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HUMORAL AND CELLULAR IMMUNOGENICITY OF MYCOBACTERIAL MYCOLIC ACIDS IN TUBERCULOSIS

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Humoral and Cellular Immunogenicity of Mycobacterial Mycolic Acids in Tuberculosis

by

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Outline of the Dissertation

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

The general introduction, that involves the description of the immune response to the infection with *Mycobacterium tuberculosis,* is given in chapter 1. This includes the role of macrophages and the different cytokines that play a role in the protective immune response to *M. tuberculosis* infection. The innate immune response at the normal infection site, the lungs, is also discussed.

In chapter 2 the investigation into the ability of *M. tuberculosis*-derived mycolic acids (MA) to elicit an immune response in human tuberculosis patients are described. ELISA and western blot analysis were used to confirm the presence of anti-MA antibodies.

In chapter 3, the preliminary experiments aimed at elucidating the role of MA in eliciting the protective immune response to *M. tuberculosis* are described and their outcome discussed. The potential of MA in preventing and /or curing tuberculosis was addressed using a murine model. A detailed description of the methods used in determining macrophage-derived cytokine profiles in the groups of experimental animals is also provided in this chapter. The correlation between the cytokine profiles obtained for mice pre-treated and post-treated with MA (as well as for control animals), and the survival of the mice in these respective experimental groups concludes this chapter.

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

List of Abbreviations:

AM	Alveolar Macrophages.
APC	Antigen Presenting Cells.
BCG	Bacillus Calmette-Guerin.
bp	Base pair.
CD	Cluster of Differentiation.
CF	Cord Factor.
CH	Contact Hypersensitivity.
CLMF	Cytotoxic Lymphocyte Maturation Factor.
cDNA	Complementary Deoxyribonucleic acid.
CFU	Colony Forming Units.
DEPC	Diethylpyrocarbonate.
DN	Double Negative.
DNA	Deoxyribonucleic acid.
dNTP	Deoxynucleotides.
DTH	Delayed Type Hypersensitivity.
DTT	Dithiotreitol.
ELISA	Enzyme-Linked Immunosorbent Assay.
Fc	Constant domain of the Immunoglobulin.
FDC	Follicular Dendritic Cell.
GM-CSF	Granulocyte Macrophage Colony Stimulatory Factor.
HIV	Human Immunodeficiency Virus.
HPLC	High Pressure Liquid Chromatography.
hsp	Heat shock protein.
IFN-γ	Interferon-γ.
IgG	Immunoglobulin of the G class.
I-κB	Inhibitor of kappa B.
IL	Interleukin.
iNOS	Inducible form of Nitric Oxide Synthase.
kDa	Kilodalton.
LAM	Lipoarabinomannan.

LJ	Löwenstein - Jensen.
LPS	Lipopolysaccharides.
MA	Mycolic acid.
MDR-TB	Multi Drug Resistant *Mycobacterium* strains.
MEM	Minimum Essential Medium.
MHC	Major Histocompatibility Complex.
mM	Millimolar.
Mn-SOD	Manganous superoxide dismutase.
MRC	Medical Research Council.
mRNA	Messenger Ribonucleic Acid.
NF-κB	Nuclear Factor- kappa B.
NK cells	Natural Killer cells.
NKSF	Natural Killer cell Stimulatory Factor.
NO	Nitric oxide.
PBS	Phosphate Buffered Saline.
PPD	Purified Protein Derivative.
RNI	Reactive Nitrogen Intermediates.
ROI	Reactive Oxygen Intermediates.
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction.
Rv	Virulent strain of *Mycobacterium tuberculosis.*
SDS-PAGE	Sodium dodecyl sulphate Poly Acrylamide Gel Electrophoresis.
STAT	Signal Transducers and Activators of Transcription.
SOD	Superoxide Dismutase.
SQ-RT-PCR	Semi Quantitative Reverse Transcriptase Polymerase Chain Reaction.
TB	Tuberculosis.
TCR	T cell receptor.
TGF-β	Transforming Growth Factor-beta.
Th1	T helper 1.
TNF-α	Tumour Necrosis Factor alpha.
V	Volts.
WHO	World Health Organisation.

Summary

Acquired protective immunity to *Mycobacterium tuberculosis* and its antigens is characterised by the induction of specific pro-inflammatory cytokines of mononuclear phagocytes. Recently it was discovered that lipids and hydrophobic protein antigens can be presented on CD1 membrane proteins to induce an immune response. In fact, mycolic acids (MA), a cell wall component of *M. tuberculosis,* were the first non-protein antigens shown to be presented by CD1. In this study it was determined whether *M. tuberculosis* derived MA would induce MA-specific antibodies in human tuberculosis patients and *M. tuberculosis* infected experimental animals. The immunoregulatory role of MA in the progression of tuberculosis was also determined.

Western blot analysis revealed that MA-specific antibodies exist in tuberculosis patients and that these antibodies recognised MA preferentially on a serum protein of ca. 90 kDa. Upon screening of 100 patient's sera with ELISA, 65 % were negative for MA-specific antibodies, whereas 20 % tested positive. The remaining 15 % of the sera gave high background values, typical of rheumatoid antibodies. The existence of MA-specific antibodies was also investigated in *M. tuberculosis* infected mice. Two out of ten Balb/c mice showed expression of MA-specific antibodies of the IgM class.

In experiments done to compare the IL-12 expression in various organs of *M. tuberculosis* infected and non-infected mice, evidence was obtained that the lung was the responsive organ in which a difference could be detected between susceptible and resistant mouse strains. In susceptible Balb/c mice, low levels of IL-12 expression were observed in all the organs except the spleen of the negative control group. After infection was introduced, the levels of IL-12 expression increased significantly in the lungs, kidneys and livers. In the more resistant C57BL/6 mice, the same results were obtained, except that the level of IL-12 expression in the lungs of non-infected mice was already high, i.e. comparable to that of infected mice. The observation that the lung was the responsive organ in susceptible mice was further corroborated in an experiment where mice were administered MA in the absence of *M. tuberculosis* infection. The spleen again remained non-responsive, while the lung showed increased expression levels of IL-12.

The role of MA in the induction of a protective immune response was investigated in mice infected with *M. tuberculosis*. The animals were treated with MA, either one week before, or during the third week after the infection. The more resistant C57Bl/6 mice infected with *M. tuberculosis* were impervious to MA administration in survival experiments. An appreciable extension of the lifespan of Balb/c mice was observed when MA was given prior to or after the infectious challenge with *M. tuberculosis*. In fact, the survival of Balb/c mice pre-treated with 25 μg of MA-serum came to the same level as that of C57BL/6 mice. Cytokine profiling, conducted via semi quantitative RT-PCR of the lungs of Balb/c mice pre-treated with MA, showed increased levels of IL-12 and IFN-γ when compared to serum control mice. This suggests an effect of MA in enhancing innate immunity. MA treatment three weeks after the infection reduced the expression levels of the cytokines IL-12 and IFN-γ in lungs. Our results suggest that the observed protection against *M. tuberculosis* after pre-treatment with MA in Balb/c mice is conferred by the cytokines IL-12 and IFN-γ. In the case of MA treatment after the infectious challenge, the resistance mechanism is apparently based on a different, as yet unknown mechanism.

Opsomming

Beskermende immuniteit teen *Mycobacterium tuberculosis* infeksies word gekarakteriseer deur die induksie van spesifieke makrofaag pro-inflammatoriese sitokines. Daar is onlangs ontdek dat presentering van lipiede en hidrofobiese proteiene aan die immuunstelsel deur 'n MHC-verwante proteïen bekend as CD1 plaasvind. Mikolsuur (MA), 'n selwandkomponent van *M. tuberculosis* in die besonder, was die eerste lipied wat aangetoon is wat deur CD1 gepresenteer word. In hierdie studie is bepaal of die MA van *M. tuberculosis* MA-spesifieke teenliggame sou kon induseer in menslike tuberkulose-pasiënte asook *M. tuberculosis* geïnfekteerde muise. Die immunoregulatoriese rol van MA in die progressie van tuberkulose is ook ondersoek.

Deur middel van Western klad-analise kon aangetoon word dat pasiënteserum MA-spesifieke teenliggame bevat, wat by voorkeur MA herken op 'n serumproteïen met 'n molekulêre massa in die omgewing van 90 kDa. Die sifting van 100 pasiëntesera m.b.v. ELISA, het aangetoon dat 20 % daarvan wel MA-spesifieke teenliggame bevat. 'n Verdere 15 % van die sera het hoë agtergrondseine gelewer, tipies van die teenwoordigheid van reumatoïde teenliggame. Die teenwoordigheid van teenligamme teen MA is ook bepaal in 'n muis model. Twee uit tien tuberculose geïnfekteerdemuise het ook MA-spesifieke teenliggame bevat, en wel van die IgM-isotipe.

'n Muismodel is gebruik om die rol van MA in die induksie van 'n beskermende immuunrespons te ondersoek. Die ekspressie van IL-12 in verskillende organe, insluitende die lewer, niere, hart, milt en longe, is eers bepaal deur middel van semi-kwantitatiewe RT-PCR. In die afwesigheid van infeksie het die vatbare Balb/c muise 'n lae IL-12 ekspressie aangetoon in al die organe, behalwe in die milt. 'n Toename in IL-12 mRNA is waargeneem in die lewer, milt en longe van Balb/c muise na *M. tuberculosis* infeksie. Dieselfde resultate is waargeneem in die negatiewe kontrole groep van die meer vatbare C57BL/6 muise, maar die ekspressie van IL-12 was reeds hoog, en van dieselfde orde-grootte as met *M. tuberculosis* infeksie. Dat die longe ook in vatbare muise die reagerende orgaan teenoor MA is, is bevestig met die waarneming dat Balb/c muise, wat met MA behandel is in die afwesigheid van *M. tuberculosis* infeksie, 'n toename in IL-12 ekspressie getoon het, terwyl die milt nie daarop gereageer het nie.

Die rol van MA in die beskerming teen tuberkulose is ondersoek in tuberkulose-geïnfekteerde muise. Muise was óf behandel met MA-serum een week voor *M. tuberculosis* infeksie, óf gedurende die derde week na infeksie toegedien is. Die meer weerstandige C57Bl/6 muise was ongevoelig vir enige effek wat MA sou kon gehad het. Oorlewingsprofiele het aangetoon dat Balb/c muise behandel met 25 µg MA voor infeksie en muise behandel met 48 µg MA-serum na infeksie se oorlewing in so 'n mate verbeter het, dat dit in ooreenstemming was met die oorlewing van *M. tuberculosis* geïnfekteerde C57BL/6 muise. Sitokienprofiele van die verskillende groepe het aangetoon dat die muise wat behandel is met 25 µg MA-serum voor infeksie, verhoogde IL-12 en IFN-γ ekspressie in die longe gehad het, vyf weke na infeksie. Hierdie wys op 'n versterking van die konstitutiewe ('innate') immuniteit deur MA. Geen korrelasie tussen oorlewing en IL-12-ekspressie is egter waargeneem by muise wat behandel is met MA-serum na infeksie nie. Die meganisme van weerstandbiedendheid in laasgenoemde groep bly dus nog onbekend, maar vind in elk geval nie plaas deur die verhoogde uitdrukking van IL-12- en IFN-γ-ekspressie nie.

