chains, or γ and δ chains. In human

and mouse peripheral blood and lymphoid organs, about 90% of the T cells express the αβ TCR, whereas only a minority (<10%) possesses the γδ TCR. During antigen recognition, optimal signalling through the αβ TCR requires aggregation with the co-receptors CD4 and CD8. CD4 binds to invariant parts of the major histocompatibility complex (MHC) class II antigen-presenting molecule and CD8 to invariant parts of the MHC class I molecule. T lymphocytes recognise mycobacterial-derived peptides presented by MHC molecules (Roitt, 1994).

In contrast to MHC, a family of non-polymorphic proteins, *i.e.* CD1 (Calabi *et al.*, 1989) functions to present lipid and glycolipid antigens to T cells (Porcelli and Modlin, 1999). CD1 proteins are coded for by up to five distinct genes (isotypes) that are conserved in different mammalian species and can be separated into two subgroups on the basis of their amino acid sequence (Porcelli, 1995). Group I, which comprises CD1a, CD1b, CD1c and CD1e, is present in humans but absent from mice and rats. Group II, which includes only CD1d, is found in all species studied thus far. The ligands identified thus far for group I CD1 molecules are predominantly from mycobacteria and the responding T lymphocytes are mostly TCRαβ$^+$ cells. Group I CD1 molecules (e.g. CD1a, b and c) present mycobacterial lipid antigens to CD4$^-$/CD8$^-$ (double negative (DN)) and CD8$^+$ human T cells (Porcelli *et al.*, 1992; Beckman *et al.*, 1994; Beckman et *al.*, 1996; Rosat *et al.*, 1999). CD1-reactive DN T cells can express either αβ or γδ TCRs. Among the group I CD1 proteins, the response mediated by the CD1b molecules is the best studied. The human CD1b protein is currently known to mediate T cell responses to three classes of mycobacterial lipids. These are free mycolates, glycosylated mycolates and diacylglycerol based phospholipids such as phosphatidylinositol mannoside (PIM) or lipoarabinomannan (LAM) (Beckman *et al.*, 1994; Sieling *et al.*, 1995; Moody *et al.*, 1997). CD1d is recognised by natural killer T (NKT) cells, a specialised T cell subset that has been found in mice and humans (Bendelac *et al.*, 1997). These cells are characterised by the expression of receptors of the NK lineage and a pattern of cell surface protein

expression typical of activated memory T cells (Bendelac *et al.*, 1997). NKT cells have a highly restricted TCR repertoire in mice (Makino *et al.*, 1995; Arase *et al.*, 1992) and humans (Porcelli *et al.*, 1993; Dellabona *et al.*, 1994). These cells are mostly $CD4^+$ or $CD8^-CD4^-$ T lymphocytes (60%/40%) (Bendelac *et al.*, 1997), although CD4 does not seem to function as a co-receptor for CD1. Studies so far have been restricted to the presentation of nonmicrobial lipids to NKT cells. To date, the only glycolipid that has been shown to strongly stimulate NKT cells is α-galactosylceramide, a glycolipid originally isolated from marine sponge because of its antitumour properties (Kawano *et al.*, 1997). To date, no bacterial antigens presented by group II CD1 molecules have been identified.

It has become increasingly clear that immunity to intracellular bacteria comprises not only MHC class II-restricted $CD4^+$ $\alpha\beta$ T cells but also MHC class I-restricted $CD8^+$ $\alpha\beta$ T cells. The efficacy of BCG, the most widely used attenuated bacterial vaccine for decades, against adult pulmonary TB is possibly insufficient due to inefficient stimulation of $CD8^+$ T cells (Kaufmann, 1998). Additional T cell subsets, such as $\gamma\delta$ T cells and CD1-restricted $\alpha\beta$ T cells play complementary, apparently minor roles.

1.3.1. MHC class II-restricted $CD4^+$ $\alpha\beta$ T cells

Animal studies indicate a major role for $CD4^+$ T cells in protection against mycobacterial infections. The depletion of $CD4^+$ T cells in an *M. tuberculosis*-infected murine model leads to the uncontrolled multiplication of bacteria (Barnes and Modlin, 1996; Toossi, 1996; Orme, 1996). $CD4^+$ T cells appear rapidly after infection. Upon adaptive T cell transfer from immunised mice, protection was provided to non-immune mice against a challenge with *M. tuberculosis*. Some sensitised T cells may enter a longer-lived phenotype and mediate the expression of immunological memory (Orme, 1988; Anderson *et al.*, 1995). Direct evidence of a protective role for $CD4^+$ T in human TB is less clear-cut. In HIV-infected patients low $CD4^+$ T cells numbers lead to reactivation of quiescent

mycobacteria and aggravation of disease (Orme *et al.*, 1993a). Moreover, the severity of TB correlates with the levels of $CD4^+$ lymphocytopenia (Jones *et al.*, 1993).

Functionally, $CD4^+$ T cells are thought to contribute to protection against *M. tuberculosis* by the antigen-specific production of cytokines, which activate infected macrophages to kill the intracellular bacteria. Recently, transgenic $CD4^{-/-}$ mice have been shown to succumb to TB, due to diminished levels of IFN-γ early in infection (Caruso *et al.*, 1999). $CD4^+$ T cells also greatly accelerate granuloma initiation, while $CD8^+$ T cells have no role in this respect (Izzo and North, 1992; Hänsch *et al.*, 1996).

Mycobacterium-reactive $CD4^+$ T cells can display cytotoxicity in addition to lymphokine production in humans and in mice (Mutis *et al.*, 1993; Kumararatne *et al.*, 1990; Munk *et al.*, 1989; Orme *et al.*, 1992), but the relevance of this effector mechanism *in vivo* remains elusive. It was shown that $CD4^+$ T cell clones specific for TB-derived purified protein derivative efficiently lyse a variety of targets using both the Fas-Fas ligand (FasL) pathway and the granule exocytosis pathway of cytotoxicity (Lewinsohn *et al.*, 1998). In another study, lysis of *M. tuberculosis*-infected monocytes by recombinant FasL resulted in the killing of intracellular bacteria (Oddo *et al.*, 1998). These experiments were performed in the absence of T cells and it remains to be determined whether the contact-dependent pathway by which $CD4^+$ cytotoxic T lymphocytes (CTLs) kill intracellular mycobacteria (Silver *et al.*, 1998) involves the Fas-FasL pathway.

1.3.2. MHC class I-restricted $CD8^+$ $\alpha\beta$ T cells

Substantial numbers of both activated $CD4^+$ and $CD8^+$ T cells migrate to the lungs of *M. tuberculosis*-infected mice (Serbina and Flynn, 1999; Feng *et al.*, 1999). Furthermore, $CD8^+$ CTLs were found in outer mantles of TB and tuberculoid leprosy granuloma lesions (Cooper *et al.*, 1989). Animals lacking

$CD8^+$ T cells were found to be more susceptible to the infection, specifically in the lungs. $CD8^+$ T cells can prolong survival of acutely challenged mice (Orme, 1987). CD8 KO mice in which the gene for the CD8 molecule had been disrupted were also found to be highly susceptible to *M. tuberculosis* infection (D'Sousa *et al.*, 1998), leading to incorrectly organised granulomas (Ladel *et al.*, 1995).

β2-microglobulin (β2m)-deficient mice fail to control experimental *M. tuberculosis* infection (Flynn *et al.*, 1992). Because these β2m-mutant mice lack cell surface MHC class Ia, MHC class Ib and CD1 molecules, functional $CD8^+$ T cells cannot develop. TAP molecules transport peptides from the cytosol to the endoplasmic reticulum for loading onto MHC class I molecules; the complex is then transported to the cell surface. Presentation of antigens by CD1 is not dependent on TAP molecules (Spada *et al.*, 1998). $Tap1^{-/-}$ mice are deficient in peptide presentation by MHC class I molecules and consequently lack $CD8^+$ T cells (Van Kaer, 1992). Behar *et al.* (1999) reported that susceptibility to *M. tuberculosis* infection was similar in $TAP1^{-/-}$ and $\beta2m^{-/-}$ mice. These data support the hypothesis that MHC class I-restricted $CD8^+$ T cells are required for control of TB.

Activated $CD8^+$ T cells directly lyse infected antigen presenting cells (APCs) and produce phagocyte-activating cytokines. MHC class I-restricted $CD8^+$ T cells from the lungs of infected mice recognised *M. tuberculosis*-infected dendritic cells (DCs) or macrophages and produced IFN-γ and TNF-α (Feng *et al.*, 1999; Serbina and Flynn, 1999). Activation by IFN-γ is crucial for the macrophage to eradicate intracellular pathogens. It is likely that $CD8^+$ T cells can contribute to protection by complementing $CD4^+$ T cells as a source of IFN-γ. Short term culture of mouse lung T cells with infected DCs resulted in $CD8^+$ T cells capable of MHC class I-restricted specific lysis of macrophages infected with live, virulent *M. tuberculosis*. These cells expressed perforin *in vivo* and specifically recognised and lysed *M. tuberculosis*-infected macrophages in a perforin-

dependent manner after a short period of *in vitro* restimulation (Serbina *et al.*, 2000).

In CD8 KO mice, increased bacterial growth in the lungs is a relatively late event, suggesting that $CD8^+$ T cells may be more important in controlling the chronic phase of the disease (A.M. Cooper *et al.*, unpublished results referred to in Orme and Cooper, 1999). The study of animal models suggests that the perforin-containing granule exocytosis pathway of cytotoxicity does not contribute to immunity against intracellular pathogens in the acute stage of infection. However, in disease in which acute infection is followed by an asymptomatic chronic stage, the presence of perforin-containing cytotoxic granules might be necessary for the containment of disease. In TB the presence of perforin-containing cytotoxic granules appears to be necessary for the containment of pathogens in the chronic stages of the disease, whereas perforin and granzymes are not critically involved in the resolution of early infection (Denkers *et al.*, 1997; Laochumroonvorapong *et al.*, 1997; Cooper *et al.*, 1997a).

1.3.3. CD1-restricted $CD8^+$ $\alpha\beta^+$ T (CD1-restricted $CD8^+$ T cells)

Human CD1-restricted $CD8^+$ T cells recognise *M. tuberculosis* phospholipid antigens presented in the context of CD1 antigen presenting molecules (Rosat *et al.*, 1999). Human CD1-restricted $CD8^+$ T cells can produce IFN-γ and lyse infected macrophages (Rosat *et al.*, 1999; Stenger *et al.*, 1997). Human CD1-restricted $CD8^+$ T cell lines that lyse *M. tuberculosis*-infected macrophages by a perforin-dependent mechanism can kill *M. tuberculosis* (Stenger *et al.*, 1997) via the cytolytic T cell granule protein granulysin (Stenger *et al.*, 1998).

1.3.4. CD1-restricted $CD4^-CD8^-$ $\alpha\beta^+$ (CD1-restricted double negative (DN)) T cells

Human DN CD1-restricted T cells release IFN-γ and express cytolytic activity upon contact with target cells pulsed with mycobacterial lipids and glycolipids *in vitro* (Porcelli *et al.*, 1992; Beckman *et al.*, 1994; Sieling *et al.*, 1995). Recently it was demonstrated that human DN CD1-restricted T cells can also recognise antigen on the cell surface of cells infected with viable *M. tuberculosis* (Stenger *et al.*, 1997; Jackman *et al.*, 1998; Gong *et al.*, 1998). Similar to $CD8^+$ CTLs, human DN CD1-restricted T cells efficiently lysed macrophages infected with *M. tuberculosis*. The cytotoxicity of DN CD1-restricted T cells was however mediated by Fas-FasL interaction and had no effect on the viability of the mycobacteria (Stenger *et al.*, 1998). This was in striking contrast to $CD8^+$ T cells that lysed infected macrophages by a Fas-independent, granule-dependent mechanism, which resulted in the killing of bacteria. The Fas-FasL pathway serves an autoregulatory role by maintaining peripheral tolerance. In the context of microbial infection this pathway may serve to deplete APCs, thus dampening the immune response to prevent it from causing extensive tissue damage. In this way, the DN T cell response may contribute mainly to the regulation of the anti-TB response.

1.3.5. TCR $\gamma\delta^+$ T Cells ($\gamma\delta$ T cells)

TCR $\gamma\delta^+$ ($\gamma\delta$) T cells are known to participate in the immune response against mycobacteria, as evidenced by their early accumulation in human disease lesions (Modlin *et al.*, 1989; Griffin *et al.*, 1991). While they constitute only a minor T cell population (1 to 5%) within lymphoid organs, $\gamma\delta$ T cells are a predominant T cell population within epithelial tissues, including the skin, gut and airways (Griffith, 1997). The $\gamma\delta$ T cell responses are highly increased in primary *M. tuberculosis*-infected children in comparison with age-matched controls

(Poccia *et al.*, 1998). This increase in γδ T cell reactivity subsides after successful antibiotic therapy, suggesting that a persistent exposure to mycobacterial antigens is required for the maintenance of T cell activation *in vivo*. Transiently increased γδ T cell numbers have been observed in the peripheral blood of healthy donors exposed to *M. tuberculosis*-infected patients, but not in the blood of chronically ill TB patients, indirectly suggesting a protective role of this subset (Ueta *et al.*, 1994).

Although these T cells are generally $CD4^-CD8^-$ but $CD3^+$ (Borst *et al.*, 1987, Nakanishi *et al.*, 1987), they have been shown to recognise antigen in the context of MHC class I or II and also non-polymorhic MHC class Ib molecules (Haas *et al.*, 1993). γδ T cells also expand *in vitro* in response to mycobacterial non-peptide phosphoantigens (Constant *et al.*, 1994; Tanaka *et al.*, 1995). New evidence showed that T cells of the major tissue γδ T cell subset recognise nonpolymorphic CD1c molecules; however, the ligand being presented to these T cells has not yet been identified (Spada *et al.*, 2000).

A role for γδ T cells is supported by functional studies demonstrating their capacity to display cytotoxicity and produce IFN-γ in response to contact with *M. tuberculosis*-infected macrophages (Tsukaguchi *et al.*, 1995). The cytotoxicity of γδ T lymphocytes can be through Fas-FasL mediated apoptosis or a granule-dependent mechanism involving pore-forming perforin and antibacterial granulysin resulting in the reduction of the viability of intracellular bacilli (Dieli *et al.*, 2000; Spada *et al.*, 2000). The presence of bactericidal granulysin suggests these cells may directly mediate host defence even before foreign antigen-specific T cells have differentiated. However, a recent evaluation using aerosol-infected γδ TCR KO mice did not support any protective role, since IFN-γ production was not affected in the lungs. A novel role for these cells appears to be in the efficient recruitment of monocytes into the lung granuloma of TB-infected mice (D'Souza *et al.*, 1997). γδ T cells were markedly activated in the

first phase of infection. In absence of the γδ T cells a much more pyrogranulomatous inflammatory response with lesions containing increased numbers of neutrophils and large foamy macrophages formed, distinct from the lymphocytic granulomas in the wild type.

1.3.6. Natural killer T cells (NKT cells)

In mice, NKT cells can be detected wherever conventional T cells are found, although their abundance varies widely in different tissues (Bendelac, 1995; Bendelac *et al.*, 1997; MacDonald, 1995; Eberl *et al.*, 1999, Hammond *et al.*, 1999), e.g. as a proportion of mature T cells 30-50% in liver, 20-30% in bone marrow, 10-20% in thymus, 3% in spleen, 0.3% in lymph node, 4% in blood and 7% in lung. Although most reports on human NKT cells have been limited to those in peripheral blood, Vα24JαQ$^+$ NKT cells are clearly present in human liver although apparently not as frequent as they are in mice (approximately 4% of hepatic T cells versus 50% in mice) (Doherty *et al.*, 1999). The distribution of NKT cells in other human tissue remains to be determined. The role of NKT cells in the immune system is not clearly defined. Whatever their physiological role, it is clear that many NKT cells are reactive to CD1 molecules (Bendelac *et al.*, 1995; Exley *et al.*, 1997). It is manifested as CD1 autoreactivity because their *in vitro* response to CD1 occurs in the absence of exogenous antigen. Even though no mycobacterial antigen has been found to bind to CD1 and stimulate NKT cells to date, it was found that mycobacterial infection increased CD1d surface expression on murine DCs and macrophages *in vitro* (K. Fischer *et al.*, unpublished results referred to in Schaible and Kaufmann, 2000).

A striking characteristic of NKT cells is their ability to produce high levels of IL-4, IFN-γ and other cytokines within a few hours of *in vivo* activation (Yoshimoto *et al.*, 1995). Upon infection with BCG, the initial IL-4 burst by CD4$^+$ NKT cells is modulated towards IFN-γ production (Emoto *et al.*, 1999). Treatment of mice with anti-CD1 antibodies slightly exacerbated murine TB at early time points and

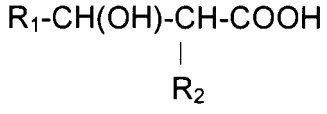
reduced the production of IFN-γ, interleukin (IL)-12 and TNF-α in the spleen (Szalay *et al.*, 1999). By contrast mice deficient in group II CD1d molecules were as resistant as wild-type mice to *M. tuberculosis* infection (Behar *et al.*, 1999). This paradox could be explained by (1) a redundancy of the immune system that allows compensation of NKT cell functions by other (T) cells in the CD1 KO mouse, or (2) the possibility that anti-CD1d antibody treatment not only blocks recognition of CD1 by NKT cells but also influences APCs directly. It remains to be determined whether NKT cells could play an important role in the host response by influencing the cytokine microenvironment during the course of an infection.