Chapter 1

The Immune Response to *Mycobacterium tuberculosis*

According to archaeological evidence, tuberculosis (TB) has afflicted humans for thousands of years. It was only in 1882 that Robert Koch was able to identify *Mycobacterium tuberculosis*, an intracellular parasite, as the causative agent for TB (Fenton and Vermeulen, 1996; Mendez-Samperio *et al.*, 1995; Elhers, 1994).

The introduction of Bacillus Calmette-Guerin (BCG) vaccine helped to keep the spreading of the disease at bay at least for some time. The appearance of HIV and *M. tuberculosis* multi-drug-resistant (MDR)-strains, was a factor that contributed to the sudden rise in TB between 1982 and 1992 (Fenton and Vermeulen, 1996). In November 1993 the World Health Organization (WHO) declared TB a global health emergency. Currently, TB is responsible for 2-3 million deaths annually, and by the year 2005 this figure can rise to 4 million deaths per year (Elhers, 1994; Fine, 1994; Andersen, 1997). A summary of some of the important events in the field of tuberculosis is represented in Figure 1.

M. tuberculosis commonly infects human and animal hosts. Infection starts upon inhalation of the bacteria into the deep lung (Toossi, 1996; Fenton and Vermeulen, 1996; Bermudez and Goodman, 1996). Mycobacteria have the unique ability to survive and to withstand the hostile environment within the phago-lysosome in the lung. This allows the bacteria to escape humoral defense mechanisms and complement cascade initiation (Pece *et al.*, 1997).

To counteract *M. tuberculosis* infection, the majority of host organisms will develop an immune response that relies on cellular mediators rather than on an antibody response against the invading bacilli (Andersen, 1997; Toossi, 1996; Fenton and Vermeulen, 1996; Bermudez and Goodman, 1996; Bendenelli and Friedman, 1989).

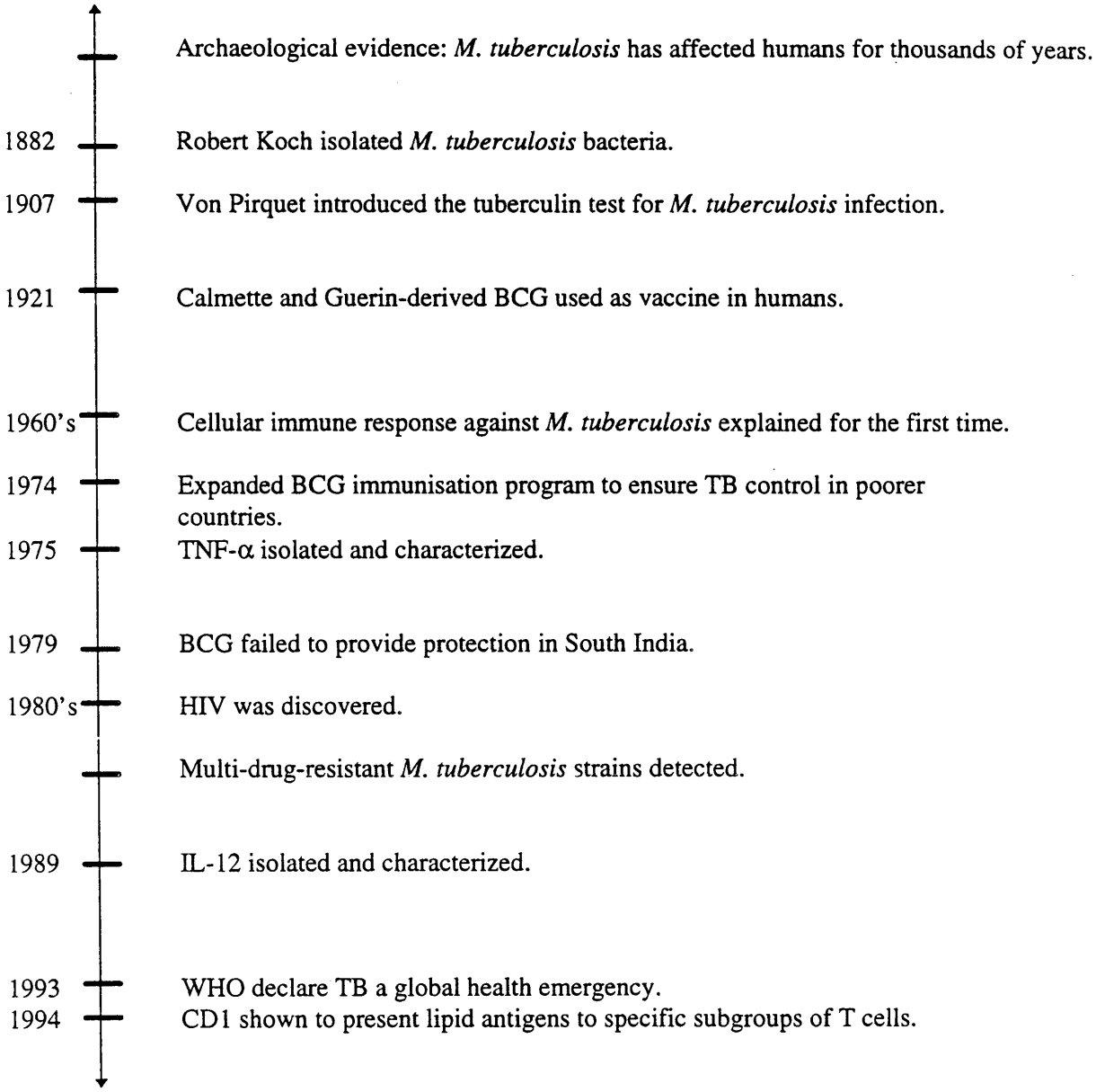


Figure 1 *Historic events in the field of tuberculosis*

Cell-mediated immunity to mycobacteria is characterized by the sensitization of T cells, and the subsequent release of pro-inflammatory cytokines such as interferon-γ (IFN-γ), Interleukin-1 (IL-1), IL-6 and IL-8 (Flynn *et al.*, 1993; Flynn *et al.*, 1995; Saunders and Cheers, 1994). These cytokines activate cells such as macrophages, and play a role in the recruitment of monocytes, natural killer cells and other T cells to the infection site (Toossi, 1996a; Fenton and Vermeulen, 1996; Rhoades *et al.*, 1995; Bermudez *et al.*, 1995; Ehlers *et al.*, 1994).

The activation of macrophages by cytokines and the recruitment of other lymphocytes to the infection site, are crucial for growth inhibition and/or killing of the virulent strains of mycobacteria. Macrophages and T cells also secrete immuno-suppressive cytokines. The balance between the activating and deactivating cytokines would therefore determine the outcome of the host defense against the pathogen (Toossi, 1996; Fenton and Vermeulen, 1996; Rhoades *et al.*, 1995; Bermudez *et al.*, 1995). An understanding of the protective immune response against infectious agents such as *M. tuberculosis* will have an impact on the development of suitable vaccines against infection.

1.1 Properties of *Mycobacterium tuberculosis*

M. tuberculosis is a rod shaped (1-4 x 0,3-0,6 μm), gram positive bacterium. These bacteria are resistant to most common antibiotics available, and are only susceptible to aminoglycosides (streptomycin), rifamycins and fluoroquinoles. They are also resistant to drying, alkali and most chemical disinfectants. It is believed that resistance is related to the unusual cell-wall structure of the bacteria (Brennan and Nikaido, 1995).

The cell envelope of *M. tuberculosis* consists of a plasma membrane (phosphatidyl-inositol-mannosides) and a cell-wall consisting of peptidoglycan linked to arabinogalactan and mycolic acids (Brennan and Nikaido, 1995; Yuan *et al.*, 1997). Peptidoglycan is also associated with various proteins in the cell-wall. Other molecules present in the cell-wall include lipoarabinomannan (LAM) and trehalose-2'-sulfates, both of which are associated with the virulence of the bacteria (Fenton and Vermeulen, 1996).

In the cell-wall, mycolic acids (MA) are linked to carbohydrates or exist as cord factor (CF) that can be isolated from free lipid extracts of mycobacteria. CF consists of a mixture of 6-6'diester of αD trehalose and natural MA (Orbach-Arbouys *et al.*, 1983; Gotoh *et al.*, 1991). MA can also be linked to glucose to form mono-mycolate (Gotoh *et al.*, 1991). It is proposed that CF might be the substance responsible for the inhibition of phagosome-lysosome fusion in macrophages (Spargo *et al.*, 1991).

1.2 Mechanism of protective immunity to *Mycobacterium tuberculosis* infection

The T cell-mediated immune response can be divided in two subclasses: T helper 1 (Th1) and T helper 2 (Th2). The cytokines that play a role in the induction and mediation of these responses are however not secreted by T cells only. The overall cytokine release by all cell types including $CD4^+$ T cells, $CD8^+$ T cells, macrophages and some stromal cells can be referred to as type I and type II immune responses (Rook and Hernandez-Pando, 1996). In murine models, immunity against *M. tuberculosis* correlates with a type I immune response.

The different subclasses of T cells play a unique role in the development of an intracellular response against the invading parasites. The $CD4^+$ T helper cells play an important role. Cytokines secreted by these T cells direct the immune response either in a humoral (Th2) or cell-mediated response (Th1). Each of the Th1 and the Th2 T cells secrete specific cytokines. Th1 is dominated by TNF-α, IL-2 and IFN-γ secretion whereas Th2 is dominated by IL-4, -5, -6, -10 and -13 secretion (Aliprantis *et al.*, 1996; Dipiro, 1997).

In a typical *M. tuberculosis* infection, Th1 cytokines are known to predominate, and cell-mediated protection is established (DiPiro, 1997). It was reported that the adoptive transfer of $CD4^+$ T cells to infected mice confers protection against *M. tuberculosis* infection (Orme *et al.*, 1987). The depletion of $CD4^+$ T cells in a *M. tuberculosis*-infected murine model lead to the uncontrolled multiplication of bacteria. This confirms that the imunological resistance to infection is mediated through the induction of a type I immune response (Barnes and Modlin, 1996; Toossi, 1996; Orme, 1996).

The major cytolytic T cell population consists of $CD8^+$ T cells. $CD8^+$ T cells also play a role in the controlling of infections but not to the same extent as $CD4^+$ T cells (Barnes and Modlin, 1996). It has been indicated that mice deficient in $CD8^+$ T cells were fully capable of controlling pulmonary mycobacterial infection by *Mycobacterium bovis* and the granulomatous responses were similar to those demonstrated in normal control mice (Xing *et al.*, 1998). This T cell subset does, however, play a role in mediating immunological memory.

The $CD8^+$ T cells recognise antigen presented on MHC class I molecules (Barnes and Modlin, 1996). Mice lacking MHC class I have been shown to be more susceptible to *M. tuberculosis* infection. This implies a crucial role for $CD8^+$ T cells in the protective immune response. $CD8^+$ T cells are also implicated in the lysis of infected cells in granulomatous lesions in the lung and other organs (Andersen, 1997; Rook and Hernandez-Pando, 1996; Barnes and Modlin, 1996; Tascon *et al.*, 1998). Immunisation of mice with *Mycobacterium vaccae* elicited $CD8^+$ T cells, which killed macrophages infected with *M. tuberculosis*. These $CD8^+$ T cells were shown to secrete IFN-γ and enhance IL-12 production (Skinner *et al.*, 1997).

T cells are characterized by their expression of αβ T cell antigen receptors (TCR) or γδ TCR (the latter occurring less frequently than in human donors than in murine models). There is strong evidence that αβ T cells participate in the immune response to *M. tuberculosis*. It has been shown that the numbers of αβ T cells expand in mice exposed to live bacteria when inoculated into the foodpads or *via* aerosol inoculation into the lungs (Janis *et al.*, 1990). Soluble extracts prepared from *M. tuberculosis* bacteria killed at 85 °C and directly disrupted by prolonged sonication (TBe), also elicited a strong proliferation of $CD4^+$ $\alpha\beta^+$ T cells (Batoni *et al.*, 1998). Activation of these T cells require the recognition of *M. tuberculosis* antigens presented by the class II major histocompatibility complex (Balaji and Boom, 1998).

It has been observed that the γδ T cells population increases during the onset of *M. tuberculosis* infection. These cells can play a role in the first line of defense against the infection, because they recognize low weight non-peptide ligands directly on the surface of infected macrophages (Andersen, 1997; Rook and Hernandez-Pando, 1997; Barnes and Modlin, 1996; Orme, 1996).

Human γδ T cells have an innate capacity to recognize *M. tuberculosis* antigens without prior exposure to these antigens. Upon activation these T cells secrete IL-2 and IFN-γ (Barnes and Modlin, 1996). Recently, it has been indicated that γδ T cells also respond to glycolipid antigens and nucleotide moieties (Tanaka *et al.*, 1995; Boom, 1996; Dauglat *et al.*, 1995). When mice with disrupted δ genes were infected with *M. tuberculosis,* an increased susceptibility to infection was observed that coincided with a down-regulation of IFN-γ in the spleen (Rook and Hernandez-Pando, 1996). It was postulated that γδ T cells are prominent at

the infection site, and require help from αβ T cells to successfully contain *M. tuberculosis* infection (Boom, 1996; Dauglat and Kaufmann, 1996).

The double negative (DN) $CD4^-$ $CD8^-$ T cells constitute another important subgroup of T cells, postulated to play a role in the defense against tuberculosis infection. These cells have been shown to be activated upon recognition of mycolic acids presented on CD1 molecules (Beckman *et al.*, 1994). The physiological function of these cells remains elusive, but it is known that DN T cells can promote cell-mediated immunity at the site of infection (Dauglat and Kaufmann, 1995; DiPiro, 1997; Barnes and Modlin, 1996; Rook and Hernandez-Pando, 1996).

Like MHC class I proteins, the CD1 protein consist of α 1, α 2 and α 3 extracellular domains associated with β_2-microglobulin. CD1 proteins are remotely homologous to MHC in their α 1 and α 2 domains. These α 1 and α 2 domains are unusually hydrophobic, and binding of lipids to CD1 is probably through hydrophobic interactions. In humans, CD1 can be divided into two groups: Group 1 consists of CD1a, -b and -c which are expressed on professional antigen presenting cells, whereas group 2 consists of human CD1d. CD1d and its murine CD1 equivalent are expressed on intestinal epithelium cells (Calabi *et al.*, 1989; Beckman and Brenner, 1995; Bendelac, 1995; Porcelli and Modlin , 1995). The group 1 CD1 molecules are not represented in mice.

The expression of CD1 on professional antigen presenting cells (APC) requires activation by granulocyte macrophage colony stimulatory factor (GM-CSF), a non-specific cytokine expressed by both Th1 and Th2 cells, and IL-4 that is associated with a Th2 response (Kasinrerk *et al.*, 1993; Thompsen *et al.*, 1995).

$NK1.1^+$ $CD4^+$ T cells, a subgroup of T cells known to express surface markers naturally associated with natural killer (NK) cells, has been implied in the recognition of glycolipid presentation on murine CD1d molecules (Kawano *et al.*, 1997). The role of these molecules in the induction of an immune response against mycobacteria is still unknown (Bendelac *et al.*, 1993; Yoshimoto *et al.*, 1995a; Dauglat and Kaufmann, 1995).

Other cells that play a role in the protective immune response against *Mycobacterium* infection, include NK cells, neutrophils, mast cells and macrophages. NK cells are part of the first line of defense against the invading mycobacteria. These cells secrete IFN-γ after TNF-α and macrophage-derived IL-12 stimulation. NK cells are known to exhibit non-MHC restricted cytotoxic activity towards the infected alveolar macrophages (Andersen, 1997, Dauglat and Kaufmann, 1995).

The primary immune response against *M. tuberculosis* invasion is however mediated by macrophages. In this dissertation the action and mechanism of macrophages and the release of macrophage-derived cytokines to initiate protective immunity against *Mycobacterium* infection in response to a *M. tuberculosis*-derived lipid antigen challenge, was investigated.

1.3 Microbicidal action of macrophages

1.3.1 The function and properties of mononuclear phagocytes in a cellular immune response

Macrophages are cells whose predominant role in the immune system is the removal and processing of particulate antigens and the presentation of their antigenic determinants to the T cell population. Typical macrophages are large cells with abundant cytoplasm often containing vacuoles. The nucleus is large, with prominent nucleoli and can vary in shape. Alveolar macrophages have a prominent spherical nucleus, as opposed to those of other macrophages that show an "indented" or kidney bean shape. Macrophages originate from blood monocytes which in turn are derived from bone marrow precursors (Roitt *et al.*, 1993; Janeway and Travers, 1996). The migration of monocytes (through blood-vessel walls) into infected organs induces macrophage differentiation (Toossi *et al.*, 1996; Fenton and Vermeulen, 1996).

Macrophages have the ability to phagocytose invading pathogens. Once in the macrophage, lysosomal enzymes such as non-specific esterase, peroxidase and lysozyme will assist in bacterial degradation. These macrophages have a well developed Golgi complex that is essential for antigen presentation on major histocompatibility complex (MHC) protein products. On the cell membrane several specific surface markers are visible including

specialized micro-organism adhesion receptors, IgG Fc-, mannosyl-, fucosyl- and cytokine receptors (Van Oss and Regelmortel, 1994) .

Macrophages are potent inducers of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and IL-12. Inhibitory cytokines secreted by macrophages include transforming growth factor-beta (TGF-β) and IL-10 (Toossi, 1996; Fenton and Vermeulen, 1996; Roitt *et al.*, 1993; Van Oss and Regelmortel, 1994).

1.3.2 The activation of macrophages

T lymphocytes control the activation of macrophages by means of IFN-γ secretion. Activated macrophages are able to fuse their lysosomes more effectively to phagosomes, that contain the ingested bacteria. This exposes the intracellular or recently ingested bacteria to a variety of lysosomal enzymes that are bactericidal. Activated macrophages also produce oxygen radicals and nitric oxide, both of which have potent antibacterial activity (Schebesch *et al.*, 1997; Desmedt *et al.*, 1998; Bonecini-Almeida, 1998).

Additional changes in the activated macrophage establish the amplification of the immune response. This includes an enhanced expression of MHC class II molecules and TNF-α receptors on the macrophage cell surface. The increased expression of MHC class II molecules will enable the macrophage to present more antigens, which in turn activate more T cells. This will lead to enhanced recruitment of activated lymphocytes. The binding of TNF-α, to its receptor on the macrophage cell-surface, induces the expression of reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) that enhance the microbicidal activity of the macrophage (Murch, 1995; Allendoerfer and Deepe, 1998; Sato *et al.*, 1998; Bilyk and Holt, 1995; Leenen *et al.*, 1994).

In vitro studies have indicated that alveolar macrophages produce nitric oxide (NO) whereas blood monocytes do not. The superior function of alveolar macrophages is confirmed in their ability to contain bacterial growth. Alveolar macrophages also show a reduced capacity to produce TGF-β that coincides with an increased expression of TNF-α, as compared to blood monocytes (Toossi *et al.*, 1996).

The production of NO is one of the effector pathways necessary for the containment of *M. tuberculosis* infection. The addition of inhibitors to NO will aggravate the infection, because NO signaling and second messenger functions also cause direct toxicity to the micro-organisms (Rook and Hernandez-Pando, 1996; Fenton and Vermeulen, 1996).

Activated macrophages are extremely efficient in destroying pathogens, but consume large quantities of energy to maintain this activated state. Unfortunately macrophages are also responsible for localized host tissue destruction. Therefore regulation of macrophage activation, by inflammatory $CD4^+$ T cells, is essential to minimize local tissue damage and energy consumption (Murch, 1995; Hansch *et al.*, 1996; Munk and Emoto, 1995; Dunn and North, 1995; Saunders and Cheers, 1995).

1.4 Cytokines secreted by activated macrophages

1.4.1 What are cytokines?

Cytokines are small peptide mediators through which cells of the immune response communicate, and are produced by many different cell types. Monokines are products from macrophages, lymphokines are products from lymphocytes and cytokines are products from all cell types. Different cell types can secrete the same cytokine, therefore the term cytokine is most often used. Cytokines generally have a molecular weight of less than 25 kDa. Although cytokines are produced in discrete sub-units, most biologically active cytokines exist in a multimeric form. This multimeric structure is often important in the association of the cytokine with its receptor. Despite the ability of many cells to produce cytokines, monocytes and macrophages tend to generate these mediators in the greatest quantities (Allendoerfer and Deepe, 1998; Sato *et al.*, 1998; Bilyk and Holt, 1995; Leenen *et al.*, 1994).

1.4.2 Interleukin-12

Interleukin-12 (IL-12) was first described in 1989. This cytokine is also known as natural killer cell stimulatory factor (NKSF) or cytotoxic lymphocyte maturation factor (CLMF). Since then the central role of this cytokine in the immune system has become well documented. The cytokine is produced by mononuclear phagocytic cells, dendrtitic cells, neutrophils and B cells in response to bacterial infection. It is known to promote the

development of T helper 1 cells (Th1) both *in vivo* and *in vitro* (Kobayashi *et al.*, 1989; Stern *et al.*, 1990; Gazzinelli *et al.*, 1993; Trinchieri and Scott, 1994; Snijders *et al.*, 1996).

Interleukin-12 has an unusual heterodimeric structure consisting of a light chain (p35) that shows homology to other cytokines and a heavy chain (p40) that shows homology to the extra-cellular portion of several cytokine receptors. This suggests that IL-12 is derived from a primordial cytokine and one chain of its corresponding receptor. These two subunits are covalently linked to form a biologically active heterodimer. The heavy chain subunit is secreted in large excess over the biologically active heterodimer. The light chain mRNA is detectable in many cell types that are unable to produce IL-12. In murine models the IL-12 heavy chain can form homodimers that have an antagonistic activity to the biologically active IL-12. The biological significance of the homodimer formation is not yet known, and was not demonstrated in human models (Trinchieri and Scott, 1994; Bost and Clements, 1995).

IL-12 can affect a variety of immune processes and is thought to be a principal regulator of cytokines and of other lymphoid cells. The cytokine is known to promote the development of a $CD4^+$ Th1 immune response (Scott, 1993; Hsieh *et al.*, 1993). Hsieh *et al.* (1993) demonstrated that IL-12 induces the differentiation of Th1 cells from uncommitted T cells thus initiates cell-mediated immunity.

Two distinct pathways exist for the induction of IL-12. The first pathway involves the T cell dependent responses to antigens such as ovum-albumen (OVA). This involves the triggering of CD40 molecules on APC. IL-12 increases in direct proportion to antigen concentration and requires TCR ligation but not CD28 co-stimulation. The second pathway occurs when bacterial antigens such as lipo-polysaccharides (LPS) or heat killed bacteria are used. This pathway does not require T cells and is completely independent of CD40 triggering (DeKruyff *et al.*, 1997). Using a CD40 knock-out *M. tuberculosis*-infected mouse model, it was shown that the protective Th1 immunity could still be achieved. In the *M. tuberculosis* model, the CD40 dependent activation of IL-12 does not seem to play an important role (Campos-Neto *et al.*, 1998).