1.4. Mycobacterial cell wall mycolic acids

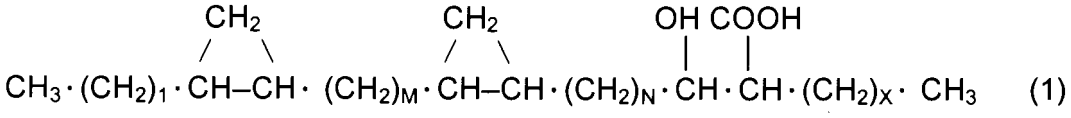
M. tuberculosis is an acid-fast bacillus characterised by a waxy cell wall. The relevant components comprise long-chain fatty acids, mostly mycolic acids (MA) and glycolipids like LAM, phenolic and peptidoglycolipids, sulpholipids and others (Brennan and Besra, 1997).

MA are high molecular weight, α-alkyl, β-hydroxyl fatty acids and are the most characteristic components of the cell envelope of *Mycobacteriaceae*, *Corynebacteriaceae*, *Rhodococci*, *Nocardiae* and members of the order *Actinomycetes* (Minnikin, 1982). In the mycobacterial cell envelope, MA are present as free lipids, such as trehalose dimycolate (TDM) or cord factor and trehalose monomycolate (TMM) (Minnikin, 1982) and mycolate phospholipids (Besra *et al.*, 1994). For the most part, they are esterified to the terminal penta-arabinofuranosyl units of arabinogalactan, a peptidoglycan-linked polysaccaride (Brennan and Nikaido, 1995). The presence of such long-chain fatty acids is largely responsible for the high hydrophobicity and very low permeability of the mycobacterial cell envelope (Lee *et al.*, 1996). The number of carbon atoms that make up the MA varies from C_{20} to C_{36} in the genus *Corynebacterium* to C_{60} to C_{90} in the genus *Mycobacterium*. MA of the *Nocardia*

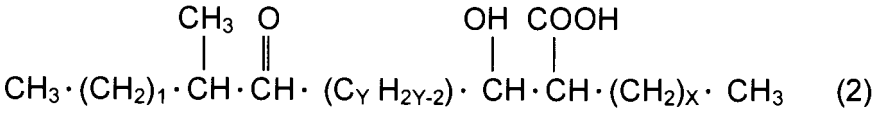
and *Rhodococcus* species have lengths ranging from C_{36} to C_{66} (Butler *et al.*, 1991). Mycobacterial MA compose about 40-60% of the dry weight of the cell wall of the bacteria (Brennan and Nikaido, 1995; Lee *et al.*, 1996).
The general formula for MA is:



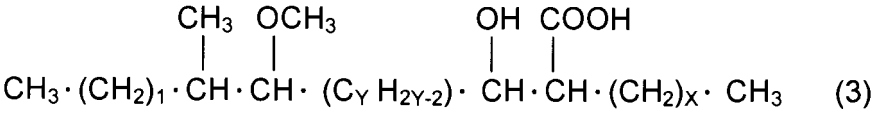
R_2 is a normal straight carbon chain with slight variation in carbon chain length between mycobacterium species and R_1 contains the different structural types including the non-oxygenated α-mycolates and mycolates having other oxygen functions (keto, methoxy, ω-carboxy, epoxy) in addition to the 3-hydroxy acid unit. Several species of mycobacteria have distinctly different MA "fingerprints" (Butler *et al.*, 1991). The MA profile of *M. tuberculosis* includes α-mycolates (no. 1 below), ketomycolates (no. 2 below) and methoxymycolates (no.3 below).



(1)



(2)



(3)

It was found that oxygenated MA are necessary for virulence of *M. tuberculosis* in mice, since a mutant strain no longer synthesising oxygenated MA, had profound alterations in its envelope permeability and was attenuated in mice (Dubnau *et al.*, 2000).

The importance of MA in mycobacterial physiology is exemplified by the fact that many of the first-line and most effective anti-TB drugs such as isoniazid, ethionamide and ethambutol interfere with either MA biosynthesis or their deposition into the cell wall (Wheeler and Anderson, 1996; Mdluli *et al.*, 1998; Banerjee *et al.*, 1994; Quemard *et al.*, 1995; Mikusova *et al.*, 1995). The ensuing lack of MA has been thought to ultimately lead to a disruption of the hydrophobic barrier, resulting in a loss of cellular integrity (Barry III *et al.*, 1998).

1.5. Aim of this study

One of the most interesting immunological findings of the past decade was the discovery that human CD1 proteins present mycobacterial lipid antigens to T cells. This came as a real surprise because immunologists had become comfortable with the notion that T cells detect only peptides in the context of MHC molecules. MA were the first non-protein antigens shown to stimulate human $CD4^-CD8^-$ T cell proliferation upon CD1 presentation (Beckman *et al.*, 1994). It was shown that MA could stimulate not only human DN T cells *in vitro*, but also $CD4^+$ T cells when presented on $CD1^+$ APCs (Goodrum, 2001). It was found that CD1 can activate not only $CD4^+$ $NK1.1^+$ T cells but also some $NK1.1^-$ $CD4^+$ T cells (Wang *et al.*, 1999). Stimulation of $CD8^+$ T cells by MA under the same circumstances was not observed (Goodrum, 2001). The recognition that CD1, an orphan family of MHC-like molecules, has an entirely new role in antigen presentation to T lymphocytes came as a surprise in a closely studied area of immunology, raising the prospect of novel, lipid-based vaccines.

In previous studies (Pretorius, 1999) pretreatment of Balb/c mice, *i.e.* mice relatively susceptible to *M. tuberculosis* (Flynn *et al.*, 1995b), with 25 µg MA-serum conjugate, as opposed to 5 µg, increased resistance to intravenous *M. tuberculosis* infection. Bacilli that reach the lungs via the respiratory route are ingested by resident alveolar macrophages whereas those that enter from the blood need to be carried into the organ after being ingested by monocytes, neutrophils or pulmonary vascular macrophages. Resident AMs tend to be less capable than other macrophages of killing microbial pathogens in general (Fels and Cohn, 1986). It is possible that this enables *M. tuberculosis* to grow more rapidly during the initial stage of infection. *M. tuberculosis* colony forming units (CFU) given by aerosol were substantially more virulent than much larger numbers inoculated *i.v.*, as shown by a faster rate of bacillary growth in the lungs and much shorter survival of the host. Earlier death of mice infected by aerosol was associated with faster development of lung pathology, even though the number of *M. tuberculosis* implanting initially in the lungs were the same for *i.v.* and *i.n.* infection (~$10^{2.4}$ and $10^{2.8}$ CFU respectively). Most of the 10^5 CFU inoculum given *i.v.* was taken up by the liver (North, 1995). The aim of the present study was to determine whether MA administered intravenously as a pretreatment would provide protection against TB to Balb/c mice infected intranasally with *M. tuberculosis* as well, using survival and the expression of cytokine messenger ribonucleic acid (mRNA) as tools.

Chapter 2

Relative quantification of messenger RNA with the reverse transcriptase polymerase chain reaction

2.1. The reverse transcriptase polymerase chain reaction (RT-PCR)

In the framework of orchestrating an immune response against *M. tuberculosis*, T cells and macrophages use non-antigen-specific, low weight molecular proteins *i.e.* cytokines, to communicate with each other and among themselves, and alter each other's behaviour. Some cytokines are regarded as protective, whereas others are generally associated with the chronic and progressive phase of TB (Mosmann *et al.*, 1986; Haanen *et al.*, 1991; Huygen *et al.*, 1994). Therefore an analysis of the cytokine expression pattern provides valuable information about the type of immune response induced in different experimental research models and in infectious disease.

Genetic information is stored in chromosomes as a sequence of nucleotides in a deoxyribonucleic acid (DNA) molecule. This information is parcelled into packets called genes, each of which carries the information for coding the amino acid sequence in a single polypeptide chain. Genetic information flows from DNA to ribonucleic acid (RNA) and from there to protein: DNA → RNA →protein. There are three major types of cellular RNA involved in protein synthesis: messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA). RNA polymerase transcribes mRNA from DNA, and its nucleotide sequence contains the information for the sequence of amino acids in the protein product. mRNA is processed after transcription by adding a tail of adenine nucleotides to one of the ends of the mRNA. The second type of RNA is tRNA. Its function is to carry

amino acids to the mRNA template, where the amino acids will be linked in a specific order. rRNA is a structural and functional component of ribosomes, the large ribonucleoprotein bodies that house the machinery for most of the reactions associated with protein synthesis (Zubay, 1993). Specific mRNAs are synthesized by the cell in response to the conditions under which it finds itself, e.g. *M. tuberculosis* infection, and the cell can control the kinds and amounts of cytokines it produces. Effects on cytokine levels can be analyzed on two different levels, these being mRNA and protein.

At the protein level, cytokines can be measured in sera, body fluids (blood, urine, peritoneal fluid, nasal lavage, bronchoalveolar lavage) and in cell culture supernatants by enzyme-linked immunosorbent assay (ELISA), using antibodies recognising the specific cytokine (Diaz *et al.*, 1995). Fresh material can be homogenised for the quantification of cytokines in the supernatant (Beck *et al.*, 1996). Proteins are the effector molecules that bring about biological activity and are therefore a more direct measure than mRNA. However, cytokines are active at very low concentrations according to a paracrine mode: their circulating levels are very low (<10 pg/ml), sometimes below the detection limit of ELISA assays (Bienvenu *et al.*, 1998). Furthermore, the measurement of cytokine release into cell culture supernatants is essentially a cumulative measure and unless time points are chosen very carefully, will not yield information on the way in which cells are responding to various stimuli and how a cascade of events is progressing. Generally the rate of production of a cytokine in this type of assay is greater than the rate of degradation (by receptor binding and by non-specific proteolysis) and thus the measurement will be representative only for the sum cumulation of events. Since we know that immune responses involve a variety of cell types and sequences of events, we may not always identify the crucial alterations in gene expression. Thus, cytokine measurements in body fluids or cell supernatants do not always provide the proper or complete picture. mRNA on the other hand, measures at a specific point in time within a tissue or organ. The majority of mRNA species are generally short-lived, existing only to convey

information from nucleus to cytoplasm and thereafter being rapidly degraded by endogenous ribonuclease. This instability is of great analytical advantage in that it allows us to pinpoint very precisely what cells are doing at a given moment in time and how they are responding to various external stimuli. Due to the apparent correlation between cytokine mRNA expression and protein production or biological activity, detection of mRNA is an alternative method of demonstrating cytokine induction (Cherwinski *et al.*, 1987; Kuhn and Goebel, 1994; Rottman *et al.*, 1995; Giguère and Prescott, 1998). Several techniques are currently available to measure changes in gene expression at the mRNA level. These include the Northern blot, the RNase protection assay, *in situ* hybridisation and the RT-PCR (Gause and Adamovicz, 1995). RT-PCR is highly sensitive and permits us to look at modulation of cytokine expression in very low numbers of cells or to look at the modulation of certain immunoregulatory cytokines, which are expressed at low levels within cells. Since it is rapid and considerably more sensitive than traditional RNA blot techniques, RT-PCR is increasingly used to detect small changes in gene expression that would otherwise be undetectable (Gause and Adamovicz, 1995).

The initial step in RT-PCR is the production of a single-stranded (ss) complementary DNA (cDNA) of the mRNA through the action of the retroviral enzyme reverse transcriptase. Following the reverse transcriptase (RT) reaction, the cDNA is amplified by the polymerase chain reaction (PCR) (Rugo *et al.*, 1992; Friedhoff *et al.*, 1993; Taniquchi *et al.*, 1994; Esnault *et al.*, 1996). The method of PCR is primer-mediated enzymic (polymerase) amplification of specific genomic cDNA sequences. The polymerase chain reaction (PCR) is divided into cycles, each of which is comprised of three discrete steps, namely denaturation of both target sequences and primers, by which all of these molecules become single-stranded; primer annealing, by which the oligonucleotide primers base-pair to a complementary sequence within the template (target) DNA; and primer extension, in which the polymerase activity of a thermostable enzyme synthesises DNA from the 3'-OH terminus of each primer

molecule which is base-paired to the template. These three steps, as illustrated in Figure 2.1, constitute "cycle 1" and the repetition of these steps, using the product as the new template for the next step, supports the exponential accumulation of the desired sequences. After only 30 cycles it is thus possible to achieve a multimillion fold amplification of template (2^{30} = 1 073 741 824), however, the reaction is not 100% efficient. Given that a geometric amplification of template occurs, a single contaminant DNA molecule is enough to generate false signals and/or false positives. Contamination in the PCR environment has been traced to aerosol formation from micropipettes, tainted stock solutions of PCR reagents, contaminated thermal cyclers, laboratory coats and, in some cases, the epidermal layers of the investigator himself (Farrell *et al.*, 1997). Therefore negative control samples, *i.e.* all the reagents described above except cDNA, must be run with each PCR.

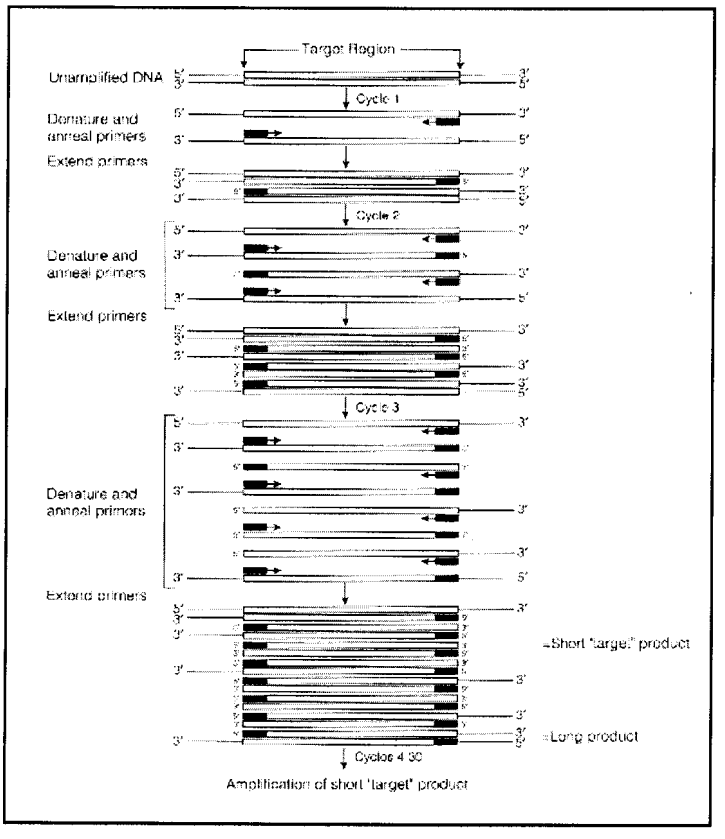


Figure 2.1: Schematic diagram of the PCR process (Zubay, 1993).

During the progression of a PCR, the reaction goes through two distinct phases, the exponential phase and the plateau phase. In the exponential phase, theoretically, every cDNA is denatured, bound by primers and copied by the polymerase. This phase occurs in the early through middle cycles of a PCR. The exponential phase is followed by the plateau phase. In the plateau phase of the reaction, components of the reaction mixture become limiting. The supply of nucleotides might be depleted, inhibitors from the reaction might accumulate, the polymerase might lose activity or primers might become limiting. Most likely, the cDNA begins to compete for primers and DNA amplification product concentration can increase to the point where single-stranded products re-anneal with each other rather than with a primer. The effect this has on quantification is dramatic. A reaction becomes less predictable as it enters and proceeds into the plateau phase. Equal amounts of template can give widely varying amounts of signals in the plateau phase but give, in general, equal signals in the early exponential phase. Differing amounts of initial template can also give equal signals in the plateau phase because they will proceed until the supply of some reagent becomes limiting. Under these conditions differences in transcript levels may be missed.

2.2. Quantification of the effect of mycolic acids on messenger RNA

Although RT-PCR offers many advantages over RNA blot methods, the possibility to accurately quantify mRNA levels by RT-PCR is not universally accepted. RT-PCR is in essence a qualitative method indicating the presence or absence of specific transcripts and is a useful method for detecting alterations in cytokine levels produced at the paracrine or autocrine level.

There are essentially three problems to contemplate when attempting to impose quantification upon an RT-PCR analysis, (a) to control for different recovery of

RNA between different samples; (b) to control for differential amplification efficiency between different samples; and (c) to prove linearity in the method of detecting PCR products.