It was demonstrated and confirmed in several laboratories that IL-12 expression mediates the initiation of a cell-mediated immune response *via* the induction of IFN-γ by T cells (Kobayashi *et al.*, 1989; Sypec *et al.*, 1993; Gazinelli *et al.*, 1993; Flynn *et al.*, 1995; Bost and Clements, 1995; Cooper *et al.*, 1995; Ladel *et al.*, 1997; Cooper *et al.*, 1997; De Jong *et al.*, 1997; Probst *et al.*, 1997). IFN-γ is known to be crucial for macrophage activation in the onset of the disease. It has been indicated that IFN-γ plays a protective role against *M. tuberculosis* infection in murine models tested (Flynn *et al.*, 1993).

Interleukin-12 was also shown to regulate many of the functions of natural killer (NK) cells. Bermudez and Young (1995) showed that IL-12 stimulated NK cells to secrete TNF-α, GM-CSF and IFN-γ. This stimulation is important in host defense against bacterial infections. IL-12 activated NK cells produce soluble factors that trigger macrophages to inhibit intracellular growth of the bacteria (Bermudez and Goodman, 1995; Orange and Biron, 1996).

Cells other than $CD4^+$ T cells and NK cells can also be affected by IL-12. Brunda *et al.* (1993) demonstrated a crucial role for $CD8^+$ T cells in mediating the anti-tumour activity of IL-12 in several murine tumour models. The effects of IL-12 are, however, dependent on the expression of IL-12 receptors on the various T cells. It has been determined that the lack in the expression of IL-12 receptors would result in immunodeficiency where intracellular pathogens are concerned (De Jong *et al.*, 1998).

Studies suggested that IL-12 enhanced the cytotoxicity of $CD4^+$ T cells against macrophages infected with *M. tuberculosis.* Administration of recombinant IL-12 to *M. tuberculosis*-infected Balb/c mice increased their resistance to the infection, confirming the protective role of IL-12 in bacterial infections such as *M. tuberculosis* (Zhang *et al.*, 1994; Cooper *et al.*, 1995; Flynn *et al.*, 1995). *In vitro* studies also demonstrated that IL-12 augmented the cytolytic activity of NK cells in HIV-positive patients toward the *M. tuberculosis*-infected monocytes (Kobayashi *et al.*, 1989; Denis, 1994; Saunders and Cheers, 1995). Furthermore human patients infected with *M. tuberculosis* have shown an increase in the amount of IL-12 secreted (Murch, 1995).

The mere phagocytosis of live or dead bacilli can activate the macrophages to produce IL-12 (Fulton *et al.*, 1996). It is very interesting that *M. tuberculosis*-derived cord factor can induce IL-12 expression in alveolar macrophages, where *M. tuberculosis* peptide antigens were unable to do so (Fulton *et al.*, 1996; Oswald *et al.*, 1997). Lipoarabinomannan (LAM), a putative virulence factor of *M. tuberculosis*, added to human mononuclear cells, induced attenuation of IL-12 and TNF-α expression (Knutson *et al.*, 1998).

Recently, it was shown that IL-10 down-regulated the ability of BCG-stimulated human mononuclear cells to produce IL-12 *in vitro*. The IL-10 effect was specific and was abolished in the presence of anti IL-10 antibodies (Mendez-Samperio *et al.*, 1998; Fulton *et al.*, 1998).

1.4.3 Tumour necrosis factor-alpha (TNF-α)

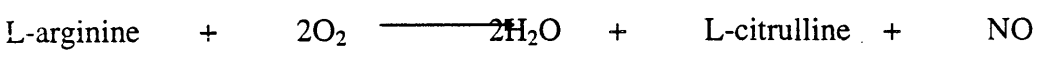
Tumour necrosis factor-alpha (TNF-α) was first isolated in 1975 by Carswell and colleagues. The cytokine is also known as cachectin. TNF-α is generally considered to be a product of activated macrophages, but is also secreted by lymphocytes, that include T cells, thymocytes, B cells, mast cells and NK cells. The production of this cytokine is partially stimulated by lipopolysacharides (LPS), but also by other cytokines and the cell-wall components of mycobacteria (Carswell *et al.*, 1975).

The cytokine is shown to be bioactive, both as a transmembrane protein and as a homotrimeric secreted protein. This protein is initially produced as a 26 kDa protein, that is then cleaved to yield a 17 kDa monomer. Three of these monomers have to combine to yield the secreted, biologically active TNF-α trimer. The receptors for TNF-α are trimers made up of either three 55 kDa (p55) or three 75 kDa (p75) monomers. The binding of TNF-α to the receptor p75 initiates the activation of lymphocyte proliferation. The receptor p55 is implicated in the mediation of apoptosis (p75 can also be involved). Mice lacking the p55 receptor show an increased susceptibility to infection with *Listeria* (Hamblin, 1993; Pasparakis *et al.*, 1996).

Some of the functions of TNF-α include: the regulation of infections, inflammatory and autoimmune phenomena, the induction of the secretion of other cytokines such as IL-1, IL-6 and granulocyte macrophage colony stimulatory factor (GM-CSF), the alteration of endothelial cells to assume a pro-coagulant profile, and the up-regulation of enzymes such as

manganous superoxide dismutase (Mn-SOD). It was also shown that TNF-α is required in the processes regulating the formation of B cell follicles, follicular dendritic cell (FDC) networks and germinal centers. TNF-α is therefore required in the physiological maturation of the humoral immune response. Membrane bound TNF-α mediates contact hypersensitivity (CH) reactions, especially in mice deficient in p55, which may indicate an immuno-suppressive role for p55 in CH (Roitt *et al.*, 1993; Hamblin, 1993; Pasparakis *et al.*, 1996; Dick *et al.*, 1996). TNF-α secreted by mast cells increased the neutrofil influx to the infection site that in turn increased the bacterial clearance rate (Malaviya *et al.*, 1996).

TNF-α is known to activate murine macrophages to produce reactive nitrogen intermediates (RNI). The induction of the RNI helps the host in the defense against bacterial infections. TNF-α induces the activation of the L-arginine dependent cytotoxic pathway, which results in the generation of nitric oxide (NO). This reaction is carried out by the inducible form of nitric oxide synthetase (iNOS) of macrophages by using L-arginine as substrate.



Dysfunction in the production of TNF-α or any regulatory element along the pathway of iNOS induction that would compromise the L-arginine-dependent cytotoxic mechanism, would increase the host susceptibility to various infectious agents (Stamler *et al.*, 1992; Chan *et al.*, 1995; Mayer *et al.*, 1997).

TNF-α plays a crucial role in the development of protective immunity in bacterial infections with mycobacteria. Secreted antigens of growing mycobacteria are important for the development of a protective cell-mediated immune response in the infected host. It was shown that infection with live *M. tuberculosis* generates a protective T lymphocyte response, but killed preparations do not. One of the *M. tuberculosis* secreted proteins (30 kDa), also known as alpha antigen or antigen 6, is known to evoke delayed type hypersensitivity (DTH) skin test responses and was shown to induce a protective immune response. Antigen-6 is a mycolyl tranferase and as such is important in *Mycobacterium* cell-wall synthesis. This 30 kDa antigen can induce the production of TNF-α on both transcriptional and post-transcriptional levels in a

murine model. Recently, it has been shown that antigen-6 can induce TNF-α secretion by human monocytes (Averill *et al.*, 1995; Aung *et al.*, 1996).

Transgenic mice unable to use TNF-α because of the expression of high levels of a soluble TNF-α receptor fusion protein, showed enhanced susceptibility to BCG and *M. tuberculosis* infection (Garcia *et al.*, 1997). It has been shown that TNF-α is a potential inducer of apoptosis during *M. tuberculosis* infection *in vitro*. Apoptosis is induced by the production of NO after TNF-α stimulation. The addition of anti-TNF-α monoclonal antibodies to this experimental model showed NO production inhibition and apoptosis ceased (Aung *et al.*, 1996; Rojas *et al.*, 1997). Furthermore, TNF-α has been demonstrated to be protective in a murine model of *Mycobacterium bovis* and is crucial for the formation of granulomas as antibodies directed against TNF-α- prevented formation of granulomas. The intracellular growth of the bacilli was also limited by exogenous administration of the cytokine (Denis, 1991; Aung *et al.*, 1996).

M. tuberculosis-infected dendritic cells (APC) were shown to produce elevated levels of TNF-α and IL-12 (Henderson *et al.*, 1997). The mechanism involved in the macrophage-mediated killing or growth restriction of the bacteria is still unclear. There is evidence for the involvement of reactive oxygen intermediates (ROI). Normal inactivated macrophages exert a base level of killing, that could be blocked by inhibiting the intrinsic L-arginine-dependent pathways by metabolic inhibition. Macrophages were fully activated by TNF-α to exert full microbicidal activity. It was demonstrated that non-virulent *Mycobacterium avium* was killed efficiently by TNF-α-treated cells, whereas virulent *M. avium* was prevented from growing. Studies suggest a clear involvement of RNI in the killing of this microbe. Addition of superoxide dismutase (SOD) protected *M. avium* from being killed by TNF-α-activated macrophages (Hibbs *et al.*, 1987; Denis, 1991).

1.4.4 Transforming growth factor-beta (TGF-β)

TGF-β is a product of blood monocytes and activated macrophages. This cytokine is produced in the cell in a latent form (110 kDa). Enhanced monocyte sialidase activity is one of the mechanisms whereby TGF-β is activated *in situ*. The biologically active cytokine is a dimeric protein with a molecular weight of 25 kDa, composed of two identical monomers of 12.5 kDa,

that are joined by disulfide bonds. The biologically active protein exists in three isoforms TGF-β1, TGF-β2 and TGF-β3 (Toossi *et al.*, 1995a; Toossi *et al.*, 1996). Of the three isoforms of TGF-β, TGF-β1 is the most potent modulator of the immune system. There are three TGF-β receptors that are found on virtually all cell types. Apparently, the type I receptor is responsible for most of the biological effects of TGF-β (Vodovotz, 1997).

TGF-β has various immuno-regulatory properties that include the regulation of cellular growth and differentiation, the suppression of lymphocyte responses to antigens and mitogens, and the modulation of the production and effects of monocyte pro-inflammatory cytokines. It is also responsible for the modulation of monocyte expression of surface immuno-regulatory molecules such as HLA-DR determinants. TGF-β is known to inhibit both IFN-γ and IL-12 expression, leading to the suppression of T cell responses. It also plays a role in the recruitment of blood monocytes to the infection site (Van Oss and Regelmortel, 1994; Toossi *et al.*, 1995b; Toossi *et al.*, 1996).