Mathematically, the reverse transcription step is very basic. There is no amplification and the sole variable is the percentage of mRNA converted into cDNA. Nevertheless, the RT step is the source of most of the variability in a RT-PCR experiment. The reverse transcriptase enzyme is sensitive to salts, alcohol or phenol remaining from the RNA isolation and carry-over from the RNA precipitation step. This can affect the yield of RNA, which can fluctuate from 5% to 90% (Ferré *et al.*, 1994). Efficiency of the reverse transcription step is measured as the percentage of RNA transcribed into cDNA. If two separate reactions have equal amounts of RNA, but their RT efficiency is unequal, the final amounts of the amplification products will be dissimilar following PCR. The most common approach to measure RNA levels by PCR was first described by Chelly *et al.* (1988). Their method is based on the co-amplification of the RNA of interest together with an endogenous mRNA as internal control. The β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs, also known as house-keeping gene transcripts, are usually constitutively expressed in most systems studied and are therefore commonly selected as internal control to serve as reference for relative mRNA expression. Comparisons between samples can be made by relating gene expression levels relative to housekeeping gene expression. After normalisation with respect to this internal control, a reasonable estimate of the RNA of interest in different samples can be obtained. β-actin was chosen as a housekeeping control for mouse lung cytokines, as it had been used before for murine mRNA quantification (Oka *et al.*, 1999).

Control of differential amplification within the PCR is best effected by the use of "MIMICS" which are sections of DNA which have been engineered to yield a PCR product using the same oligonucleotide primers as the cDNA of interest but incorporate additional spurious DNA such that the final PCR product is of

different size than the cDNA of interest. This competitive PCR (Siebert and Larrick, 1992) provides an intra-tube control on each PCR reaction such that cDNA product can be differentiated from the higher molecular weight "MIMIC" product on simple agarose gel electrophoresis. The method involves co-amplification of sample DNA and known amounts of "MIMIC". When target DNA and "MIMIC" are at equal concentrations, co-amplification results in equal amounts of both products with bands of equal density. Since the concentration of the "MIMIC" is known, this gives the concentration of the target (unknown) DNA. Thus, in an analysis of PCR products in a gel by densitometry, relative expression is given by (OD cytokine cDNA×OD housekeeper "MIMIC") divided by (OD housekeeper cDNA×OD cytokine "MIMIC"). However, the production of "MIMICs" for competitive RT-PCR for the simultaneous quantification of transcripts of several cytokines is both expensive and time consuming. Also, it has been reported that competitor DNAs do not always have the same amplification kinetics as the target DNA (Reyes-Engel *et al.*, 1996). In addition, considerable amounts of cDNA are needed to carry out the competitive PCR amplification at the various dilutions needed. This is a disadvantage as the specimens available are often small and many different experiments need to be carried out at the same time.

In an alternative approach, dilution series of the target product are prepared as external controls and used to generate calibration curves (Heid *et al.*, 1996; Horikoshi *et al.*, 1992). A linear relationship between the amount of cDNA put into the PCR and the resulting PCR product is present only in the exponential phase of PCR. Generally, this means that this linear relationship is found with relatively low cycle numbers and relatively low amounts of cDNA. This can be assessed by calculating the correlation of the line. Using linear regression the slope of the line is calculated. The correlation (r^2) can be used to establish the accuracy of the line. For each sample this regression analysis is performed for the amount of mRNA encoding the product of interest (here called *X*) and for the amount of mRNA encoding a housekeeping enzyme (here called *H*). The ratio of the slopes

($_X$ divided by $_H$) is then calculated for each sample. From this, effects on the expression of *X* can be calculated. This type of analysis can only be made if for each sample and for each *X* the efficiency of PCR is the same. Obviously, this means that the number of cycles should be the same for each *X* and for *H*. This type of analysis involves the use of slopes (with an intrinsic dose-response relationship), instead of the more commonly used analysis of single measurements of PCR products (resulting from a single amount of cDNA).

This alternative approach was used to investigate the relative changes in the steady-state expression of various genes when *M. tuberculosis*-infected Balb/c mice were pretreated with MA (refer to Chapter 3). The next sections describe the investigation to determine the effect of the exponential character of the PCR on the quality of semi-quantitative results.

2.3. Materials

Ethidium bromide (Boehringer Mannheim, Germany)

Oligo deoxythymidine (dT) primers (Life Technologies Inc., Scotland)

Deoxynucleotides (dNTPs) (Roche Molecular Systems, USA)

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

Amplitaq Gold (Roche Molecular Systems, USA)

Tris-borate/EDTA (TBE) buffer: 0.089 M Tris-(Hydroxymethyl)-aminomethane (Merck, Darmstadt, Germany), 0.089 M boric acid (Merck, Darmstadt, Germany), 0.002 M ethylenediaminetetra-acetic acid (EDTA) (Merck, Darmstadt, Germany) (pH 8.00)

Agarose (Promega, USA)

2.4. Methods

2.4.1. The reverse transcriptase reaction (RT-reaction)

In the RT-reaction all the mRNA of a combined sample from a few of the total RNA samples isolated as described in chapter 3 was reverse transcribed to ss cDNA. From all the types of RNA isolated, only the mRNA has a poly adenylate (poly A) tail. By using oligo(dT) primers only the mRNA is used as a template for the production of first strand cDNA. The enzyme used for this reaction was the Superscript RNase H Reverse Transcriptase. Following the protocol supplied by the manufacturer (Life Technologies Inc.), total RNA (5 μg) and the oligo(dT) primer were heated to 70 °C for 10 min to ensure RNA denaturation. The reaction mixture was cooled on ice to enable annealing of the oligo(dT) primer. The dNTPs necessary for the elongation of the cDNA were added. The enzyme was added after the mixture was heated to 37 °C. The reaction was terminated after one hour incubation at 37 °C, by heat inactivation of the enzyme at 75 °C for 2 min.

2.4.2. β-actin PCR:

The β-actin master mixture contained 2 mM magnesium chloride, 0.2 mM of each deoxyribonucleotide, 0.04 U/μl reverse transcriptase (RT) enzyme and 5 ng/μl of each β-actin primer (forward: 5'–CTCCATCGTGGGCCGCTCTAG–3' and reverse: 5'-GTAACAATGCCATGTTCAAT–3' as in Ma *et al.*, 1994) and varying volumes of the cDNA prepared in section 2.4.1. for the different experiments. The heat-stable Amplitaq Gold DNA Polymerase enzyme was added before the PCR cycling was initiated by an incubation step for 10 min at 94 °C. At this temperature,

the enzyme becomes activated. After the enzyme was activated, three cycles consisting of steps of:

- 45 sec at 94 °C
- followed by 75 sec at 60 °C and
- 105 sec at 72 °C

were run to initiate the synthesis of the second cDNA strands.

The subsequent amplification cycle consisted of the following shorter steps:

- 35 sec at 94 °C,
- 45 sec at 60 °C and
- 75 sec at 72 °C.

This cycle was repeated a number of times as required for obtaining the desired result.

PCR products were subjected to electrophoresis on a 2 % agarose gel containing 0.5 µg/ml ethidium bromide. The samples were prepared by adding 4 µl of dye consisting of 0.025 % bromophenol blue and 15 % ficoll, to each 20 µl sample. The samples were subjected to electrophoresis for 1 hour and 20 minutes at 67 V in TBE buffer. The DNA was visualized with a Spectroline Model TC 312A transilluminator. Densitometry was performed using National Institute of Health (NIH) Image software on an Apple computer.

2.4.3. PCR amplification validation

A β-actin master mixture containing cDNA prepared as described in section 2.4.1. was divided into volumes of 20 µl . Samples were placed in alternating wells of the heat block of the Perkin Elmer PE9700 PCR machine as in the diagram on the next page:

Rows	1	2	3	4	5	6	7	8	9	10	11	12
A	X		X		X		X		X		X	
B		X		X		X		X		X		X
C	X		X		X		X		X		X	
D		X		X		X		X		X		X
E	X		X		X		X		X		X	
F		X		X		X		X		X		X
G	X		X		X		X		X		X	
H		X		X		X		X		X		X

The samples were amplified for 26 cycles.

2.4.4. Linearity of densitometry

Several β-actin PCR reactions performed on cDNA were combined. Different volumes of the combined sample were loaded onto a 2 % agarose gel to evaluate the linearity of densitometry.

2.4.5. Influence of cDNA concentration and PCR cycle number

Volumes of 0.1, 0.3, 0.5, 0.7, and 0.9 µl of the cDNA prepared in section 2.4.1. were amplified for 22, 23 and 24 PCR cycles at 60 °C.

2.5. Results

2.5.1. Validation of PCR machine reproducibility

To evaluate the uniformity of amplification and data-analysis, β-actin was amplified in aliquots of the same sample in the sample positions illustrated under 2.4.3. As shown in Table 2.1., amplification of β-actin was found to have a coefficient of variance of 8.21 %. This was deemed satisfactory for quantitative determinations using PCR.

Table 2.1: Densitometry values of β-actin cDNA amplified with PCR from aliquots of the same sample in marked positions in the Perkin Elmer PE9700 PCR machine.

Rows	1	2	3	4	5	6	7	8	9	10	11	12
A	125		129		144		132		143		124	
B		122		135		119		126		137		133
C	135		140		134		129		142		138	
D		135		149		131		145		158		155
E	122		146		151		152		156		131	
F		130		138		133		149		149		140
G	127		133		137		138		145		145	
H		113		127		112		121		120		130

Average	**135**
Standard deviation	**11**
Coefficient of variance	**8.21%**

2.5.2. Linearity of densitometry

The PCR reaction consists of 2 distinct phases, *i.e.* an exponential and a plateau phase. A linear relationship between the amount of cDNA and the resulting product applies only to the exponential phase of the PCR. Direct densitometry of ethidium bromide stained products in a gel was used to analyze PCR products to establish the efficiency and the range of linearity for the method. To evaluate whether the relationship between the density of ethidium bromide stained amplified bands and the concentration of DNA put o the gel was linear, different volumes of the same β-actin amplified sample were loaded onto a 2 % agarose gel and analysed by electrophoresis and densitometry.

As shown in Figure 2.2, the digital images analysed with the NIH Image software densitometry showed a linear relationship between input cDNA and the specific densitometry values obtained before reaching a plateau at higher concentrations. The very dark bands associated with the higher cDNA concentrations actually started to show a downward slope. As shown in Figure 2.2 this was found to be the case with the cDNA of different β-actin PCR's. For this reason a non-linear PCR amplification graph could be also be due to the limiting effect of very dark bands. Therefore linearity needs to be established for each sample under experimental conditions since all will contain a different unknown concentration of cDNA.

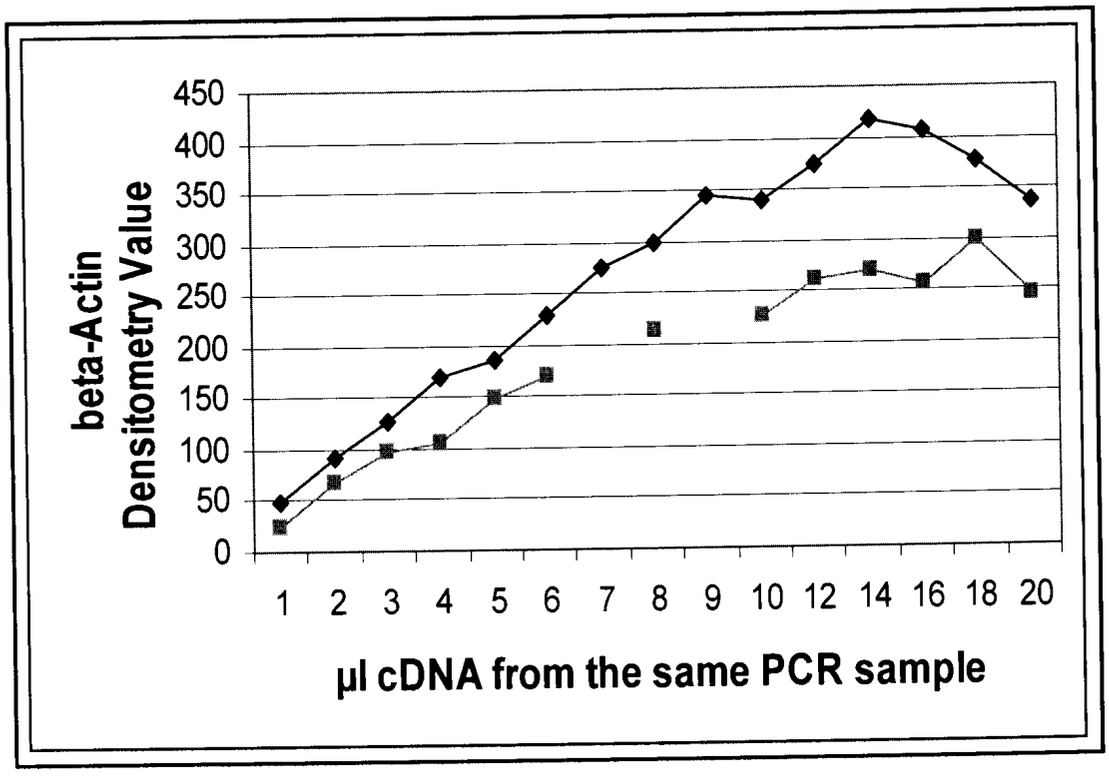


Figure 2.2: Relationship between input β-actin cDNA and the measured densitometry value.

2.5.3. Influence of cDNA concentration and PCR cycle number

The influence of the rate and extent of amplification on the interpretation of semi-quantitative data was verified by amplifying the β-actin gene at various cDNA concentrations of the same sample for 22, 23 and 24 cycles. Densitometric readings of bands for the different concentrations at increasing number of PCR cycles are shown in Table 2.2 and Figure 2.3.

Table 2.2: β-actin densitometry values for various cDNA concentrations at different PCR cycles.

µl cDNA	PCR Cycle Densitometry Value		
	22*	23*	24*
0.1	20	66	87
0.3	49	93	122
0.5	53	108	145
0.7	93	147	168
0.9	132	171	167
Concentration Correlation Coefficient for the different PCR cycles	**0.98**	**0.96**	**0.88**

*: Number of PCR cycles

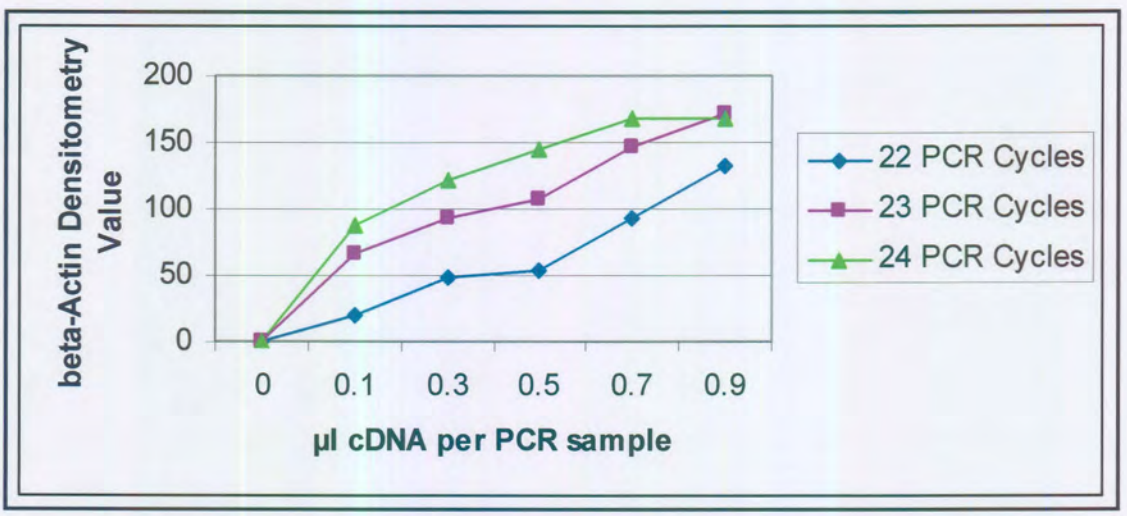


Figure 2.3: β-actin densitometry values for various volumes of input cDNA at different numbers of PCR cycles.

PCR products accumulated in a linear fashion when 0.1 to 0.9µl cDNA of this specific sample were amplified for 22 or 23 cycles, whereas the plateau phase was reached after 24 cycles of amplification.

The effect of different cDNA starting concentrations and different numbers of PCR cycles on the β-actin factor (normalisation factor) is shown in Table 2.3.

Table 2.3: Normalisation factors for various cDNA concentrations at different PCR cycles.

Volume of sample cDNA (µl)	**Normalisation factor for different PCR Cycles**		
	22*	**23***	**24***
0.1	1.00	1.00	1.00
0.3	0.41	0.71	0.71
0.5	0.38	0.61	0.60
0.7	0.22	0.45	0.52
0.9	0.15	0.39	0.52

* : Number of PCR cycles.

As the amplification process nears the plateau phase of PCR due to increases in starting cDNA or PCR cycle, differences in the normalisation factor between the various samples in Table 2.2 becomes smaller. Therefore the closer the PCR conditions are to the plateau phase of amplification, the less responsive the assay becomes towards different transcript levels in samples.

2.6. Discussion

The RT-PCR is a highly sensitive method allowing the detection of mRNA transcripts from small quantities of sample tissue due to the exponential amplification process.

Advantageously, the co-amplification of a housekeeping gene (β-actin) compensates for variability in cell number, RNA isolation, reverse transcription and PCR amplification efficiency (Livak *et al.*, 1995; Heid *et al.*, 1996) which allows accurate comparison of different preparation conditions. However, the co-amplification of cytokine with β-actin is not always possible due to differences in annealing temperatures of the various cytokine mRNAs. For this reason, the different cytokine mRNAs (β-actin as well) under investigation in Chapter 3 were run in separate PCRs. Since target sequences are theoretically amplified some 2^{35}-fold, small variations in amplification efficiency may lead to significant changes in PCR product yield (Gilliland *et al.*, 1990). The reason for inter-sample variations of amplification efficiency is poorly understood, but non-homogenous heat distribution within the thermal block cycler might play an important role. In the absence of internal controls it is necessary that all samples are amplified with the same efficiency. Coefficients of variation between 10 and 20 % have been reported for the analysis of replicate portions of the same sample on different occasions (Zimmerman and Mannhalter, 1996). In real-time PCR, the inter-sample variation in amplification efficiency of the LightCycler® system (Roche Diagnostics) is small, with duplicate samples typically differing by less than 10 % (Blaschke *et al.*, 2000). The inter-sample coefficient of variation in amplification efficiency of the Perkin Elmer PE9700 PCR machine used in this experiment was found to be 8.21 %, which is well within the published ranges for quantitative PCR analysis.

In the approach used in Chapter 3 to quantify cytokine mRNA a linear relationship is needed between different concentrations of input cDNA and the

end product. First, it was found that a linear relationship did exist between the intensity of ethidium bromide stained cDNA and cDNA concentration using a Spectroline Model TC 312A transilluminator and NIH soft ware before reaching a plateau at higher concentrations. Secondly, the normalisation factors became less responsive to input cDNA as the PCR became less linear at higher cycles. For this reason, comparable cytokine levels could prove to be different if the PCR is run at a lower cDNA concentration or PCR cycle. Therefore to ensure maximum sensitivity it is vital to run each PCR at the lowest possible input cDNA and PCR cycle.

Chapter 3

The influence of MA on the lung cytokine response during *M. tuberculosis* infection

3.1. The cytokine response during tuberculosis infection

The complex network of cytokine interactions between macrophages and T cells is a fascinating interplay of positive and negative signals. A detailed analysis of murine T cell clones first clearly established the existence of two distinct T cell subsets. These two populations were termed T helper (Th)1 and Th2. Th1 and Th2 patterns of cytokine secretion correspond to activated effector phenotypes generated during an immune response. They do not exist among naïve T cells. Work in mice demonstrated that Th1 and Th2 cytokines act as autocrine growth factors, amplifying a specific response and promoting the differentiation of naïve T cells to that particular subset (Lichtman *et al.*, 1987). Therefore, once a T cell response progresses along a Th1 or Th2 pathway, it tends to become polarised in that particular direction. The Th1 and Th2 patterns of cytokine production were originally described among mouse $CD4^+$ T cell clones (Mosmann *et al.*, 1986; Cherwinski *et al.*, 1987) and later among human T cells (Del Prete *et al.*, 1991). Mouse Th1 cells secrete mainly interleukin (IL)-2 and IFN-γ, whereas Th2 cells secrete mainly IL-4, IL-5, IL-6, IL-10 and IL-13. Some cytokines such as IL-3, TNF-α and granulocyte-macrophage colony stimulating factor (GM-CSF) are secreted by both T cell subsets. Human Th1 and Th2 cells produce similar patterns, although the synthesis of IL-2, IL-6, IL-10 and IL-13 is not as tightly restricted to a single subset as in mouse T cells.

It is important to keep in mind though, that the Th1/Th2 dichotomy is a simplified version of reality. T cells expressing cytokines of both types (coded as Th0) represent a heterogeneous population of partially differentiated effector cells consisting of multiple discrete subsets (Abbas *et al.*, 1966), indicating that Th1 and Th2 do not represent two absolute and mutually exclusive states. Furthermore, the terminology referring to "Th1" or "Th2" responses is misleading, since other cell types also produce many of the Th1/Th2 cytokines. For example, IFN-γ is produced by all T cells stimulated in response to mycobacterial infection, *i.e.* $CD4^+$ T cells, $CD8^+$ T cells, $\gamma\delta$ T cells, CD1 restricted T cells (Schaible *et al.*, 1999) and NK cells. IL-10 and TNF-α are also produced by macrophages. Recent evidence indicates that a similar dichotomy in cytokine profile can be observed among $CD8^+$ CTLs (Tc1, Tc2) (Mosmann and Sad, 1996) and T cells expressing the $\gamma\delta$ antigen receptor (Ferrick *et al.*, 1995).

The trigger to develop into Th1 or Th2 effectors appears to depend to a large extent on the cytokines that naïve T cells are exposed to during primary activation. IL-12, produced by activated macrophages, is the principal Th1-inducing cytokine. Recently activated, uncommitted $CD4^+$ T lymphocytes express receptors for IL-12. When bound it leads to the production of IFN-γ by T cells (Jacobson *et al.*, 1995; Hou *et al.*, 1994; Kaplan *et al.*, 1996). IFN-γ then further promotes Th1 differentiation by enhancing IL-12 secretion by macrophages and by maintaining expression of IL-12 receptors on the T cells (Trinchieri, 1995; Guler *et al.*, 1996). Therefore, IL-12 and IFN-γ act together on macrophages and lymphocytes to generate the production of Th1 populations. Binding of IL-4 to its receptor on recently activated uncommitted $CD4^+$ T lymphocytes leads to the production of more IL-4, which can then act in an autocrine fashion on the lymphocytes (Hou *et al.*, 1994; Seder and Paul, 1994; Ryan *et al.*, 1996). As the Th2-inducing effect of IL-4 dominates over other cytokines, Th2 differentiation is initiated upon reaching threshold levels of IL-4. If IL-12 is not present in the activating environment, or if T cells are poorly responsive to IL-12, the activated lymphocytes will become Th2 effectors.

In spite of the simplification embedded in the Th1/Th2 model, evidence shows that the ability to fight infectious agents often depends on which pattern (Th1 or Th2) dominates. A Th1 dominant response was found to be protective against TB, whereas a Th2 dominant response was associated with the chronic and progressive phase of TB in the experimental mouse model (Haanen *et al.*, 1991; Huygen *et al.*, 1994).

The cytokines produced by Th1 and Th2 were found to cross-regulate each other in that one type of cytokine pattern inhibits the induction of the other (Fitch *et al.*, 1993) and this provides an explanation for the inverse relationship observed between Th1 and Th2-directed responses (Mosmann *et al.*, 1986). IFN-γ, secreted from Th1 cells, is known to regulate the differentiation of Th1 cells and to inhibit the proliferation of Th2 cells (Gajewski *et al.*, 1989). On the other hand, IL-4 secreted from Th2 cells, has been shown to regulate the differentiation of Th2 cells (Mosmann and Coffmann, 1989) and IL-10 has been shown to inhibit the function of Th1 cells by inhibiting Th1 cytokine synthesis (Fiorentio *et al.*, 1989).

Macrophages are indispensable cells in the regulation of Th1/Th2 cell responses. Mononuclear phagocytes are able to produce both pro-inflammatory cytokines, e.g. TNF-α and IL-12, and anti-inflammatory cytokines, e.g. IL-10 (Lucey *et al.*, 1996). The delicate balance between pro- and anti-inflammatory cytokines is considered to determine the outcome of disease (Rook and Hernandez-Pando, 1996).

3.1.1. IFN-γ

IFN-γ is produced by all T cells stimulated in response to mycobacterial infection, *i.e.* $CD4^+$ T cells, $CD8^+$ T cells, γδ T cells, CD1-restricted T cells (Schaible *et al.*, 1999), NK cells and to a lesser extent by macrophages (Fultz *et al.*, 1993). IFN-γ

exerts important effects on both monocyte/macrophages and lymphocytes, which generally result in macrophage activation and T cell differentiation towards a Th1 type of immune response (Nathan *et al.*, 1984; Gajewski and Fitch, 1988).

TB infection in mice in which the gene encoding IFN-γ was disrupted, was not contained and infected animals died sooner from the infection than the control mice. IFN-γ KO mice developed neither mature granulomas nor protective immunity after experimental infection with *M. tuberculosis* Erdman strain, whereas administration of recombinant IFN-γ prolonged their survival (Cooper *et al.*, 1993; Flynn *et al.*, 1993). The injection of IFN-γ into mice lead to an increase in resistance against subsequent lethal challenge with *M. tuberculosis* and *M. bovis* (Denis, 1991). Depletion of IFN-γ *in vivo* by anti-IFN-γ monoclonal antibody treatment enhanced the susceptibility of mice to a lethal challenge with *M. tuberculosis* (Banerjee *et al.*, 1986, Denis, 1991).

IFN-γ also enhances the secretion of several cytokines, such as TNF-α, which induces granuloma formation (Gazzinelli *et al.*, 1992), and IL-12, which enhances the cell's cytotoxic activity (reviewed in Trinchieri, 1995). IFN-γ also maintains the expression of functional IL-12 receptors on Th cells (Szabo *et al.*, 1995). In addition IFN-γ inhibits macrophage secretion of IL-10 (Mosmann, 1994).

3.1.2. IL-12

IL-12 is held to be a marker of active disease in pulmonary TB (Taha *et al.*, 1997). The main source of IL-12 during infection is the macrophage that is stimulated by the phagocytic process (Fulton *et al.*, 1996) and by the presence of TNF-α and IFN-γ in the local environment (Flesch *et al.*, 1995). In mice in which the IL-12 molecule had been knocked out by gene disruption, the infection expanded progressively in the lungs, and the granuloma response was negligible (Cooper *et al.*, 1997a). IL-12 mediates the initiation of a protective response by

activating T cells, NK cells and NKT cells to produce IFN-γ (Lieberman and Hunter, 2002; Emoto *et al.*, 1999) and the subsequent production of TNF-α (Trinchieri and Scott, 1994; Flynn *et al.*, 1995b; Cooper *et al.*, 1997b; Emoto *et al.*, 1997). Another potential role for IL-12 lies in enhancement of cytolytic activity of $CD4^+$ T cells and NK cells against macrophages infected with *M. tuberculosis* (Boom *et al.*, 1992; Denis, 1994).

3.1.3. TNF-α

TNF-α is another important known mediator involved in the protective response to mycobacteria. This cytokine is generally considered to be a product of activated macrophages, but is also secreted by lymphocytes (Tracey and Cerami, 1993).

The critical role of TNF-α is not only in the activation of T cells and macrophages but also in the local organisation of granulomas by regulating leukocyte movement via triggering of production of chemokines from tissue-resident cells. TNF-α has been shown to be an important co-factor for the induction of IFN-γ by murine T cells and NK cells (Bermudez *et al.*, 1988). TNF-α induces the production of several macrophage products such as GM-CSF, which shows microbicidal activity, and NO (reviewed in Tracey, 1994). TNF-α is one of the well-characterised mediators that sensitises *M. tuberculosis*-infected macrophages to undergo apoptosis (Rojas *et al.*, 1999; Keane *et al.*, 1997). Apoptosis is induced by the production of NO after TNF-α stimulation. Apoptosis of *M. tuberculosis*-infected macrophages functions as a protection mechanism against dissemination of bacterial infection (Fratazzi *et al.*, 1997; Rojas *et al.*, 1997). It has been shown that attenuated strains of *M. tuberculosis* are more potent inducers of apoptosis than the virulent strains (Keane *et al.*, 1997) and that macrophages from mice resistant to mycobacterial infection are more susceptible to *M. tuberculosis*-induced apoptosis (Rojas *et al.*, 1997).

In TNF-α KO mice aerosolised with *M. tuberculosis*, the granulomas in the lungs were enlarged, diffuse structures with extensive regions of necrosis and neutrophilic infiltration in the alveoli where bacilli were neutralised inefficiently, as compared to the well-structured T cell and macrophage accumulations found in wild-type (WT) mice. Lymphocytes were restricted to the perivascular and peribronchial areas rather than co-located with macrophages in granulomas in TNF-α KO mice (Bean *et al.*, 1999).

In WT mice, anti-TNF-α antibody treatment enhanced the susceptibility to the mycobacteria. The addition of anti-TNF-α monoclonal antibodies resulted in inhibited NO production and impaired apoptosis (Aung *et al.*, 1996; Rojas *et al.*, 1997) and also prevented the formation of granulomas. This was accompanied by massive replication of bacilli (Bermudez and Kaplan, 1995). In contrast, WT mice treated with recombinant-TNF-α (r-TNF-α), showed increased resistance to *M. tuberculosis* infection, associated with limited intracellular growth of the bacilli (Aung *et al.*, 1996).

3.1.4. GM-CSF

The hematopoietic system is capable of rapid expansion and maturation of specific cells necessary for launching a localised inflammatory response. Activated Th cells and activated macrophages regulate this inducible hematopoietic activity. They secrete a number of cytokines that stimulate proliferation and differentiation of different white blood cells involved in the immune response. Among these cytokines is GM-CSF. GM-CSF acts directly on bone marrow cells and stimulates the expansion of granulocytes and macrophages (Boom, 1996).

Murine CD1 expression can be up-regulated by GM-CSF with IL-4 acting synergistically with GM-CSF (Mandal *et al.*, 1998). Other cytokines such as IFN-γ, IL-1 and TNF-α do not affect CD1 expression. The same applies to IL-4 when given alone (Kasinrerk *et al.*, 1993; Porcelli *et al.*, 1992; Porcelli, 1995).

Strong expression of GM-CSF was found in tuberculous granulomas (Bergeron *et al.*, 1997), suggesting that the infection may contribute to CD1 protein expression.

Similar to the effects of IFN-γ (Rook *et al.*, 1986; Flesch and Kaufmann, 1987), GM-CSF has been shown to enhance macrophage antimicrobial activity in both the mouse and human system (Weiser *et al.*, 1987; Reed *et al.*, 1987), which represents one of the major protective effector functions against mycobacterial infections (Kaufmann, 1995).

3.1.5. IL-10

IL-10 is primarily produced by macrophages in response to infection with *M. tuberculosis* (Barnes *et al.*, 1993; Tsukaguchi *et al.*, 1999; Othieno, 1999) and is also produced in smaller quantities by Th2 lymphocytes. IL-10 is an immunosuppressive cytokine that down-regulates Th1 activity through macrophage deactivation and the blocking of IFN-γ release by Th1 lymphocytes (Bogdan *et al.*, 1991; Koppelman *et al.*, 1997; Gazzinelli *et al.*, 1992; Murray *et al.*, 1997; Hsu *et al.*, 1995). IL-10 inhibits macrophage activity by inhibiting TNF-α and NO production (Bogdan *et al.*, 1991). IL-10 promotes secretion of soluble TNF receptor 2 (TNFR-2) (Dickensheets *et al.*, 1997). TNFR-2 forms an inactive complex with TNF-α and thus neutralises the activity of TNF-α (Balcewicz *et al.*, 1998). Macrophages isolated from IL-10-deficient mice overproduce cytokines, prostaglandins and NO following stimulation (Murray and Young, 1999). Thus IL-10 appears to play a general role as regulator of inflammation, fine-tuning strong responses to pathogens or antigenic stimulation.

3.1.6. IL-4

IL-4 is considered to be a prototypical Th2 cell cytokine. Mice lacking IL-4 exhibit a deficiency in Th2 responses, indicating that IL-4 itself is necessary for Th2 development (Kaplan *et al.*, 1996; Kopf *et al.*, 1993; Kuhn *et al.*, 1991; Shimoda *et al.*, 1996; Takeda *et al.*, 1996). Depressed protective responses have been ascribed to IL-4. It is involved in down-regulating and opposing development of a Th1-type cell response by inhibiting Th0 to Th1 development. Its effect is linked to down-regulation of secretion of IFN-γ and enhanced production of antibodies, thus affecting a switch between cellular and humoral immunity (Mosmann and Coffmann, 1989a). Although antibodies to various antigens of the tubercle bacillus exist in all cases of TB, B cells are thought to be irrelevant for the control of mycobacterial growth as immune serum does not consistently transfer any protection against infection with *M. tuberculosis* (Chan and Kaufmann, 1994; Glatman-Freedman and Casadevall, 1998).

3.2. Hypotheses

1. MA (25 μg) protects *i.v.* and *i.n. M. tuberculosis*-infected Balb/c mice against tuberculosis.
2. The protective effect of MA is more pronounced when *M. tuberculosis* infection is effected intranasally rather than intravenously.
3. MA as such can induce cytokine expression in the lungs of Balb/c mice.
4. The protective effect of 25 μg MA correlates with a protective Th1 cytokine profile in the lungs of Balb/c mice infected intranasally with *M. tuberculosis*.

3.3. Materials

3.3.1. Cultures

M. tuberculosis H37Rv ATCC 27294 - a virulent strain, originally isolated from an infected human lung. The culture was purchased in lyophilised form from the American Type Culture Collection (ATCC), Maryland, USA.

3.3.2. Media

The following media were used for the cultivation of *M. tuberculosis*:

Löwenstein-Jensen (LJ) medium (slants) (MRC, Pretoria, R.S.A.) and Middlebrook 7H-10 agar medium (plates) (Difco, U.S.A).

A detailed composition of the ingredients necessary for the preparation of the media as well as the conditions recommended for their sterilisation, are given in the Laboratory Manual of TB Methods, TB 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 TB Institute of the Medical Research Council of South Africa, in Pretoria. The sterility of all the media was confirmed by eye, before they were used in the experiments, after incubating them at 37 ^{0}C for 24 h.