At low concentrations TGF-β has pro-inflammatory functions, stimulating the mRNA expression of monokines such as IL-1, platelet growth factor, IL-6 and basic fibroblast-growth factor (Toossi *et al.*, 1996). TGF-β production is induced by both the purified protein derivative (PPD) of *M. tuberculosis,* and by a *Mycobacterium* cell-wall constituent, lipoarabinomannan (LAM). The role of TGF-β in tuberculosis infection includes the down-regulation of TNF-α and IL-1 locally. Because TNF-α and IL-12 play a crucial role in the containment of the bacteria, an increase in TGF-β will decrease the capacity of the mononuclear phagocytes to contain the mycobacteria in the granuloma. T cell clonal proliferation and cytokine production in response to *M. tuberculosis* infection or its products my be limited by this cytokine. TGF-β was shown to suppress T cell function in *M. tuberculosis*-infected cells. The addition of natural inhibitors of TGF-β restored T cell function (Toossi *et al.*, 1995a; Toossi *et al.*, 1995b; Hirsh *et al.*, 1997).

Contrary to the results stated above, it has been indicated that TGF-β can induce fibroblast synthesis, and can recruit fibroblasts to the site of infection. An increase in fibroblasts may enhance the containment of the bacteria (Toossi *et al.*, 1995; Toossi *et al.*, 1995; Dahl *et al.*, 1996; Ellner, 1997). In this regard the secretion of TGF-β will have an impact on the outcome

of the disease. These results corroborate the fact that the secretion of the cytokine is concentration-dependent. If secreted in low concentrations, a protective effect will be observed. If secreted at high concentrations the positive effects of cytokines such as IL-12 and TNF-α will be abrogated.

All three isoforms of TGF-β are known to regulate the production of nitric oxide (NO). These isoforms could suppress the capacity of murine macrophages to produce NO *in vitro*. The most potent effects of this cytokine occurred at the level of the expression of NO synthetase (NOS). In macrophages, TGF-β1 reduced the stability and rate of translation of NOS mRNA and increased the rate of the degradation of NOS protein. The transcriptional effects of TGF-β were thought to be mediated by altering the binding of and/or activity of any of the numerous transcription factors that induce transcription of the NOS gene (Vododotz, 1997).

1.5 Cytokines secreted by NK- and T cells.

1.5.1 Interferon-gamma (IFN-γ)

Interferon-γ is a cytokine that was initially shown to be secreted by activated T cells and natural killer (NK) cells (Flynn *et al.*, 1993; Ogasawara *et al.*, 1998). IFN-γ mediates the activation of macrophages as a mechanism for controlling pathogens such as *Leishmania major, Leishmania donovani, Leishmania monocytogenes* and *M. tuberculosis* (Flynn *et al.*, 1995; Kemp *et al.*, 1997). It was shown that the exogenous administration of recombinant IFN-γ leads to the migration of large numbers of Th1 lymphocytes to the site of infection, thus initiating granuloma formation (Bonecini-Almeida *et al.*, 1998). Moreover, IFN-γ gene knock-out mice were not able to control *M. tuberculosis* infection (Flynn *et al.*, 1993). The intracellular pathogens are most likely controlled by IFN-γ induced reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) (Sato *et al.*, 1998).

As mentioned before, IL-12 is known to induce IFN-γ expression in T cells and NK cells. IL-12 is not the only cytokine that can modulate the expression of IFN-γ. The expression of IFN-γ is also regulated by IL-18, and it has been postulated that IL-12 and IL-18 could synergise in enhancing IFN-γ expression (Barbulesco *et al.*, 1998; Micallef *et al.*, 1996; Kohno *et al.*, 1997). In an experiment done with IL-12 p40 knock-out mice infected with

M. tuberculosis, IFN-γ mRNA expression decreased even though IL-18 levels were equivalent to those of the control mice (Cooper *et al.*, 1997). They could also demonstrate that mice, in the absence of biologically active IL-12, have no control of bacterial growth within the macrophage (Kobayashi *et al.*, 1997).

1.6 The development of a protective immune response to tuberculosis infection

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

Once within the lung, small particles are carried *via* the air stream to all parts of the lung where the *M. tuberculosis* bacteria are taken up by alveolar macrophages by means of complement activation or mannose-mannose receptor interaction. Mannose is expressed by most of the virulent bacteria and the mannose receptor is better known as macrophage entry protein (Andersen, 1997). The bacteria can either be killed or survive within the macrophage. As the mycobacteria slowly grow at the primary focus of infection in the lung, the macrophages are able to break down some of the phagocytosed organisms. The resulting antigens are then presented on MHC class II molecules to specific T cells (Andersen, 1997; Fenton and Vermeulen, 1996; Rhoardes *et al.*, 1995; Bermudez and Goodman, 1995; Eigler *et al.*, 1995).

The interaction between the macrophage-presented antigen and the specific T cell stimulates the macrophages to secrete IL-1, IL-6 and chemokines. IL-1 induces proliferation of neighbouring T cells, that in turn secrete IFN-γ. The secretion of these cytokines and chemokines would lead to the recruitment of cells to the infection site that initiate the inflammatory process (Ellner, 1994; Porter *et al.*, 1993; Andersen, 1997).

The inflammation process is divided into three stages. At first the bacteria and the macrophages appear to have a symbiotic relationship. This is followed by the first stage of the innate immune response, which is characterised by the activation of alveolar macrophages leading to the secretion of cytokines such as IL-12 (Andersen, 1997; Kaufmann and Andersen, 1998; Vanham *et al.*, 1997). Two weeks after the infection (in murine models) cytokine release is triggered in both T cells, including $CD8^+$-, DN-, NKT- and $CD4^+$ T cells, and macrophages. Secretion of cytokines activates macrophage bacteriostatic activity, recruits lymphocytes to the infection site and activates T cells to secrete IL-2 and IFN-γ (Andersen, 1997; Ellner, 1997; Rhoardes *et al.*, 1995). IFN-γ acts as a chemotaxin, that attracts monocytes and other lymphocytes to the infection site. Activated monocytes secrete IL-1 and TNF-α. Once the monocytes reach the infection site they cannot leave the site. This results in the final stage of inflammation - granuloma formation, involving the accumulation of macrophages, T cells and NK cells.

Under the influence of cytokines, some of the macrophages become elongated and weakly phagocytic. They are known as epitheliod cells. Some of the macrophages may also fuse with each other and become giant cells with multiple nuclei (Fenton and Vermeulen, 1996; Eigler *et al.*, 1995; Janeway and Travers, 1996). The giant cells usually form the center of these granulomas and are surrounded by T cells, many of which are $CD4^+$. The T cells main function is the regulation of the macrophages and the prevention of wide spread tissue damage (Janeway and Travers, 1996).

1.7 The role of macrophage-derived cytokines in granuloma formation - A summary

The interplay of T cell- and macrophage-derived cytokines is important for the control of *Mycobacterium tuberculosis* infection in the lung. Formation of granulomas for containment of the bacterial growth, is dependent on the production of specific cytokines especially macrophage-derived cytokines, that include IL-12, TNF-α and TNF-β (Pece *et al.*, 1996; Toossi, 1996; Andersen, 1997). After infection, the bacteria either invade the macrophages or are killed. In the latter case macrophages present the mycobacterial antigens to T cells, that in turn secrete cytokines that are both chemotraxins (recruitment of blood monocytes to the

infection site) and activators of monocytes (Bermudez and Goodman, 1995; Porter *et al.*, 1993).

Activated monocytes will secrete IL-12, IFN-γ and TNF-α. IL-12 secretion is important in mediating the immune response towards the Th1 mode, and is known to enhance the cytotoxicity of $CD4^+$ T cells against the macrophages infected with *M. tuberculosis*. IL-12 will also recruit NK cells to the infection site, that are known for their cytolytic activity towards the *M. tuberculosis*-infected monocytes (Flynn *et al.*, 1995; Cooper *et al.*, 1997).

The activated macrophages and NK cells secrete yet another cytokine, TNF-α. This cytokine is very important in the formation of granulomas and the subsequent confinement of the bacteria within the macrophages. It also plays a role in the differentiation of macrophages to form giant cells. TNF-α is known to induce iNOS, that is responsible for the formation of nitric oxide and other RNI. Elevated NO levels will either kill the bacteria or inhibit the growth of the bacteria (Malaviya *et al.*, 1996; Averill *et al.*, 1995; Aung *et al.*, 1996).

Mycobacteria have acquired specific mechanisms allowing them to survive and multiply in the macrophage. Even under these circumstances a protective immune response can develop. Specific antigens, secreted by the multiplying and growing bacteria can stimulate cytokine secretion. These include antigens present in the purified protein derivative (PPD) fraction and antigen-6 (30 kDa). Both these antigens are known to induce TNF-α secretion that in turn activates granuloma formation (Stamler *et al.*, 1992; Fenton *et al.*, 1996). The secretion of protein antigens also activates the production of TGF-β. This cytokine is known to down-regulate IL-12, IFN-γ and TNF-α at high concentrations (Toossi *et al.*, 1995). At low concentrations TGF-β has pro-inflammatory functions, stimulating the mRNA expression of monokines such as IL-1, platelet growth factor, IL-6 and basic fibroblast-growth factor (Toossi *et al.*, 1996).

One could argue that the more mycobacterial antigens are secreted, the more monocytes, NK cells and T cells are activated. This would lead to an amplification in the secretion of other pro-inflammatory cytokines, such as TNF-α, at the infection site. The increased amounts of TNF-α will not only influence the infected tissue but also healthy, uninfected tissue

surrounding the granuloma. In this regard the secretion of TGF-β is essential. TGF-β will down-regulate the production and effects of TNF-α, ensuring the survival of healthy tissue. There is therefore a very fine balance between the pro-inflammatory and the immuno-suppressive cytokines secreted at the infection site. This balance is necessary for the maintenance of healthy lungs.

1.8 Aim of this study

The general description of the immune response to *Mycobacterium tuberculosis,* as described in the previous section, was based on research and observations done on protein antigens in general. Furthermore, the search for new vaccines to *M. tuberculosis* infection is based on protein-derived antigens and antibiotics. It has been shown that subsets of T cells are activated when CD1 presented mycolic acids are recognised. This has led to the assumption that mycolic acids can also have an influence on the immune system.

In this study it was attempted to prove that *M. tuberculosis*-derived MA have an influence on the development of the immune response to *M. tuberculosis* infection. In view of their aliphatic structure and the absence of aromaticity, similar to other lipid components, they should have very weak or limited immunogenic properties. To investigate the immunogenicity of MA in their native state, the antibody response in human *M. tuberculosis* patient sera and *M. tuberculosis*-infected mice were investigated. The results obtained are discussed in chapter 2.

In an attempt to determine the underlying mechanism of the cytokine regulation by MA in a murine model, survival studies and subsequent cytokine profiling were carried out. The aim of the project was to investigate the potential of MA to prevent or cure tuberculosis by correlating the macrophage-derived cytokine profiles to the survival provided by mycolic acids (MA) treatment either before or after the infection of mice with *M. tuberculosis*. This included the determination of IL-12 expression in various organs after *M. tuberculosis* infection, and an investigation of the ability of MA to induce IL-12 at the infection site. The results obtained will be discussed in chapter 3.

Chapter 2
Antibodies Against *Mycobacterium tuberculosis*-derived Mycolic Acids

1. Introduction

Mycobacterium tuberculosis, an intracellular bacterial pathogen is known to commonly infect human and animal hosts. The pathogen is phagocytosed by alveolar macrophages upon infection. Due to the unique cell-wall composition of the bacteria and the ability of the bacilli to inhibit phagosomal-lysosomal fusion, the pathogen can survive and grow within the host macrophage (Spargo *et al.,* 1991).