3.3.3. Experimental animals

Six weeks old female Balb/c (a TB-susceptible strain) mice were used. The South African Vaccine Producers in Johannesburg inbred the mice for 11 generations. Female Balb/c mice of corresponding age were used for the collection of serum necessary for the preparation of MA/mouse serum conjugates. The animals were maintained at the Animal Facilities of the Medical Research Council in Pretoria. Mice were kept in a glove isolator in a temperature and humidity controlled room.

3.3.3.1. Cages

Mice were maintained under positive pressure in a glove isolator manufactured by Labotec, South Africa. 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.

Mice were housed in groups of eight in transparent polypropylene cages of 450 cm^2 with tight fitting stainless steel lids. Wooden shavings, after autoclaving, were provided as nesting material.

Temperature and humidity were set at 20 °C (+/-1 °C) and 40% (+/-10 %), respectively. Lighting was provided by means of fluorescent tubes. A light-dark cycle of alternating 12 hour periods was set up.

3.3.3.2. Feed and water

Mice cubes manufactured by EPOL and autoclaved tap water were provided *ad libitum*.

3.3.3.3. Sanitation

Animal rooms, mouse cages, the glove isolator and water bottles were cleaned and decontaminated once a week using Bronocide (Essential Medicines, R.S.A.). Water bottles were autoclaved after washing.

3.3.3.4. Identification of the experimental animals

Individual identification of mice was accomplished by making earmarks.

3.3.4. Reagents

3.3.4.1. Saponification, extraction, derivatisation and high-performance liquid chromatography (HPLC) analysis of MA

Potassium hydroxide (Saarchem, RSA, Analytical Grade)

Methanol (BDH, UK, HPLC Grade)

Hydrochloric acid (Saarchem, RSA, Analytical Grade)

Potassium bicarbonate (BDH, UK, Analytical Grade)

Para-bromophenacylbromide dissolved in acetonitrile and crown ether (Pierce Chemical Co, Illinois USA)

High Molecular Weight Internal Standard (C-100) (Ribi ImmunoChem Research Company, Hamilton, MT.)

Chloroform (Saarchem, RSA, Analytical Grade)

Methylene chloride (BDH, UK, HPLC-Grade)

Methanol (BDH, UK, HPLC grade)

Double distilled deionised water prepared in a Milli-Q^{TM} reagent-grade water system.

3.3.4.2. Preliminary countercurrent purification of extracted MA from crude bacterial extracts

Refer to Goodrum *et al.* (2001).

3.3.4.3. Washing and diluting of mycobacteria

NaCl (Saarchem, RSA, Chemically Pure)
Tween 80 (Merck, RSA, Chemically Pure)

3.3.4.4. Semi-quantitative RT-PCR

Ethidium bromide (Boehringer Mannheim, Germany)

Formamide and formaldehyde (BDH, UK)

Tris (Hydroxymethyl)-aminomethane (Merck, Germany)

EDTA (Merck, Germany)

Sodium acetate (Merck, Germany)

TRI-reagent (Molecular Research Centre Inc, USA)

Formazol (Molecular Research Centre Inc, USA)

MOPS (3-(*N*-morpholino) propanesulfonic acid) (Sigma Chemicals, USA)

Diethyl pyrocarbonate (DEPC) (Sigma Chemicals, USA)

RNasin (Promega, Madison, WI, USA)

Oligo (dT) primers (Life Technologies Inc., Scotland)

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

Amplitaq Gold (Roche Molecular Systems, USA)

Tris-borate/EDTA (TBE) buffer: 0.089 M Tris-(Hydroxymethyl)-aminomethane (Merck, Darmstadt, Germany), 0.089 M boric acid (Merck, Darmstadt, Germany), 0.002 M ethylenediaminetetra-acetic acid (EDTA) (Merck, Darmstadt, Germany) (pH 8.00)

Agarose (Promega, USA)

3.3.4.5. Reagents used for the preparation of organ homogenates

NaCl (Saarchem, Chemically Pure)
Tween 80 (Merck, Chemically Pure)

3.3.4.6 Decontamination of blood samples and organ homogenates

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

i) Sodium hydroxide pellets (4 g) (Merck, Germany) in 100 ml distilled water;

ii) Sodium citrate (2.9 g) (Merck, Germany) in 100 ml distilled water.

After autoclaving at 121 °C for 15 minutes, both solutions were mixed and 1.0 g N-acetyl-L-cysteine powder (Becton Dickinson and Company, U.S.A.) was added.

Phosphate buffer 0.15 M, pH 6.8:

iii) Anhydrous Na_2HPO_4 (9.47 g) (Merck, Germany) in 1000 ml distilled water;
iv) Anhydrous K_2HPO_4 (9.07 g) (Merck, Germany) in 1000 ml distilled water.

Solutions (iii) and (iv) (50 ml of each) were mixed and the pH was adjusted to pH 6.8 by using solution (iii) to raise the pH or solution (iv) to lower it.

3.4. Methods

3.4.1. Preparation of MA

M. tuberculosis-derived MA were extracted, purified and derivatised as described by Butler *et al.* (1991) and purified according to Goodrum *et al.* (2001). Briefly, bacteria were scraped from LJ slants and re-suspended in 25 % potassium hydroxide in methanol-water (1:1). The suspension was homogenised by vortexing in the presence of sterile glass beads. Prior to the saponification, the density of the bacterial suspensions was adjusted to a density corresponding to a McFarland standard 4. The cells were subsequently saponified in an oven at 70 °C, for 16 hours. After allowing the sample to cool, MA were extracted by adding 1.5 ml 50 % (v/v) HCl per 2 ml sample to adjust the pH to 1. The lower phase was transferred to a roundbottom flask, after chloroform-water (1:1) extractions. The chloroform was evaporated on a Buchi rotary evaporator under vacuum at 80 °C.

Aqueous potassium carbonate (K_2CO_3, 2 %) was added (100 µl per 2 ml 25 % potassium hydroxide in methanol-water (1:1) initially used) and the mixture dried at 80 °C on a Buchi rotary evaporator under vacuum. The material obtained from the large-scale extraction *i.e.* the crude bacterial extract, was stored dry, at 4 °C in 4 ml amber WISP vials. This crude saponified extract was used to purify MA in a biphasic, tri-component solvent system consisting of 42 % (v/v) chloroform, 39 % (v/v) methanol and 19 % (v/v) 0.2 M NaCl by countercurrent separation as described by Goodrum *et al.* (2001). Purity analysis was done by HPLC after the MA sample was derivatised by the addition of para-bromophenacylbromide in acetonitrile and crown ether (100 µl per 2 ml 25 % potassium hydroxide in methanol-water (1:1) initially used). In order to increase the accuracy of the HPLC determination of MA, the High Molecular Weight Internal Standard (C-100) was introduced into the countercurrent-purified MA before saponification. A sample of 0.5 mg of the countercurrent-purified MA was introduced into a WISP vial containing 5 µg of the High Molecular Weight Internal Standard (C-100). After saponification and extraction with the same reagents as described previously, but starting with only 2 ml 25% potassium hydroxide in methanol-water (1:1), 100 µl para-bromophenacylbromide was added. 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 lower phase 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 against the internal standard.

3.4.2. Serum preparation

To obtain serum, mice were bled from the tail vein and the blood collected into sterile Eppendorf tubes. The collected blood was incubated at 37 ^{0}C for one hour and then left at 4 ^{0}C overnight for the clot to retract. The serum was recovered by centrifugation (in a Beckman J-6 centrifuge, at 1000 g for 15 min), pooled and aliquoted in volumes of 1.0 ml and stored frozen at -70 °C until used.

3.4.3. Immunisation of mice with MA-serum conjugate

Six week old female Balb/c mice were immunised with only serum or with MA-serum conjugate one week before *i.n.* or *i.v.* infection with *M. tuberculosis*. 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. 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. Control serum was prepared by adding 2 % pure chloroform (v/v) to the serum before the mixture was sonicated as described for the MA-serum conjugate. In order to remove chloroform, nitrogen was blown over the surface of the conjugate mixture until no chloroform odour could be detected. The sample was maintained for 1 hour at room temperature to allow air bubbles formed during sonication to escape before it was injected into the mice.

The MA-serum conjugate containing 25 µg MA was prepared by the sonication method as described above. The 5 µg MA-serum conjugate was prepared by dilution of the 25 µg MA-serum conjugate using chloroform-treated mouse serum. To verify the presence of MA in the mouse serum conjugate, HPLC analysis was performed.

3.4.4. Infection of mice with *M. tuberculosis*

M. tuberculosis H37Rv was cultivated at 37 °C on LJ medium slants for 2 weeks. The cells of *M. tuberculosis* H37Rv, harvested from LJ slants, were suspended in the diluting buffer (0.01 % v/v Tween 80 in 0.9 % m/v NaCl) and homogenised. 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 (approximately OD of 1.0; using a Beckman DU 65 spectrophotometer, at 486 nm). After the confirmation

of the total direct bacterial count in an autoclaved suspension using 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 10^4 – 10^6 cells/ml. Bacterial viable counts of the samples were determined by counting CFU in 1:10 and 1:1 dilutions on Middlebrook 7H-10 agar after incubation at 37 °C for two to three weeks.

Hundred thousand (10^5) *M. tuberculosis* cells were introduced into mice, 1 week after the administration of the MA-serum conjugates. The infection was performed in a biosafety cabinet class III in the PIII facilities at the TB Institute of Medical Research Council in Pretoria. In the *i.n.* infected group of mice, the bacterial suspensions prepared in sterile saline were introduced into the nostrils of mice anaesthetized with 5 % diethylether, 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. Prior to the introduction of the bacterial suspensions via *i.v.* route, the mice were heated for 5 minutes in a heating box until vasodilation of the tail veins could be observed. Once this was observed, the respective bacterial suspensions were introduced in aliquots of 100 µl per mouse. Control animals received an equivalent volume of sterile saline, *i.e.*, 60 µl introduced *i.n.* or 100 µl administered *i.v.*

3.4.5. Experimental set-up

Balb/c mice were divided into eight groups and treated as indicated in Table 3.1.

Table 3.1: Experimental set-up for the pretreatment of Balb/c mice with MA before *i.v.* or *i.n.* infection with *M. tuberculosis.*

Group No.	Infection	MA administration	Mice per group
1	Saline only, *i.n.*	Serum only, *i.v.*	10
2	*M. tuberculosis, i.n.*	Serum only, *i.v.*	15
3	*M. tuberculosis, i.n.*	5 µg MA, *i.v.*	15
4	*M. tuberculosis, i.n.*	25 µg MA, *i.v.*	15
5	Saline only, *i.n.*	25 µg MA, *i.v.*	15
6	*M. tuberculosis, i.v.*	Serum only, *i.v.*	10
7	*M. tuberculosis, i.v.*	5 µg MA, *i.v.*	10
8	*M. tuberculosis, i.v.*	25 µg MA, *i.v.*	10

The animals used in experiments for the cultivation of mouse organs to test for the presence of *M. tuberculosis* were withdrawn from the experiment, 14 and 35 weeks after infection with *M. tuberculosis* H37Rv. Five weeks after *M. tuberculosis* infection, 5 mice of each *i.n.* infected group were sacrificed and the required organs were removed and analysed for the presence of various cytokines/interleukins. The remaining mice were retained for survival studies.

3.4.6. Assessment of pathology of the experimental animals

3.4.6.1. Mass

Individual mass measurements of all the experimental animals were carried out at seven-day intervals, at the same time of the particular day. These measurements were carried out using a Sartorius electronic scale (with a range of 0.00 - 200.00 g and accuracy of 0.01 g) and a plastic beaker to contain the mice.

3.4.6.2. Organ Indexes

The organ index gives an indication of the relation of the mass of the removed organs to the mass of the mouse at the particular time-point. 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. The approximate number of CFU present in the isolated organ was calculated by multiplying the number of CFU obtained for 1 mg of the relevant organ homogenate by the total mass of the organ. The plates were examined and counted after 24 days and 28 days incubation at 37 °C. Two types of organ homogenates, *i.e.,* with or without decontamination were plated for lungs and spleen extracted 14 weeks after infection with *M. tuberculosis* for cultivation to verify the presence of *M. tuberculosis*. One mouse of each group was sacrificed 14 weeks after *M. tuberculosis* infection. The lungs and spleens removed 35 weeks after *M. tuberculosis* infection were decontaminated prior to the preparation of ten fold dilutions and plating. Six mice were sacrificed after 35 weeks: two mice from the untreated *i.n.* infected group and four mice from the group pretreated with 25 µg MA before *i.n.* infection.

3.4.7. Preparation of organ homogenates

To compare the spread of *M. tuberculosis* in the organs of Balb/c mice immunised with MA and infected *i.n.* or *i.v.* with *M. tuberculosis* H37Rv, the number of mycobacteria present in the blood, lungs and spleen of mice, selected at random from the different experimental groups, was determined.

The specimens were prepared as follows:

i) The animals were bled and the blood was introduced into sterile tubes with heparin. 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 ^{0}C.

ii) The animals were sacrificed by cervical 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.

iii) The spleens and the lungs were transferred into sterile Potter Elvejhem homogenizers into which aliquots of 300 µl sterile saline were introduced. After homogenation, the suspensions were diluted with the sterile saline to reach a concentration of 1 mg wet organ tissue/ml saline.

iv) The homogenates were divided into two equal parts. The first part was diluted in the diluting medium. For the determination of the concentration of *M. tuberculosis* in various mouse organs, serial dilutions of the organ

homogenates were made in 0.01 % v/v of Tween 80 (Merck, Chemically Pure) prepared in 0.9 % m/v NaCl (Saarchem, Chemically Pure). The diluent was distributed in 9.0 ml aliquots into test-tubes, autoclaved and stored at 4 ^{0}C. The second part was decontaminated according to the method specified in section 3.4.8. Serial dilutions were prepared in the diluting medium.

v) In the course of these experiments the following dilutions of blood samples and organ homogenates were prepared:

blood : undiluted, 10^{-1} and 10^{-2} dilutions

lungs (direct and decontaminated homogenate): undiluted, 10^{-1}; 10^{-2} and 10^{-3} dilutions

spleens (direct and decontaminated homogenate): undiluted, 10^{-1}; 10^{-2} and 10^{-3} dilutions

vi) From the undiluted blood samples and from the 1 mg/ml non-decontaminated and decontaminated organ homogenates aliquots of 100 µl were plated onto Middlebrook 7H-10 agar plates. Sterile glass rods were used for spreading the homogenate suspensions and blood on the surface of agar plates. Duplicate Middlebrook 7H-10 agar plates were prepared for each dilution by evenly spreading the introduced aliquots with the sterile glass rods. Each tube with diluted organ homogenate or blood sample was vortexed prior to plating.

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

3.4.8. Decontamination of blood samples and organ homogenates

Decontamination of blood samples and of spleen and lung homogenates was performed as follows:

Aliquots (1 ml) of the blood samples or spleen or lung homogenates were introduced into 10 ml centrifuge tubes. Into each tube an equal volume of N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) was added. Caps on the test tubes were tightened securely and the contents were 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 1.0 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. The samples were diluted and applied to Middlebrook H-10 agar plates as described above.

3.4.9. Methods used in semi-quantitative RT-PCR determination

3.4.9.1. Preparation of the lungs used for RNA extraction

The lungs originating from both infected and uninfected mice were used for the RNA extraction experiments. Mice were sacrificed by rapid cervical dislocation. The lungs were aseptically removed from each mouse and kept at -72 °C after snap freezing in liquid nitrogen.

3.4.9.2. RNA extraction from control and infected organs

RNA was isolated from the lungs using the TRI-Reagent protocol based on an acid guanidium thiocynate-phenol-chloroform extraction, a method first developed by Chomczynski and Sacchi (1987). The isolated RNA was quantified using a Shimadzu UV-Visible Recording Spectrophotometer model UV-160, at a wavelength of 260 nm. Integrity of the isolated RNA was determined using a denaturing formaldehyde gel (Maniatis, 1982). These denaturing conditions prevent degradation of the RNA by RNases. The water used was DEPC-treated. Ethidium bromide was added to 3 µg of RNA sample, before it was loaded on the gel, at a concentration of 0.5 ng/ml. The ethidium bromide intercalates into the deoxyribonucleic acid (DNA) and can be visualised with UV light. Pure, undegraded RNA gives three rRNA bands (the 28S rRNA, 18S rRNA and the 5S rRNA) on agarose gel electrophoresis. Only completely intact RNA was used for the reverse transcriptase reaction (Maniatis, 1982).

3.4.9.3. The reverse transcriptase reaction (RT-reaction)

In the RT-reaction all mRNA of the total RNA sample is reverse transcribed to ss cDNA. Before the RT reaction 5 µg total RNA (5 µg) was co-precipitated overnight at –20 °C with 3 pmol Oligo (dT)12-18 using 0.1 M NaOAc, pH 5.5 and absolute ethanol (1:2). After centrifugation the pellet was washed with 70% ethanol in 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), heated to 70 °C for 10 minutes, followed by a 3 hour incubation at 37 °C. The RT reaction was performed with Superscript™ RNase H reverse transcriptase as recommended by the manufacturer. Total RNA (5 µg) and the oligo(dT) primer were heated to 70 °C for 10 min to ensure RNA denaturation. The reaction mixture was cooled on ice to enable annealing of the oligo(dT) primer. The dNTPs necessary for the elongation of the cDNA were added. The Amplitaq Gold DNA polymerase enzyme was added after the mixture was heated to 37 °C. The

reaction was terminated after one hour incubation at 37 °C by heat inactivation of the enzyme at 75 °C for 2 min.

3.4.9.4. β-actin and Cytokine PCR

The reaction mixture for PCR of the cytokines had a final volume of 20 µl. Several different volumes of cDNA were assayed for each sample, to reach non-saturating concentrations of the amplification product in the RT-PCR. The objective was to pinpoint the volume of cDNA required to match a particular amplified cytokine relative to a reference product with linear regression within the non-saturated area of each sample. The "amount of cytokine/x µl RT" was then converted to the "amount of cytokine/1 µl". The mRNA for each cytokine was then normalised against β-actin gene expression, to standardise results with respect to the initial RNA yield. In order to establish an mRNA correction factor, the β-actin expression of a chosen sample was set equal to 1 and the unit value obtained for each of the remaining specimens was divided by the unit value for this specific sample. This resulted in a mRNA correction factor for the specimen series (based on the β-actin transcript level) of each specimen. Then the unit value of cytokine transcript expression for each specimen was divided by the mRNA correction factor, resulting in a normalized value for the expression of each cytokine for each specimen. All of the RNA specimens from each animal were reverse transcribed at the same time using the same RT reagents and all the PCR amplifications for each cytokine were performed at the same time using the same PCR reagents.

The reaction mixture used for the different cytokines appears in Table 3.2:

Table 3.2: Cytokine reaction mixtures.

Cytokine	Magnesium Chloride	Deoxyribo-nucleotides	Forward Primer	Reverse Primer	Amplitaq Gold Enzyme
β-actin	2 mM	0.2 mM each	5 ng/µl	5 ng/µl	0.04 U/µl
IL-12	1.5 mM	0.2 mM each	5 ng/µl	5 ng/µl	0.04 U/µl
IFN-γ	1.5 mM	0.2 mM each	12.5 ng/µl	12.5 ng/µl	0.04 U/µl
TNF-α	1.5 mM	0.2 mM each	12.5 ng/µl	12.5 ng/µl	0.04 U/µl
IL-10	1.5 mM	0.2 mM each	6.25 ng/µl	6.25 ng/µl	0.04 U/µl
IL-4	1.5 mM	0.2 mM each	12.5 ng/µl	12.5 ng/µl	0.04 U/µl
GM-CSF	2 mM	0.2 mM each	5 ng/µl	5 ng/µl	0.04 U/µl

The heat stable Amplitaq Gold enzyme was added before the PCR was initiated by an incubation step for 10 min at 94 °C. At this temperature, the enzyme becomes activated.

After the enzyme was activated, three cycles consisting of steps of:

- 45 sec at 94 °C
- followed by 75 sec at x °C and
- 105 sec at 72 °C

were run to initiate the synthesis of the second cDNA strands.

The subsequent amplification cycle consisted of the following shorter steps:

- 35 sec at 94 °C,
- 45 sec at x °C and
- 75 sec at 72 °C.

This cycle was repeated an exact number of times, optimised for each different cytokine.

The primer sequences, annealing temperatures and number of cycles used for each cytokine appear in Table 3.3:

Table 3.3: Primer sequences, annealing temperatures and PCR cycles.

Cytokine	Primer Sequence	Fragment Size	Annealing Temperature x °C	PCR Cycles y
β-actin[1]	Forward: 5'–CTCCATCGTGGGCCGCTCTAG–3'	133 Base Pairs	60 °C	27
	Reverse: 5'-GTAACAATGCCATGTTCAAT–3'			
IL-12[2]	Forward: 5'–CCACTCACATCTGCTGCTCCACAAG–3'	266 Base Pairs	60 °C	33
	Reverse: 5-ACTTCTCATAGTCCCTTTGGTCCAG–3'			
IFN-γ[4]	Forward: 5'–CATTGAAAGCCTAGAAAGTCTG–3'	267 Base Pairs	60 °C	28
	Reverse: 5-GCTTTTTCCTACGTAAGTACTC–3'			
	Reverse: 5-CACCTAGGTGCTCGGCTTCCC–3'			
TNF-α[3]	Forward: 5'–GTCTACTTTAGAGTCATTGC–3'	275 Base Pairs	48 °C	28
	Reverse: 5-GACATTCGAGGCTCCAGTG–3'			
IL-10[4]	Forward: 5'–CCAGTTTTACCTGGTAGAAGTGATG–3'	324 Base Pairs	60 °C	37
	Reverse: 5-AACTCAGACCTGAGGTCCTGGATCTGT–3'			
IL-4[4]	Forward: 5'–CATCGGCATTTTGAACGAGGTCA–3'	240 Base Pairs	60 °C	36
	Reverse: 5-CTTATCGATGAATCCAGGCATCG–3'			
GM-CSF[3]	Forward: 5'–TTCCTGGGCATTGTGGTCT–3'	411 Base Pairs	67 °C	34
	Reverse: 5-TGGATTCAGAGCTGGCCTGG–3'			

1. Ma *et al.*, 1994
2. Chong *et al.*, 1996
3. Benavides *et al.*, 1995
4. Reiner *et al.*, 1993

Negative control samples, *i.e.* all the reagents described above except cDNA, were run with each cytokine PCR.

PCR products were assessed as described in 2.4.2.

3.4.9.5. Statistical analysis

Log-rank tests of survival data were performed using the STATA-6 statistical software package. The cytokine mRNA production data and the mycobacterial counts were analysed using Student's t-test. P-values of 0.05 to 0.1 were regarded as marginally significant with P-values below 0.05 as significant. Differences within the different experimental groups were determined by calculating the mean and the standard deviation (STD) of the different individual samples in the experimental group.

3.5. Results

3.5.1. Bacterial suspension and MA-serum conjugates

The dose of *M. tuberculosis* introduced into the infected animals was 2.5 x 10^5 CFU/mouse. An attempt was made to administer 25 or 5 µg MA to mice as pretreatment according to the results of Pretorius (1999). It was established with HPLC that the aimed dose of 25 µg MA serum conjugate per mouse comprised 26.0 ± 2.4 µg MA whereas the aimed dose of 5 µg MA serum conjugate per mouse comprised 7.2 ± 1.4 µg MA.

3.5.2. Survival study and recorded masses of experimental animals

The survival rate of Balb/c mice infected *i.v.* or *i.n.* with 2.5 x 10^5 CFU *M. tuberculosis* per mouse after *i.v.* immunisation with 0, 5 or 25 µg MA is shown in Figure 3.1.

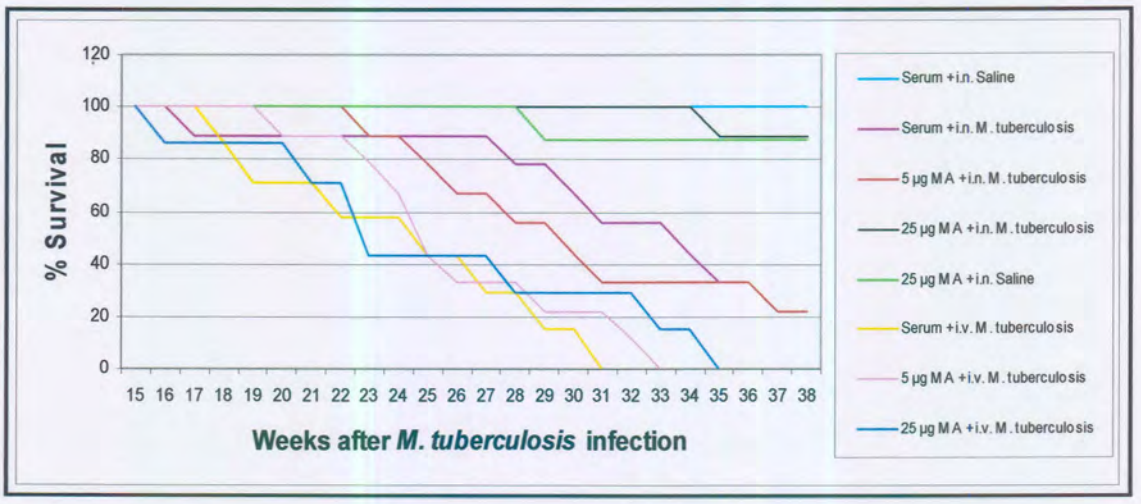


Figure 3.1: Survival rate of Balb/c mice infected *i.v.* or *i.n.* with 2.5 x 10^5 CFU *M. tuberculosis* per mouse after *i.v.* immunisation with 5 or 25 µg MA.

The protective effect of 25 µg MA observed with *i.v. M. tuberculosis* infection was not as pronounced as previously observed (Pretorius, 1999). In previous *i.v.* infected Balb/c mice experiments the protection provided by 25 µg MA lasted for ± 30 weeks, after which the mice started to get sick and died. In this experiment *i.v.* infected mice pretreated with 25 µg MA already started dying after 15 weeks. The protective effect of 25 µg MA was not evident in this experiment since there was no significant difference in the survival curves of the *i.v.* infected mice receiving no treatment or 5 or 25 µg MA. Whereas the *i.n.* infected mice

pretreated with 25 µg MA showed a significant increase in survival (P=0.0039) with the 5 µg MA showing no significant increase in survival.

M. tuberculosis also seemed to be less virulent via the *i.n.* route than via the *i.v.* route in this experiment (P=0.0063). Mice infected *i.v.* with *M. tuberculosis* died earlier in this experiment than mice that received *i.n.* infection.

Administration of 25 µg MA increased the resistance of *i.n.* infected mice to disease when compared to placebo pretreated *i.n.* infected mice and *i.n.* infected mice pretreated with 5 µg MA. After a period of 38 weeks, 8 of 9 mice of the *i.n.* infected mice pretreated with 25 µg MA survived as opposed to only 2 of 9 mice of the placebo pretreated *i.n.* infected mice and *i.n.* infected mice pretreated with 5 µg MA. Although the non-infected mice immunised with 25 µg MA were not infected, one mouse died after 35 weeks. It was not possible to assess whether the mouse died of TB since the mouse was eaten by the others. Mice infected with *M. tuberculosis* usually lose about 5 g body mass before dying. This mouse only lost 1 g before dying; therefore this mouse probably died due to some cause other than TB. Progressive weight loss is characteristic of TB (Dannenberg, 1994). As shown in Figure 3.2, the average mass loss pattern of mice in the different experimental groups correlated with the pattern of survival. In particular, the non-infected groups clustered together as the best growers, followed by the intranasally infected groups. The intravenously infected groups clustered as the poorest performers in respect of weight gain. Pretreatment with 25 µg MA followed by intranasal infection made the group score almost with the cluster of the uninfected groups.

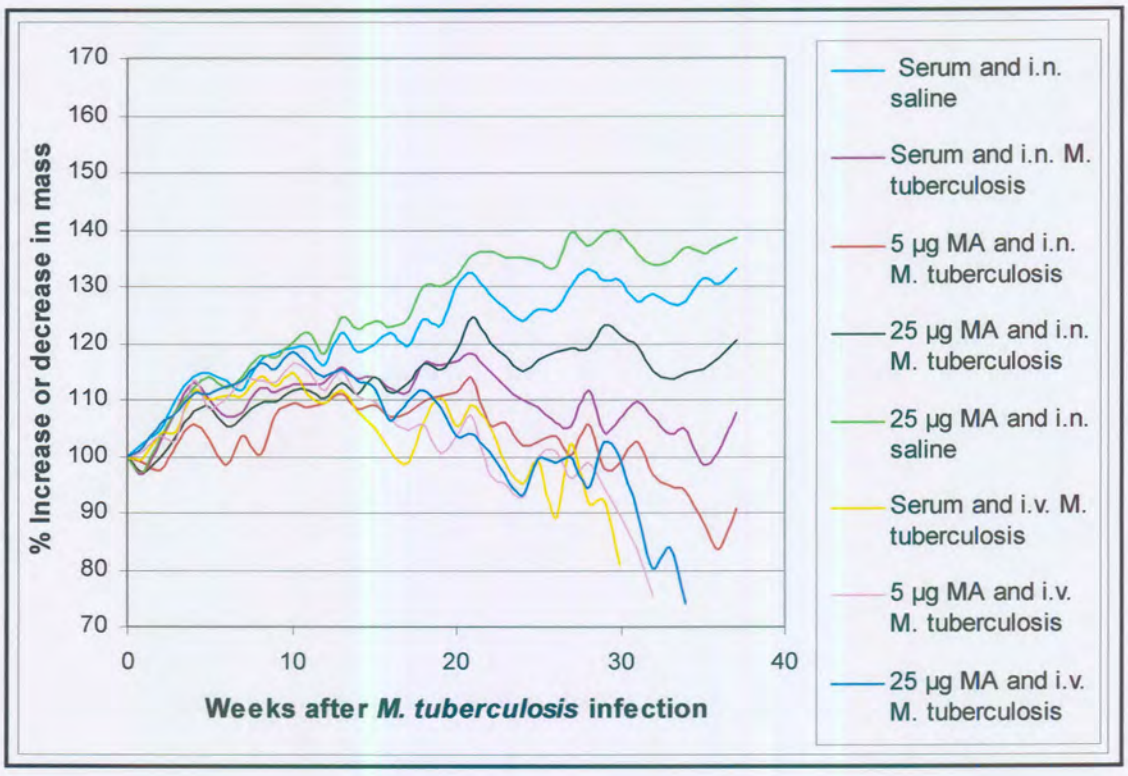


Figure 3.2: Average mass loss of Balb/c mice over a period of 35 weeks, pretreated with MA before infection *M. tuberculosis* infection.

3.5.3. Mycobacterial counts of organs removed for cultivation to verify the presence of *M. tuberculosis*

In order to compare the effect of the route of administration of mycobacteria and the contribution of pretreatment with MA on the dissemination of *M. tuberculosis* in Balb/c mice, randomly selected mice were sacrificed 14 and 35 weeks after the *i.n.* or *i.v.* infection with *M. tuberculosis* and their lungs and spleens analyzed for the number of mycobacteria present. The organ indexes and CFU counts are shown in Table 3.4.

Table 3.4: Colony number in lung and spleen extracts prepared 14 and 35 weeks after *M. tuberculosis* infection to verify the presence of *M. tuberculosis*.

			Lung			Spleen		
Pretreatment (*i.v.*)	**Infection (*i.v.* or *i.n.*)**	**Week after infection**	**Direct inoculation (CFU)**	**Inoculation after de-contamination (CFU)**	**Index[1]**	**Direct inoculation (CFU)**	**Inoculation after de-contamination (CFU)**	**Index[1]**
Serum	**Saline**	**14**	Negative	Negative	0.7	Negative	Negative	0.4
Serum	***i.n.***	**14**	9.7×10^{6}	8.0×10^{4}	1.6	3.1×10^{6}	7.2×10^{4}	1.0
5 µg MA	***i.n.***	**14**	2.1×10^{6}	1.9×10^{6}	1.3	1.8×10^{5}	6.2×10^{3}	0.8
25 µg MA	***i.n.***	**14**	2.7×10^{6}	4.8×10^{5}	1.8	1.1×10^{5}	1.5×10^{4}	0.8
25 µg MA	**Saline**	**14**	Negative	Negative	0.7	Negative	Negative	0.4
Serum	***i.v.***	**14**	-	3.2×10^{6}	1.8	9.2×10^{5}	2.9×10^{5}	2.1
5 µg MA	***i.v.***	**14**	2.4×10^{7}	1.0×10^{7}	2.2	9.8×10^{7}	3.2×10^{7}	3.7
25 µg MA	***i.v.***	**14**	-	8.8×10^{5}	1.6	1.5×10^{6}	3.8×10^{4}	2.2
None	**None**	**14**	Negative	Negative	0.8	Negative	Negative	0.5
Serum[2]	***i.n.***	**35**	-	1.9×10^{6}	2.64	-	4.83×10^{5}	1.38
25 µg MA[3]	***i.n.***	**35**	-	$1.0\times10^{6}\pm 7.6\times10^{5}$	1.85± 0.83	-	$4.8\times10^{4}\pm 3.6\times10^{4}$	1.21± 0.49

1. (Weight of organ (g)/weight of mouse (g)) × 100.
2. Results are the average obtained from two animals.
3. Results are the average obtained from four animals ± standard deviation.

Fourteen weeks after infection with *M. tuberculosis*, the lungs of *i.v.* and *i.n.* infected animals were similar in size but were considerably larger than those of non-infected control animals (P=0.005). The spleens isolated from the mice infected *i.n.* with *M. tuberculosis* were on average twice as large as the "control" spleens (P=0.005), but considerably smaller than the spleens isolated from the *i.v.* infected mice (P=0.025), which were five to eight times larger than the "control" spleens (P=0.005).

immunised with 25 µg MA before *i.n.* infection maintained on average their original size. The spleens of untreated, *i.n.* infected mice did not differ significantly in size from those of mice pretreated with 25 µg MA before *i.n.* infection, 35 weeks after infection with *M. tuberculosis*. Although not significant, the spleens from these mice appeared to be slightly larger on average at 35 than at 14 weeks after *M. tuberculosis* infection.

None of the lungs, spleens or blood of the animals in non-infected control groups were infected. This result confirms the absence of cross-infection between the experimental animals assigned to various groups and maintained in the same isolator. Furthermore, none of the animals from infected groups had any *M. tuberculosis* or other bacteria in their blood. This confirms the absence of any form of bacteraemia in the experimental animals.