Acquired protective immunity to *M. tuberculosis* infection is characterised by a protective cellular immune response mediated by T helper 1 (Th1) cells. Th1 cells secrete IL-2 and IFN-γ upon activation and play a role in the induction of a cellular- and a primary antibody immune response. The primary antibody response is mediated by IFN-γ and is characterised by the secretion of IgM and IgG2a (Orme *et al.*, 1993a; Mosmann and Coffman, 1989).

During the first two weeks after the commencement of *M. tuberculosis* infection, the growing bacteria secrete antigens that can be presented on major histo-compatibility (MHC) molecules (Andersen *et al.*, 1991 and 1995). It is during this phase that T cells are activated. *Mycobacterium* antigens include cell-wall proteins, polysaccharides and lipids. The presence of antibodies directed to various protein antigens such as antigen-6, heat shock protein 65 (hsp 65) and purified protein derivatives (PPD) was confirmed in several laboratories (Toossi, 1996; Fine, 1994; Silva *et al.*, 1996; Mendez-Samperio *et al.*, 1995).

In the progression of the *M. tuberculosis* infection it was proposed that a T helper 2 (Th2) or humoral immune response follows the Th1 immune response. The preferential secretion of IL-4 characterises the Th2 response and is important in controlling isotype switching in the development of an antibody response of the IgG1 isotype (Orme *et al.*, 1993).

There is however controversy in literature whether a Th1 to Th2 switch really takes place *in vivo*. It was indicated based on the secretion of particular cytokines, that Th2 cells may be

sequestered at the site of infection in patients with severe tuberculosis (TB). It could not, however be shown that a systemic immune response was elicited upon *M. tuberculosis* infection as such. Rather, it is believed that there is a stage in the progression of infection that mimics a Th0 response, resulting in the secretion of both Th1 and Th2 cytokines at the infection site (Reviewed by Andersen, 1997; Douglat and Kaufmann, 1996). Even if a Th0 mimic response is applicable during infection, the presence of Th2 cytokines will still favour antibody production to mycobacterial antigens.

The recent discovery that CD1 molecules can present mycobacterial lipid antigens to specific sub-populations of T cells sparked new interest in the role that lipid antigens play in the induction of an immune response to an invading intracellular parasite such as *M. tuberculosis* (Beckman and Brenner, 1995; Bendelac, 1995; Porcelli *et al.*, 1989 and 1992). Initially, it was indicated that CD1 presentation of mycolic acids (MA) activated a specific T cell sub-population that plays a role in the induction of a cellular immune response (Porcelli and Modlin, 1995; Sieling *et al.*, 1995). Murine CD1 reactive NK1.1$^+$ T cells have been shown to release large quantities of Interleukin-4 (IL-4) upon interaction with CD1-expressing antigen presenting cells (APC). NK 1.1$^+$ T cells are a subgroup of T cells that express surface markers normally associated with natural killer (NK) cells (Yoshimoto *et al.*, 1995b; Porcelli and Modlin, 1995; Douglat and Kaufmann 1996).

Secretion of IL-4, as mentioned before, will promote the activation of the non-protective Th2 mode of immune regulation (Bendelac *et al.*, 1995; Yoshimoto *et al.*, 1995a; Powrie and Coffmen, 1993; Boom, 1996), but which does elicit an antibody response. In fact, antibodies directed to the lipid fraction of the cell-wall of *M. tuberculosis* were already indicated before the role of CD1 in the induction of an immune response became known. These include antibodies directed to a phenolic glycolipid and cord factor (CF). Cord factor is a molecule that consists of 66'-trehalose covalently linked to MA residues (Orbach-Arbouyes *et al.*, 1983; Guillermard *et al.*, 1993; Cho *et al.*, 1992; He *et al.*, 1991; Kashima *et al.*, 1995; Simmoney *et al.*, 1995).

Kato *et al.* (1972 and 1974) has reported that the immunisation of mice with *Mycobacterium*-derived cord factor conjugated to proteins such as BSA did result in antibody production

against CF. There was however evidence that the sugar moiety defined the antigenic specificity of the glycolipid (Kato *et al.*, 1972 and 1974).

The purpose of this study was to investigate whether and in what form MA were immunogenic. The question of whether MA-specific antibodies were present in human patients infected with *M. tuberculosis* was also addressed. MA could become immunogenic when associated with the cell-wall of the bacteria, secreted as cord factor or associated with a transporter protein.

2. Materials and Methods

2.1 Materials

2.1.1 Mycolic acids

M. tuberculosis-derived MA were purified according to the protocol described in patent application no. SA 95/1464 (PCT/GB 96/00416).

The *M. tuberculosis* H37 Rv (ATCC 27294) strain was used as the source of MA. This was a virulent strain, originally isolated from an infected human lung, purchased in lyophilized form, from the American Type Culture Collection (ATCC), Maryland USA.

Materials used include Methanol (AR), Chloroform (AR), Acetone (AR) and sodium chloride purchased from Merck, Darmstadt, Germany.

2.1.2 Electrophoresis

Acrylamide/Bis, N'N'-Bis (AR)-methylene-acrylamide and sodium dodecyl sulphate (SDS) were purchased from BDH Laboratory Supplies, Dorset, England.

Tris (hydroxy methyl)-aminomethan (Tris); glycerol, bromo-phenol blue, glycine, TEMED (N,N,N',N'-Tetramethylethylenediamine) and ammonium persulphate were products obtained from Merck, Darmstadt, Germany.

Low Molecular Weight Markers were supplied by Pharmacia Biotechnology, Pistacaway, New Jersey, USA.

2.1.3 Western Blot

CAPS (3-[Cyclohexylamino]-1 propane sulphonic acid), 4-chloronaphtol and peroxidase conjugated goat anti-Human IgG and IgM (whole molecule) were purchased from Sigma Chemical Co., St. Louis, USA.

Methanol (AR), Sodium chloride (AR), Tris and Tween-20 were all products from Merck, Darmstadt, Germany.

Hydrogen peroxide (urea stabilised type) was purchased from BDH Laboratory Supplies, Dorset, England.

PVDF membrane was a Millipore product called Immobilon-P Transfer membrane.

2.1.4 ELISA

ELISA plates were purchased from Sterilab, (Sterilin 611F96).

Products form Sigma Chemical Co., St. Louis, USA were: O-phenylene diamine, anti-human γ-chain specific IgG peroxidase conjugate, and anti-mouse, γ- and μ-chain specific IgG peroxidase conjugate.

Hydrogen peroxide was a product obtained from BDH Laboratory Supplies, Dorset, England.

Sodium chloride, potassium chloride, potassium dihydrogen phosphate, disodium hydrogen phosphate, citric acid, trisodium citrate, potassium dihydrogen phosphate, disodium hydrogen phosphate, citric acid, trisodium citrate and casein were purchased from Merck Darmstadt, Germany.

Inbred male Balb/c mice were obtained from the South African Institute for Medical Research (SAIMR), Johannesburg, RSA.

2.2 Methods

2.2.1 Sera tested

Patient sera: Patient sera were obtained from the SA Medical Research Council (MRC), Pretoria. The samples were centrifuged at 3000g for 10 min at 4 °C using a BHG Hermle model Z3200 centrifuge. The obtained sera were heat-inactivated at 56 °C for 30 min and stored at -72 °C.

***M. tuberculosis*-infected mouse sera**: *M. tuberculosis* bacilli (5 x10^4) were injected *via* the tail vein of the male Balb/c (8-11 weeks of age) mice. Blood from control and *M. tuberculosis*-infected Balb/c male mice was obtained from the tail vein 110 days after the

infection was introduced. The blood was allowed to clot at 4 °C for 30 min to 1 hour. Sera were obtained by centrifugation in a microfuge (Eppendorf 54145) at 4°C at 12 000g for 10 min.

Bacteria: *M. tuberculosis* H37 Rv (ATCC 27294) was cultured on LJ-slants at 37 °C for 3 to 6 weeks. For each experiment, bacteria of a total count varying between 10^4 - 10^6 bacteria per ml were suspended in 0.89 % sterile saline, washed twice by centrifugation (2000 x g for 15 min) and re-suspended in saline (0.89 % sodium chloride). Bacterial counts (viable count) of the samples were determined by counting colony forming units (CFU) in 1:10 to 1:1 000 000 dilutions on Middlebrook 7H10 agar after 4 to 6 weeks of incubation at 37 °C. Total direct count was determined by means of a haemocytometer at 1:100 dilution.

2.2.2 SDS-PAGE Electrophoresis

MA Purification: *M. tuberculosis*-derived MA were extracted and derivatised as described by Butler *et al.* (1991), with minor modifications. Briefly, bacteria were scraped from LJ slants and re-suspended in 25 % potassium hydroxide in methanol-water (1:1). The suspension was vortexed in the presence of glass beads and homogenised.

The cells were subsequently autoclaved at 120 °C for 30 min. This was followed by the addition of 1.5 ml 50 % (v/v) HCl per 2 ml sample to adjust the pH to pH 1. After chloroform-water (1:1) extractions, the lower phase was transferred to a new vial. The sample was evaporated at 85 °C in a heat-block evaporator, under a flow of nitrogen and a volume of 100 µl of 2 % K_2CO_3 dissolved in methanol-water (1:1) was added to the sample before evaporation to dryness. From this crude saponified extract, MA were purified by countercurrent separation (King *et al.*, 1962). A biphasic, tri-component solvent system was used consisting of 42 % (v/v) chloroform, 39 % (v/v) methanol and 19 % (v/v) 0.2 M NaCl.

Purity analysis was done by HPLC on derivatised MA samples. Para-bromophenacylbromide (Pierce, Rockford, Illinois, USA) in acetonitrile and crown ether was added (100 µl per 2 ml sample), the samples were vortexed and heated for 20 min at 85 °C, and concentrated HCl-methanol water (1:2:1) added to adjust the pH to 1. The bottom layer was removed and

evaporated to dryness at 85 °C under a stream of nitrogen. The dried residues were re-suspended in methylene chloride and were quantified by HPLC against an internal standard.

Preparation of homologous MA-serum conjugate: In view of the highly restricted solubility of MA, conjugates had to be prepared to ensure suspension in aqueous solutions. To prepare the MA-serum conjugate, 250 µg purified MA stored under acetone, were dissolved in chloroform (20 µl) by vortexing and added to fresh mouse serum (1 ml). The mixture was sonicated on a Branson sonifier B-30 for 50 pulses at 20 % duty cycle. Chloroform was evaporated under a flow of nitrogen.

Mouse serum and MA-mouse serum conjugate were diluted 1:4 with Laemmli-buffer (0.5 M Tris-HCl, pH 6.8, 10 % Glycerol, 10 % (w/v) SDS and 0.05 % bromophenol blue). The mouse serum and MA-mouse serum conjugates were separated in separate lanes on a polyacrylamide (SDS-PAGE) gel (Owl system - 1.5 mm x 160 mm x 140 mm). The gel consisted of a 4 % stacking gel and a 6 % separating gel in electrode buffer (30 mM Tris pH 8, 200 mM Glycine and 17 mM SDS).

The non-reducing SDS-PAGE gel was initially run at a voltage of 6O V for 1h, after which the voltage was turned up to 100 V for 2-3 h. The gel was run in an electric field created by the Electrophoresis Constant Power Supply (ECPS 2000/300) from Pharmacia Biotechnology.

2.2.3 Western Blot

After the separation of the mouse serum proteins, the gel was equilibrated in CAPS buffer (1 mM CAPS, pH 9) for 15 minutes. A PVDF membrane was equilibrated in methanol and then washed with CAPS buffer. The proteins from the gel were transferred to the membrane with a Biorad Transblot-SP semi-dry transfer cell (power supply ECPS 2000/300 from Pharmacia Biotechnology).

Strips on the membrane were cut out and blocked in 1 % fat free milk solution in TBS pH 7.4 (20 mM Tris, 55 mM NaCl) which contained 0.05 % Tween-20. Each strip of PVDF membrane contained 1 lane mouse serum, and 1 lane MA-serum conjugate. As a control, a membrane strip consisting of one lane standard Low Molecular Weight Markers, one lane

mouse serum and one lane MA-serum conjugate was used. The control strip was Coomassie stained for 1 min. The other PVDF strips were individually probed with either patient serum (diluted 1:6 in blocking buffer) or control human serum (diluted 1:6) at 4 °C for 16 h.

The membrane strips were then incubated with a mixture of heavy- and light-chain specific anti-human IgG and IgM peroxidase conjugates (each diluted 1:500 with blocking buffer) at room temperature for 3h. The blots were developed by adding the substrate (0.03 mM 4-chloronaphtol, 3 % (v/v) H_2O_2 in 20 ml methanol).

2.2.4 ELISA.

ELISA plates were coated with MA at a concentration of 3 µg per well in hot PBS. A volume of 30 ml PBS pre-warmed to 85 °C in a water bath was used to suspend 1 mg of MA. Plates were coated with 100 µl/well of this hot solution. After reaching room temperature the plates were incubated for 12 hours at 4 °C and the excess buffer was flicked out before the plates were used.

Patient sera used in the normal ELISA were diluted 10 times and kept at 4 °C until used. Sera used in the inhibition ELISA were treated as follows: Patient sera and control human sera were incubated diluted 1:1 with control mouse serum or with MA-mouse serum conjugate for 1 hour at room temperature. These samples were then diluted 10 times before they were used in the ELISA.

The buffer used for blocking, diluting and washing of the plates was 0.5 % Casein/PBS, pH 7.4. ELISA plates were blocked with 200 µl per well blocking buffer for 2 hours at room temperature, followed by a single wash step. After blocking, the plates were coated with 50 µl/well of the pre-diluted serum samples (diluted with wash buffer) and incubated for 1 hour at room temperature on an orbital shaker. After 3 washing cycles, the plates were incubated for 30 minutes at room temperature with 50 µl/well of an anti-human IgG and IgM heavy chain specific peroxidase conjugate mixture that was diluted 1/1000 with wash buffer, prior to use. The plates were developed with 10.mg OPD plus 8 mg hydrogen peroxide in 10 ml 0.1 M citric acid buffer, pH 4.5, at 50 µl per well. The OD_{450} was recorded after 25 minutes on a SLT 340 ATC ELISA reader. A 690 nm reference filter was used.

2.2.5. Statistical analysis

Data in all figures are expressed as the mean ± the standard deviation. Statistical analysis was performed using Student's t test, and the results were considered significant at **p** values lower than 0.05.

3. Results

3.1 Western blot analysis of human tuberculosis patient serum.

Initial studies were designed to determine whether MA, a *Mycobacterium* cell-wall constituent, will elicit MA-specific antibodies during *M. tuberculosis* infection in humans. Should this be the case, a next logical question would be whether a preferred blood protein would act as a carrier or presenter of the MA to the immune system. This would require an analysis of sera exposed to purified MA. Mouse serum proteins were used as MA-carrier to avoid cross-reactivity of goat anti-human IgG and IgM conjugate to human serum proteins. Pure, isolated *M. tuberculosis*-derived MA, dissolved in chloroform, were conjugated to mouse serum and separated by means of gel electrophoresis. Similarly treated mouse serum, but with no MA, was used as the negative control.

The chloroform-treated mouse serum and MA-serum conjugates were separated on a non-reducing 6 % SDS-PAGE gel. The separated serum proteins were transferred from the gel to a PVDF membrane. The membrane was cut into strips, each containing either a mouse serum or a mouse MA-serum conjugate lane. Each strip was probed with either patient or control human serum, followed by immunoglobulin indicator antibody conjugate and substrate. Patient sera consisted of a pool of equal volumes of the sera obtained from five individual patients. Control sera consisted of the pooled sera of three individuals. The membrane strips were probed with patient and control sera as indicated in Figure 2.1.

In lanes 3, 5, 7 and 8, all of which were probed with patient sera, a band of ± 80 kDa was observed (indicated with a thin arrow in Figure 2.1). This band is visible on both the MA-serum conjugate and the mouse serum control lanes, indicating a patient serum specific band. This band was absent in the control human serum lanes (lanes 4 and 6). The recognition of antigen in both the MA-mouse serum and the mouse serum control might be attributed to the development of auto-immune antibodies in the patient during the *M. tuberculosis* infection.

By comparing the patient sera to the control sera (lanes 5 and 6), both with MA-mouse serum as the antigen, a patient sera specific band of ± 90 kDa was observed (indicated with a thick arrow in Figure 2.1). The absence of a clearly visible band in the control serum (lane 6) indicates MA-specific antibody recognition. Comparison of this 90 kDa band to the

Coomassie stained control (lane 2) indicated that the band represented a minor serum protein, because it did not correlate with a Coomassie stained band. Changes in the mobility of this serum protein in the gel, due to the protein-MA interaction, cannot be ruled out.

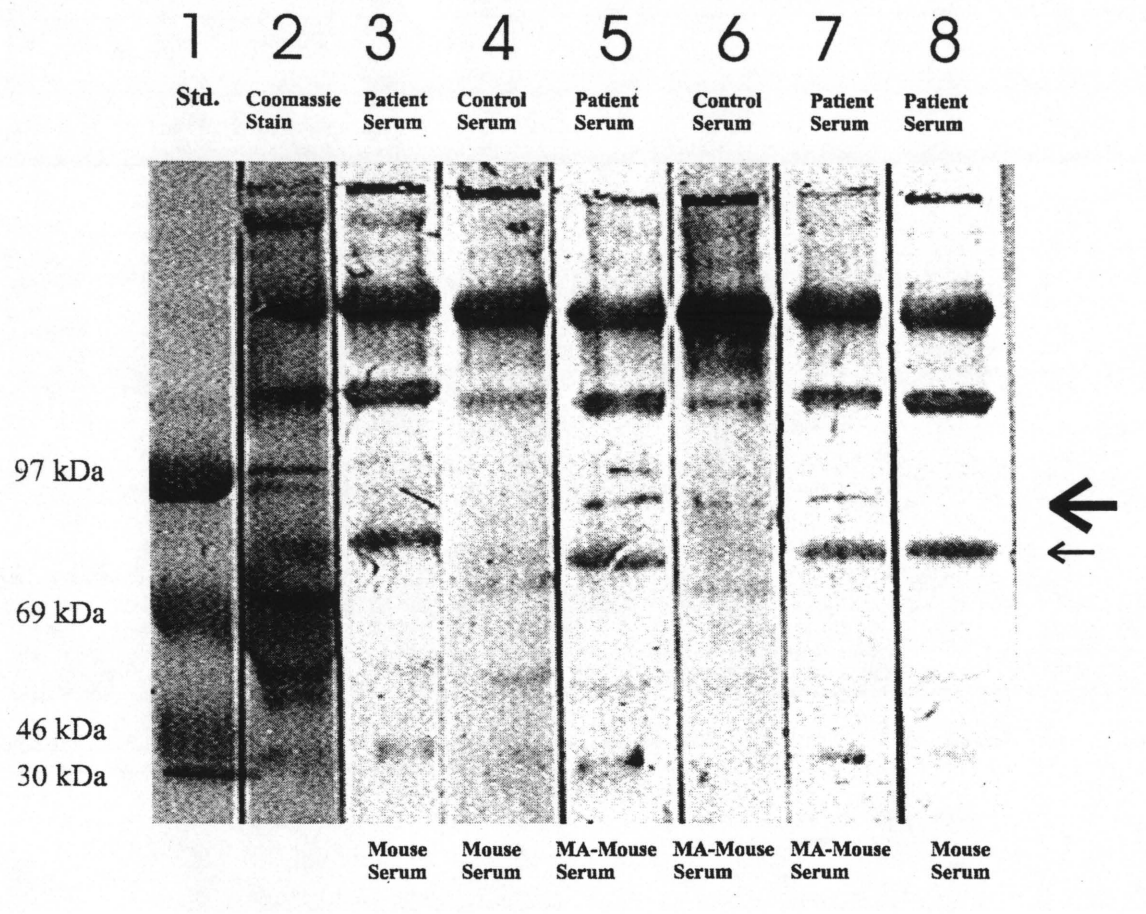


Figure 2.1. *Detection of MA-specific antibodies in pooled patient and control sera and their antigenic targets in mouse sera exposed or not exposed to MA. Patient serum was obtained from M. tuberculosis-infected patients. Lanes 1 and 2 are Coomassie stained standard markers and separated mouse serum proteins respectively. The rest of the lanes contained either mouse serum or MA-serum conjugate as indicated at the bottom. The antigen (mouse serum proteins, with or without MA) was probed with patient or control human sera, as indicated on top.*

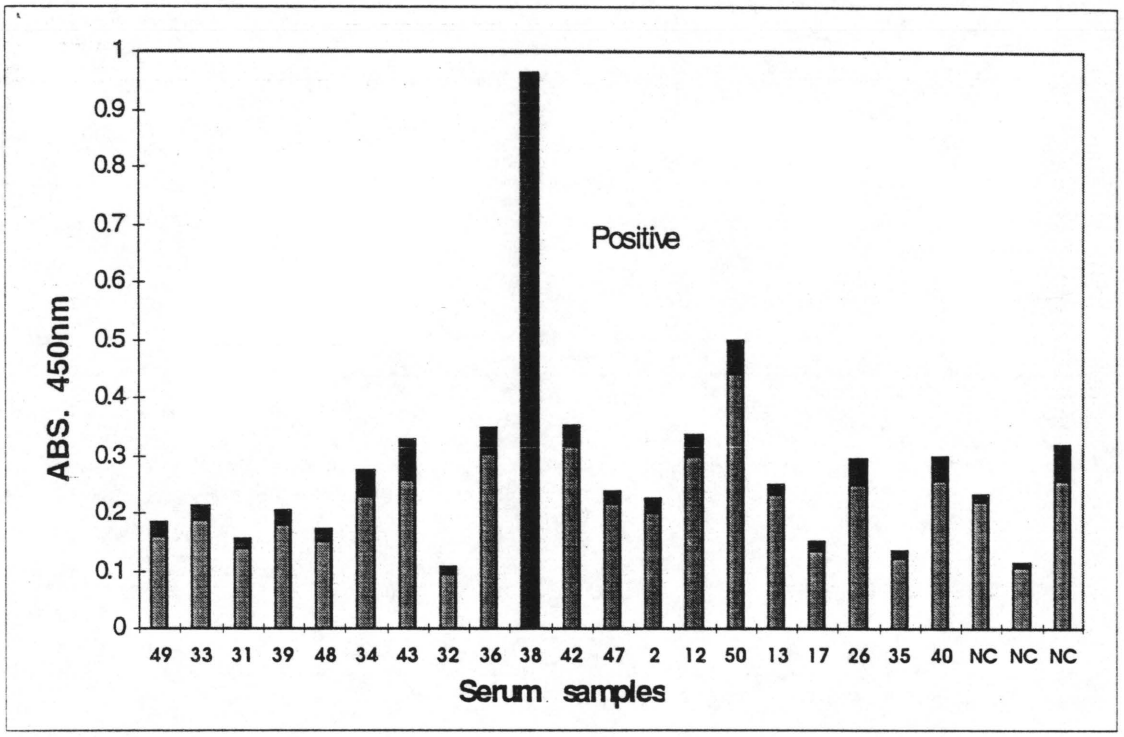
The Western blot was repeated six times with different patient and control sera, but the MA-specific band observed in Figure 2.1 could not be seen again. This indicated that the observation in Figure 2.1 was either an artifact or the presence of such antibodies in *M. tuberculosis* patients occur at a very low frequency. Due to small sample volumes and high concentration of the serum needed to complete the Western blot, individual patient sera could not be screened. Because only a few samples could be screened in one gel using PAGE and Western blot analysis, it was decided to screen patient sera for the presence of anti-MA-specific antibodies first by means of a MA-specific ELISA.