In the present study *i.v. M. tuberculosis*-infected mice died earlier than *i.n. M. tuberculosis*-infected mice. Lung and spleen CFUs/mouse determined 14 weeks after infection with *M. tuberculosis* did not correlate with observed survival rates, but lung CFUs were determined in only one mouse per group. Bacterial loads cannot be statistically analysed since only one mouse of each group was sacrificed 14 weeks after *M. tuberculosis* infection and the standard deviation appears to be high between mice within the same group 35 weeks after infection. This implies that the usefulness of the CFU data is limited to a qualitative confirmation of infection only. The results imply that pretreatment of mice with 25 µg MA followed by *i.n.* infection with *M. tuberculosis*, did not sterilize the lung and spleen, even though the disease appeared to be brought under control.

3.5.4. Cytokine mRNA profiling in the lungs of MA pretreated Balb/c mice five weeks after *i.n.* infection with *M. tuberculosis*

With survival as the endpoint, significant protection by pretreatment with 25 µg MA was observed in Balb/c mice infected *i.n.* with *M. tuberculosis* (P=0.0039) (Section 3.5.2). To determine whether this short-term protection could be linked to a protective cytokine profile, the expression of cytokines in mice infected with *M. tuberculosis* after exposure to MA was determined by means of semi-quantitative RT-PCR.

The RNA of samples of the different experimental groups (Section 3.4.5) was found to be intact. They did not contain any DNA, since an equivalent amount of RNA of each sample amplified with β-actin primers gave no detectable products.

In all negative control reactions, cDNA templates were replaced with sterile water to check for contamination and no detectable product was generated.

The relative cytokine expression levels of each sample in the different groups are shown in Figures 3.3 to 3.7. The expression levels of different cytokines are relative and can therefore not be compared with those of other cytokines. Although cytokine mRNA was determined in 5 samples from each group, the mRNA level of some samples was too low to be detected with RT-PCR under the specified conditions. In some others, the levels of detectable cytokines were so low that the data could not be adequately interpreted with densitometry evaluation. For example, most IL-4 mRNA determinations could not be visualised due to very low levels and for those bands that were visible the obtained densitometry values did not allow accurate and reliable mathematical and statistical interpretation of the results. The use of a more sensitive technique like real-time PCR might prove to be more successful for IL-4 mRNA determinations.

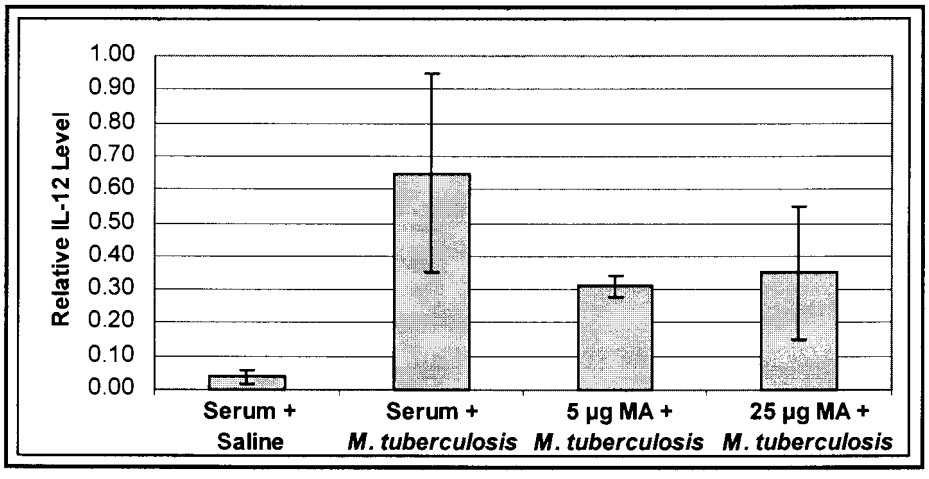


Figure 3.3: Relative IL-12 mRNA levels in the lungs of *i.n.* TB-infected Balb/c mice pretreated with MA, as assessed with semi-quantitative RT-PCR. Bars represent average values obtained from 4, 5, 4 and 5 mice respectively per group.

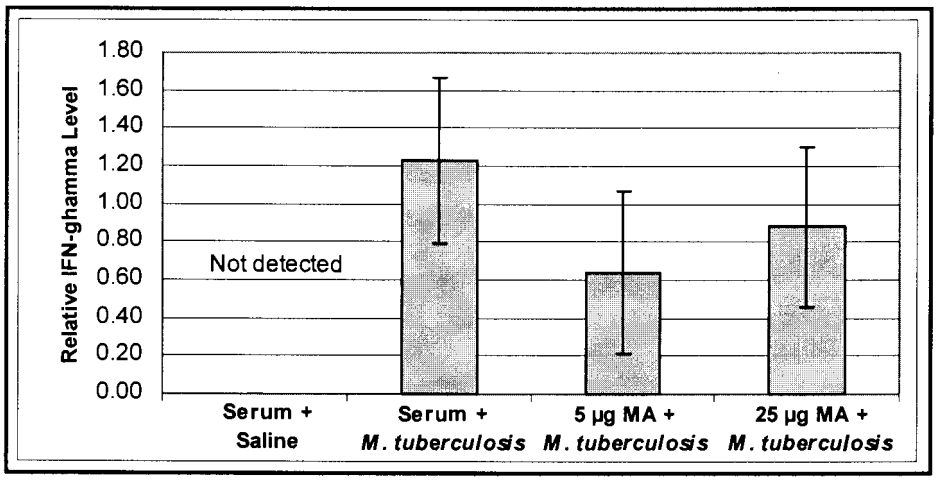


Figure 3.4: Relative IFN-γ mRNA levels in the lungs of *i.n.* TB-infected Balb/c mice pretreated with MA, as assessed with semi-quantitative RT-PCR. Bars represent average values obtained from 0, 5, 3 and 4 mice respectively per group.

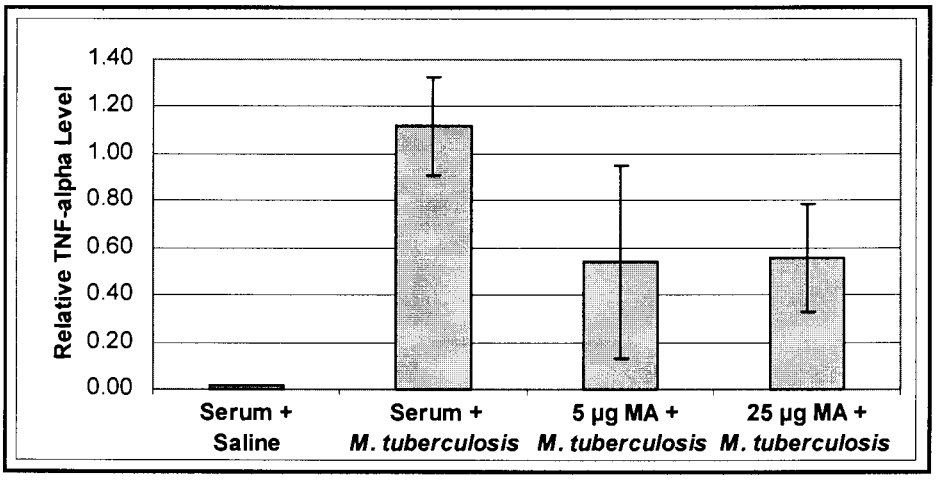


Figure 3.5: Relative TNF-α mRNA levels in the lungs of *i.n.* TB-infected Balb/c mice pretreated with MA, as assessed with semi-quantitative RT-PCR. Bars represent average values obtained from 2, 5, 5 and 5 mice respectively per group.

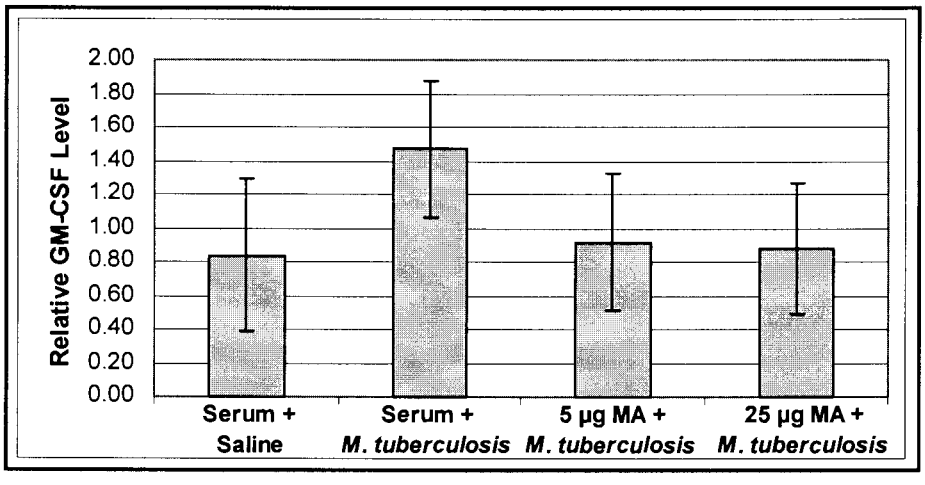


Figure 3.6: Relative GM-CSF mRNA levels in the lungs of *i.n.* TB-infected Balb/c mice pretreated with MA, as assessed with semi-quantitative RT-PCR. Bars represent average values obtained from 4, 3, 4 and 5 mice respectively per group.

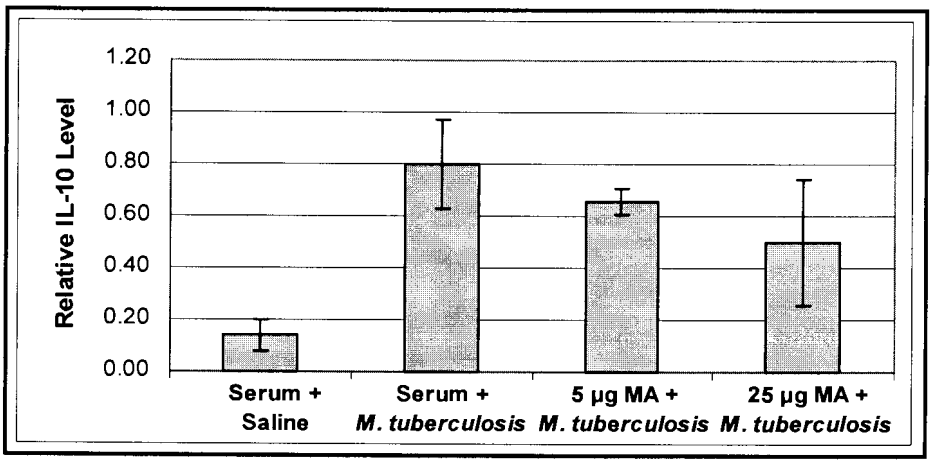


Figure 3.7: Relative IL-10 mRNA levels in the lungs of *i.n.* TB-infected Balb/c mice pretreated with MA, as assessed with semi-quantitative RT-PCR. Bars represent average values obtained from 5, 4, 3 and 4 mice respectively per group.

As shown in Figures 3.3 to 3.7, the average cytokine mRNA expression was generally much higher in the lungs of infected, untreated Balb/c (positive control) mice as opposed to non-infected, untreated mice (negative control). No IFN-γ mRNA was found in the negative control, most probably due to the very low levels of expression of this cytokine. The household genes in this group were comparable to those of the other groups, which underlines the argument. Intranasal infection with *M. tuberculosis* induced the expression of IL-12 (P=0.005), TNF-α (P=0.005) and IL-10 (P=0.005). The increase in GM-CSF mRNA expression in the lungs of infected mice, as compared to uninfected mice, was only marginally significant (P=0.1).

IL-12, IFN-γ, TNF-α, GM-CSF and IL-10 mRNA expression did not differ significantly between mice pretreated with 5 or 25 µg MA before *i.n.* infection with *M. tuberculosis*. As opposed to the positive control, the average expression of cytokine mRNA, 5 weeks after *i.n. M. tuberculosis*-infection was lower in the lungs of mice pretreated with MA for IL-12 (5 and 25 µg MA), IFN-γ (5 µg MA),

IL-10 (25 µg MA), TNF-α (5 and 25 µg MA) and GM-CSF (25 µg MA), but the decrease was only marginally significant for the 5 µg MA pretreatment for IL-10 and GM-CSF, as shown in Figures 3.4 to 3.7. The average expression of IFN-γ mRNA in infected mice pretreated with 25 µg MA didn't differ significantly from either the positive control or the mice pretreated with 5 µg MA. Most of the mRNA of these cytokines was still significantly higher though than the background level of the negative control, but the increase above background for the 5 µg MA pretreatment for TNF-α was only marginally significant. No difference was found between 5 and 25 µg MA pretreatment and the negative control for GM-CSF mRNA.

In conclusion, whereas TB infection clearly triggered the cytokine expression of IL-12, IFN-γ and TNF-α, this was not increased by MA pretreatment. In contrast, the results show these pro-inflammatory cytokines were down-regulated by MA pretreatment suggesting that the protective effect of MA pretreatment is to be found in other mechanisms of resistance to TB. To support this, no correlation whatsoever could be found between the expression of inflammatory cytokines IL-12, IFN-γ, TNF-α, GM-CSF and IL-10 that could in any way be correlated to the survival data of intranasally infected mice.

3.5.5. Cytokine profiling in the lungs of non-infected Balb/c mice six weeks after treatment with 25 µg MA

The ability of MA to stimulate the synthesis of cytokine mRNA in the lungs of non-infected Balb/c mice, six weeks after administration of 25 µg MA was also evaluated by determining the expression of cytokine mRNA with RT-PCR. Mice were injected *i.v.* with 25 µg MA absorbed onto serum or with serum only, 1 week before receiving saline *i.n* as a placebo for infection. No bands were obtained for IFN-γ, probably due to the very low levels of this cytokine at this particular time-point in non-infected mice. As shown in Figure 3.8, the mRNA levels of only

IL-10 were significantly up-regulated six weeks after administration of 25 µg MA. IL-12 mRNA was marginally up-regulated.

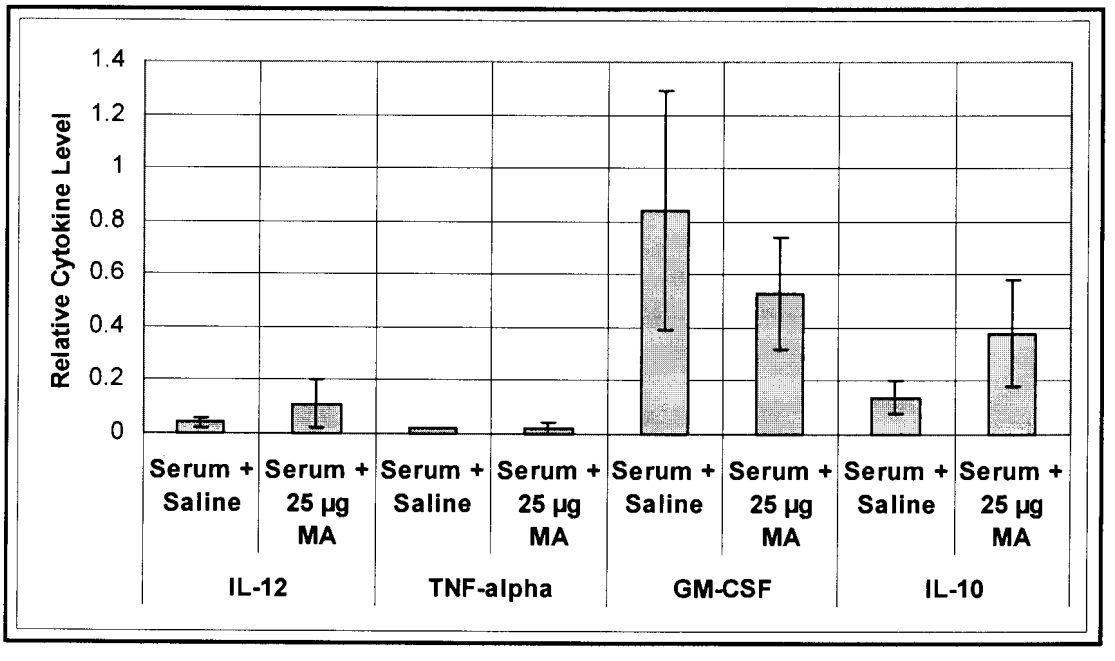


Figure 3.8: The effect of MA pretreatment on IL-12, TNF-α, GM-CSF and IL-10 mRNA levels in the lungs of uninfected Balb/c mice, six weeks after pretreatment. Bars represent average values obtained from 4, 3, 2, 2, 4, 3, 5, and 3 mice respectively per group.

An interval time study would have been far better to determine the cytokines that become induced by MA treatment in uninfected mice, but the experiment aimed only at determining a possible cytokine profile that correlated to survival as a lead to a mechanism for protection. With only IL-10 showing some upregulation with MA pretreatment, it remains difficult to find any mechanistic clue for the biological effect of MA at the cytokine level.

3.6. Discussion

In previous studies, MA was found to exert immunoregulatory functions through the stimulation of human double negative $CD4^+$ T cells (Beckman *et al.*, 1994; Goodrum, 2001) when presented by $CD1^+$ APCs. MA also marginally increased the survival of mice treated with an optimum intravenous dose of 25 µg before intravenous infection with *M. tuberculosis* (Siko, 1999) which coincided with increased expression in the lung of IL-12 and IFN-γ mRNA (Pretorius, 1999). It was noted that the cytokine response to MA only occurred in the lung, and not the spleen. It was therefore anticipated that MA would provide a much more prominent protection to TB when infection occurred through the lungs. In an attempt to determine the underlying mechanism of cytokine regulation by MA in a murine model infected via the natural route of infection, *i.e.* intranasal, survival studies and subsequent lung cytokine profiling were carried out in Balb/c mice, *i.e.* mice susceptible to TB (Flynn *et al.*, 1995b and Flynn and Bloom, 1996).

Mouse TB is a chronic disease of the lungs as evidenced by the finding that it was mainly in the lungs that infection caused progressive pathology (Dunn and North, 1995). The subsequent acquisition of host immunity was systemic, *i.e.* capable to resolve infection in the liver, spleen and kidneys, both in susceptible and resistant mouse strains (Medina and North, 1996) and irrespective of whether infection was initiated via the respiratory or *i.v.* route (North, 1995). The number of bacilli that implant initially in the lungs seems to have a significant effect on survival rate. The survival times of Balb/c mice injected *i.v.* with 10^5, 10^6 and 10^7 CFU *M. tuberculosis* shortened progressively. Giving mice a 100 times larger inoculum of *M. tuberculosis* was found to cause 100 times more bacilli to implant in the lungs, which are theoretically capable of initiating 100 times more infectious foci from which pathology can develop (unpublished results in Medina and North, 1998). Although not tested, the mice infected *i.v.* with *M. tuberculosis* in the present study most probably died earlier than *i.n. M. tuberculosis*-infected mice due to higher levels of initial *M. tuberculosis* implantation in the lungs.

A dose of 25 µg MA rendered intranasal *M. tuberculosis*-infected mice completely resistant to TB for the duration of this study, *i.e.* forty weeks. Unfortunately, the bacterial loads in the lungs and the spleens of mice did not correlate with the observed survival rates of the mice whether they were pretreated with either 5 or 25 µg MA or not. Although 25 µg as opposed to 5 µg MA was confirmed to be the optimum dose to bring TB under control, *M. tuberculosis* was not eradicated from the lungs.

The sustained production of protective cytokines in the lungs of C57BL/6 mice, as opposed to only transient expression of the protective cytokines in A/J mice, was found to be the reason for the increased susceptibility of A/J mice to *M. tuberculosis* infection. C57BL/6 mice survived twice as long as A/J mice after *M. tuberculosis* infection, even though organisms grew at similar rates in the lungs of these mice (Actor *et al.* 1999). A protective immune response to *M. tuberculosis* infection appeared to be dependent on the development of a Th1 immune response involving the secretion of cytokines such as IL-12, IFN-γ and TNF-α (Sieling, 1994), whereas a Th2 immune response involving the secretion of cytokines such as IL-10 and IL-4, related to unfavourable disease outcomes in TB of humans and mice. Therefore we investigated whether the protective effect of 25 µg MA versus the non-protective effect of 5 µg MA could be explained in terms of the production of protective cytokines.

Although it has been shown by some that chronic TB infection is associated with more IL-4 mRNA in the lung suggesting an involvement in the immunopathological changes seen in chronic disease (Muller *et al.*, 1993; Hernandez-Pando *et al.*, 1996, Hernandez-Pando *et al.*, 1997; Seah, 2000), IL-4 expression in samples from infected individuals was shown by others to be lower than in those from uninfected controls in several studies (Lin *et al.*, 1996; Fenhalls *et al.*, 2000). The confusion may be partly attributed to the diverse methodologies used and inherent difficulties in performing IL-4 assays. IL-4 is

biologically active at very low concentrations (Lewis *et al.*, 1988; Brown and Hural, 1997) which often fall below the detection limit of available immunoassays. Similarly, it has a correspondingly low mRNA copy number, making quantification by RT-PCR and RNA *in situ* hybridisation difficult too (Robinson *et al.*, 1994; Tang and Kemp, 1994; Kotake *et al.*, 1996; Torres *et al.*, 1998). IL-4 mRNA could not be detected in most of the samples in this experiment and when bands could be obtained, a linear amplification in signal was not generated. A recent study has shown that even enhancing the sensitivity of amplicon detection fails to overcome the problem of non-linear amplification kinetics at the higher number of cycles required to detect IL-4 with single round PCR (Actor *et al.*, 1998).

Infection with *M. tuberculosis* induced mRNA expression of the pro-inflammatory cytokines IL-12, IFN-γ and TNF-α and the anti-inflammatory cytokine IL-10. The beneficial effect of 25 µg MA pretreatment leading to a survival rate equal to that of non-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 infected mice were not increased by pretreatment. Mycolic acids seem to silence the immune response since pretreatment of intranasal TB-infected Balb/c mice with either 5 or 25 µg MA reduced the average expression of all these cytokines in the lungs.

Mycolic acids treatment without infection appeared to stimulate the immune system via the macrophages by means of a long term up-regulation IL-10 mRNA expression and also IL-12, although this was only marginally significant. IL-12 and IL-10 are primarily monokines, *i.e.* macrophage secreted cytokines (Chong et al., 1996; Barnes *et al.*, 1993; Tsukaguchi *et al.*, 1999; Othieno, 1999). Oswald *et al.* (1997), found that TDM, a MA-containing glycolipid present in the mycobacterial cell wall, induced the expression of IL-12 mRNA in mouse macrophages. This argues in favour of a biological effect of MA on macrophages rather than on T lymphocytes.

By the absence of reliable IL-4 mRNA expression data, an unequivocal conclusion could not be drawn on the possibility that MA pretreatment could have biased the T helper mode to the protective Th1 type. Such bias can only reliably be determined by the measurement of the IFN-γ /IL-4 ratio for each individual mouse. However, no supporting evidence for a Th1 bias induced by MA pretreatment was evident from the cytokine profile that could reliably be determined. The beneficial effect of MA pretreatment on the survival of TB-infected mice is thus more likely due to the induction of innate immune mechanisms, or else a physical blocking of some essential receptor required for uptake of *M. tuberculosis* in the macrophages. Supporting evidence for an effect of MA on innate immunity was given by A.C. Stoltz (2002), who showed that isolated murine macrophages pretreated with MA were less able to support intracellular survival of *M. tuberculosis* than control-treated marocrophages. Others found that intravenous injection of TDM factor resulted in the enhancement of early granuloma formation (Asano *et al.*, 1993; Matsunga *et al.*, 1996) and lead to the development of local immunity against against airborne infections with tubercle bacilli (Bekierkunst, 1986; Ribi *et al.*, 1976). TDM, but not 2,3,6,6'-tetraacyl trehalose 2'-sulfate, a structurally similar glycolipid without MA, enhanced NK cell activity, $\gamma\delta$ T cell migration to the lungs and CD1d1 expression (Tabata *et al.*, 1996; Ryll *et al.*, 2001). Although CD1d-deficient mice do not suffer from exacerbated TB as compared with wild-type mice, NKT cells predominated in the granulomatous reaction to deproteinised *M. tuberculosis* cell-wall preparations (Behar *et al.*, 1999). In addition granulomas did not form in NKT cell deficient Jα281$^{-/-}$ mice (Apostolou *et al.*, 1999). Granuloma formation could also be suppressed *in vivo* by injection of anti-cord factor IgG antibody that recognised MA (Fujiwara *et al.*, 1999). Possibly MA stimulate NK and NKT cells to mediate an early T cell-independent pathway of protective granuloma formation and cellular infiltration in response to mycobacteria before the activation and differentiation of T cells, which are necessary to amplify the initial innate response (Smith *et al.*, 1997).

Chapter 4

Concluding Discussion

Mycolic acids were the first non-protein antigens shown to exert immunoregulatory functions through stimulation of double negative and $CD4^+$ T cell proliferation (Beckman *et al.*, 1994 and Goodrum, 2001). It has been shown that cord factor or TDM (trehalose dimycolate, containing 2 MA moieties esterified to one molecule of trehalose) and MA administered before *M. tuberculosis* infection was initiated, had a protective effect on the development of disease (Bekierhurst, 1986; Ribi, 1976; Pretorius, 1999; Siko, 1999). MA and cord factor induced *in vivo* production of IL-12 mRNA within hours coinciding with IFN-γ mRNA induction (Pretorius, 1999; Oswald *et al.*, 1997). Cord factor also induced TNF-α secretion (Oswald *et al.*, 1999; Ryll *et al.*, 2001). Soon after infection macrophage-derived pro-inflammatory cytokines IL-12 and TNF-α already initiate events that curb mycobacterial growth, either because they help recruit new monocytes into the lesions, or because they directly activate macrophages. Mice deficient for these cytokines show very early exacerbation of mycobacterial growth in all organs and readily succumb to infection (Ladel *et al.*, 1997; Flynn *et al.*, 1995a; Cooper *et al.*, 1997b). At this early stage, IFN-γ is already involved in the inhibition of unrestrained growth of *M. tuberculosis*, as mice deficient for IFN-γ die even faster than those lacking IL-12 (Cooper *et al.*, 1997b; Cooper *et al.*, 1993; Flynn *et al.*, 1993; Bancroft, 1993). Although significant expression of IL-12 and IFN-γ was observed after TDM challenge no IL-4 was expressed, suggesting that TDM challenge may favour dominance of the Th1 response via the local cytokine profile. These mice indeed did not produce any anti-TDM antibodies that might reflect a Th2 response (Yamagami *et al.*, 2001).

During the first 3 – 4 weeks of *M. tuberculosis* infection, Balb/c mice, *i.e.* mice that are relatively susceptible to *M. tuberculosis* (Flynn *et al.*, 1995a), predominantly generate a Th1 response, whereas in the following weeks they also generate a Th2 response. A specific effector response (Th1 or Th2), once generated, can convert to another effector population. Th1 cells could readily convert to Th2 cells, while the opposite phenotype switch was much more difficult to produce. Recent work has demonstrated that Th2 cells in Balb/c mice lose responsiveness to IL-12 through down-regulation of the IL-12 receptor β chain, thereby preventing IFN-γ mediated feedback to macrophages and augmentation of IL-12 production. They can convert to Th1 cells if sufficient IFN-γ is present in the extracellular environment (Szabo *et al.*, 1997). Mice deficient in IL-12, IFN-γ, TNF-α or inducible NO synthase (iNOS, NOS2) revealed that an absence of strong Th1-mediated immunity correlates most closely with disease progression characterised by exacerbated bacterial growth, significant tissue necrosis and premature death (Flynn and Chan, 2001). The contribution of Th2 cytokines during mycobacterial infection is less clear. Although some studies have suggested that excess production of IL-4 and IL-10 might contribute to the chronic nature and progression of tuberculosis (Appelberg *et al.*, 1992 and Lang *et al.*, 2002), mice deficient in these cytokines have only shown subtle and inconsistent changes in bacterial growth (Turner *et al.*, 2001; Erb *et al.*, 1998; Jacobs *et al.*, 2000; Murray and Young, 1999). IL-4 is well known for being able to downregulate protective IFN-γ and it is entirely plausible that IL-4 could have contributed to the loss of the protective IFN-γ response seen in these mice. The absence of IL-4 in KO mice did allow earlier and increased expression of IFN-γ supporting the hypothesis that IL-4 indeed inhibits IFN-γ production, but this did not lead to an increased antimycobacterial effect in the lung (Saunders *et al.*, 2000). The inability to detect alterations in the course of *M. tuberculosis* infection in IL-4-deficient mice, may however, also be due to the compensatory effects of other Th2 cytokines such as IL-13 which has been shown to have many overlapping functions with IL-4 and also competes for binding to the IL-4 receptor (Shirakawa *et al.*, 2000). IL-10 may also compensate for the loss of function of

IL-4. However, IL-10-deficient mice have also been shown not to be more susceptible to infection with *M. tuberculosis* (North, 1998). It is not excluded that a switch from Th1 to Th2 may have very little influence on the progression of long-term disease in *M. tuberculosis*-infected mice. Instead, the relative strength of the initial Th1 response to infection could determine latency or active disease in TB (Caruso *et al.*, 1999; Scanga *et al.*, 1999; Serbina and Flynn, 1999). Thus, whereas it is generally accepted that Th2 gene activation relates to unfavourable disease outcomes in TB of humans and mice, it is not known whether this is a cause or consequence of disease.

Pretreatment of Balb/c mice infected intranasally with *M. tuberculosis* with 25 µg MA significantly increased the survival of mice (P = 0.0039), whereas pretreatment with 5 µg MA had no effect in the present study. We investigated whether the protective effect of 25 µg MA against TB could be explained by induction of a longer lasting Th1 response in Balb/c mice at a time-point of 5 weeks, in comparison to the cytokine profile that was associated with the non-protective effect of 5 µg MA.

The type of immune response was determined by using semi-quantitative RT-PCR run on the mRNA of cytokines characteristic of the different immune responses such as IL-12, IFN-γ and TNF-α for the development of a Th1 response (Sieling *et al.*, 1994) and IL-4 and IL-10 for the Th2 response (Sher and Coffmann, 1992 and Moore *et al.*, 1993). Quantitative competitive RT-PCR (QCRT-PCR) is commonly accepted as a reliable method for quantifying differences in mRNA. However, QCRT-PCR has many drawbacks. The assay is labour- and reagent-intensive requiring the generation of a large series of reactions with differing competitor:target ratios in order to assay a single sample. Amplification efficiency of competitors can vary significantly from that of wild type target due to size differences or the formation of competitor/wild type heteroduplexes (Becker-Andre and Hahlbrock, 1989; Kanangat *et al.*, 1992; McCullough *et al.*, 1995; Henley et al., 1996; Souaze *et al.*, 1996). For a more

affordable experiment with fewer reactions a well-established non-competitive RT-PCR method was used that utilises standard curves to quantify mRNA. The standard curves were obtained from a linear fit of the exponential amplification of different concentrations of template with the same number of PCR cycles (Abbott *et al.*, 1998; Wang *et al.*, 1989; Murphy *et al.*, 1990; Kanangat *et al.*, 1992; Melby *et al.*, 1993; Rasmussen *et al.*, 1995; Gallez-Hawkins *et al.*, 1997). Maximum sensitivity was obtained by using the lowest possible number of PCR cycles and template concentrations to generate the standard curves.

The beneficial effect of 25 µg MA pretreatment in TB-infected mice as opposed to the 5 µg MA pretreatment could not be explained by the prolonged induction of a protective cytokine response, as the levels of IL-12, IFN-γ and TNF-α mRNA in infected mice were not increased. Although 25 µg MA pretreatment protected *M. tuberculosis* -infected Balb/c mice against TB, the lungs were not sterilised from mycobacteria.

IL-12 and IFN-γ mRNA was rapidly produced after MA and TDM treatment of mice (Pretorius, 1999; Oswald *et al.*, 1997). The induction of IL-12 in infected macrophages lends strong support to the emerging paradigm that cells of innate immunity rather than clonally distributed adaptive lymphocytes, decode the pathogenic context and call for the appropriate recruitment and differentiation of cytokine-driven responses. The receptor for mycobacterial MA has not yet been identified, but toll-like receptors or scavenger receptors on innate APC are likely candidates (Moody *et al.*, 1999). In a primary response, antigen-specific T cells are present at very low frequency and other cells may provide the early IFN-γ. It is known that IL-12 activates NK and NKT cells to produce IFN-γ (Lieberman and Hunter, 2002; Emoto *et al.*, 1999). IFN-γ, in turn, activates macrophages by enhancing macrophage secretion of reactive intermediates such as nitric oxide, which are necessary for the killing of intracellular mycobacteria in murine macrophages (Chan *et al.*, 1992; MacMicking *et al.*, 1997). During an

inflammatory response, the production of IL-12 is strictly regulated by both positive and negative feedback mechanisms, with IFN-γ primarily representing the most potent upregulator of IL-12 production (Kubin *et al.*, 1994; Ma *et al.*, 1996). Presently IL-10 is the best studied negative regulator of the IL-12/IFN-γ pathway production (D'Andrea *et al.*, 1993) and very important to control the potent proinflammatory activities of IL-12 that can generate significant toxicity and tissue damage if unchecked. The slightly delayed expression of IL-10, as compared to that of IL-12 and other proinflammatory cytokines, confirms its likely role as a specific downregulator of the IL-12 response. Interestingly, several reports have indicated that IL-12 itself can induce T cells to produce IL-10, suggesting a mechanism in which IL-12 may limit its own production (Finkelman *et al.*, 1994; Gerosa *et al.*, 1996; Daftarain *et al.*, 1996; Meyaard *et al.*, 1996; Jeannin *et al.*, 1996; Windhagen *et al.*, 1996). In support of this, the expression of IL-12 mRNA together with IL-10 was found to remain elevated in mice even 6 weeks after treatment with 25 µg MA in this study.

In conclusion, although we were able to show that 25 µg MA could protect mice against the development of TB, this did not appear to be due to a prolonged adaptive Th1 response or due to prolonged suppression of the adaptive Th2 response. Instead, we believe that the beneficial effect of MA pretreatment on the survival of TB-infected mice is more likely due to the induction of innate immune mechanisms resulting in the increased differentiation, maturation and activation of infected macrophages and other cells of innate immunity.

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