3.2 Screening of individual patient-and control sera by ELISA.

In the Western blot analysis only pooled patient sera could be examined due to small sample volumes. To determine if MA-specific antibodies were present in individual patient sera, the ELISA technique was used. Patient sera, diluted 1:10 times in casein PBS buffer, were screened on ELISA plates coated with pure MA to a concentration of 3 μg per well. To exclude the possibility of non-specific binding of antibodies to the plates, the samples were also tested on non-coated plates, blocked with casein.

To enable identification on the resulting graphs, all patient sera were allocated separate numbers. Control sera used were allocated alphabetical letters. Control and patient sera regarded as negative gave a base level signal that varied between 0.2 and 0.4 at a wave length of 450 nm on the plates coated with MA (Figures 2.2 a and c). In Figure 2.2a only one patient (patient 38) tested positive with an OD_{450} of 0.8, which was two times higher than the base level on the MA coated plates. In figure 2.2c, patient 14 appears positive as well, and the identification of patient 38 as positive is confirmed. To exclude the possibility of non-specific antibody interaction with the ELISA plate, the experiment was repeated on non-coated plates (Figure 2.2 b and d). All the sera tested gave low signals of ± 0.1, thus confirming the presence of MA-specific antibodies in patient 38. Figure 2.2 is an example of ELISA results obtained in these experiments and Figure 2.3 is representative of the results obtained of all the sera tested.

A. Plates coated with MA:



B. Non-coated plates:

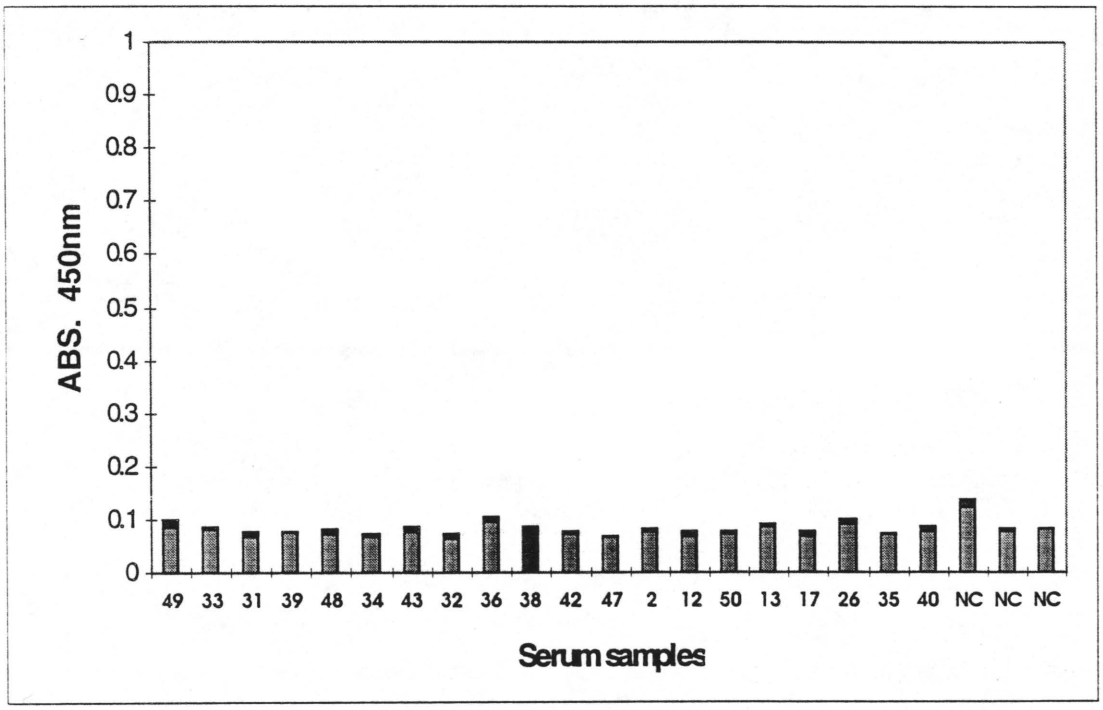
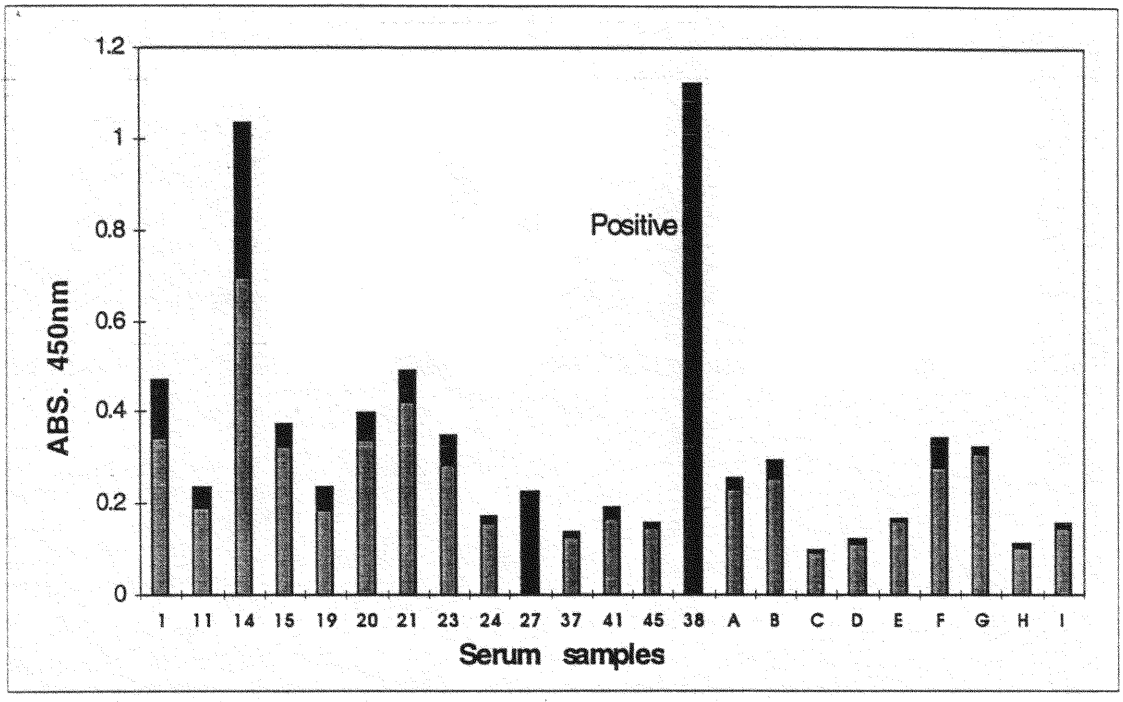


Figure 2.2. *Detection of MA-specific antibodies in various individual human patient sera on* MA *coated ELISA plates (2.2 a and c). All sera were diluted 1:10 times. Each patient was allocated a specific number and the negative controls were indicated with NC or capital alphabetical letters. The same sera were also screened on uncoated ELISA plates (2.2 b and d).*

C. Plates coated with MA:



D. Non-coated plates:

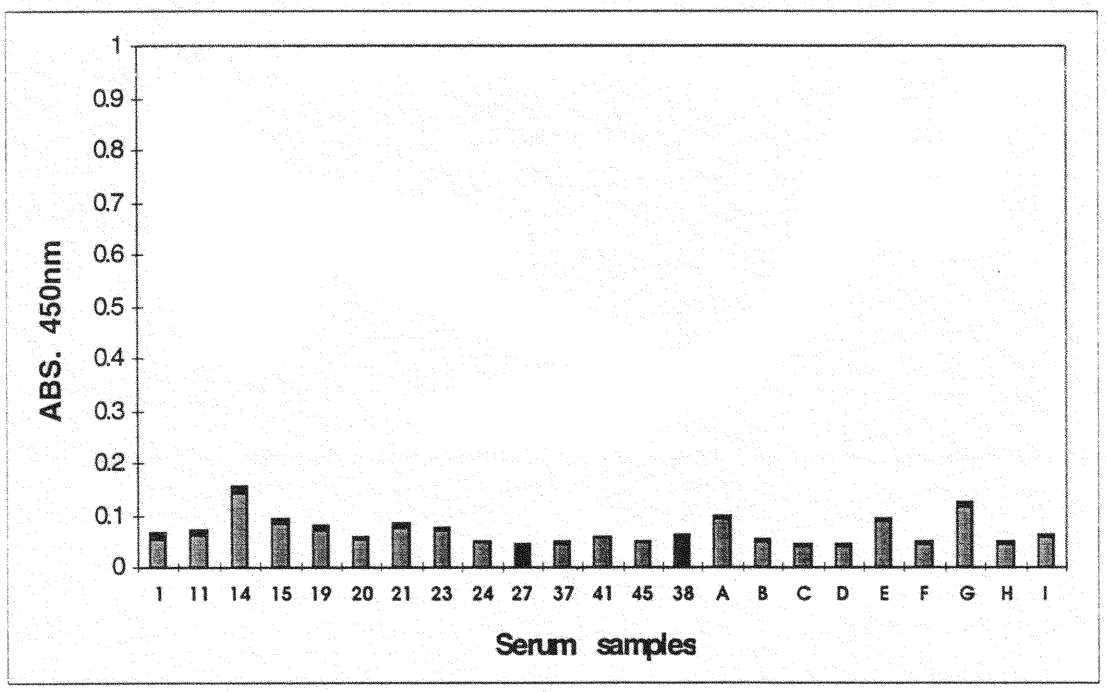


Figure 2.2. *Detection of MA-specific antibodies in various individual human patient sera on* MA *coated ELISA plates (2.2 a and c). All sera were diluted 1:10 times. Each patient was allocated a specific number and the negative controls were indicated with NC or capital alphabetical letters. The same sera were also screened on uncoated ELISA plates (2.2 b and d).*

The ELISA was repeated for 120 different patient sera including control human serum. The combined results from all these sera are presented in Figure 2.3. Signals that were more than two times higher than the background signal on PBS plates and the control human sera were regarded as positive. The ELISA results indicated that 20 % of the patient sera tested have MA-specific antibodies, confirming the low frequency of antibodies observed in the Western blot experiments. The average OD_{450nm} (0.84) of the positive group sera differed significantly from the average (0.18) of the negative control ($p < 7.14 \times 10^{-3}$). In 15 % of the sera tested, significantly high back-ground signals were observed as compared to negative control sera ($p < 1.4 \times 10^{-3}$), indicating the presence of rheumatoid antibodies. The remaining 65 % patient sera tested negative for anti-MA-specific antibodies.

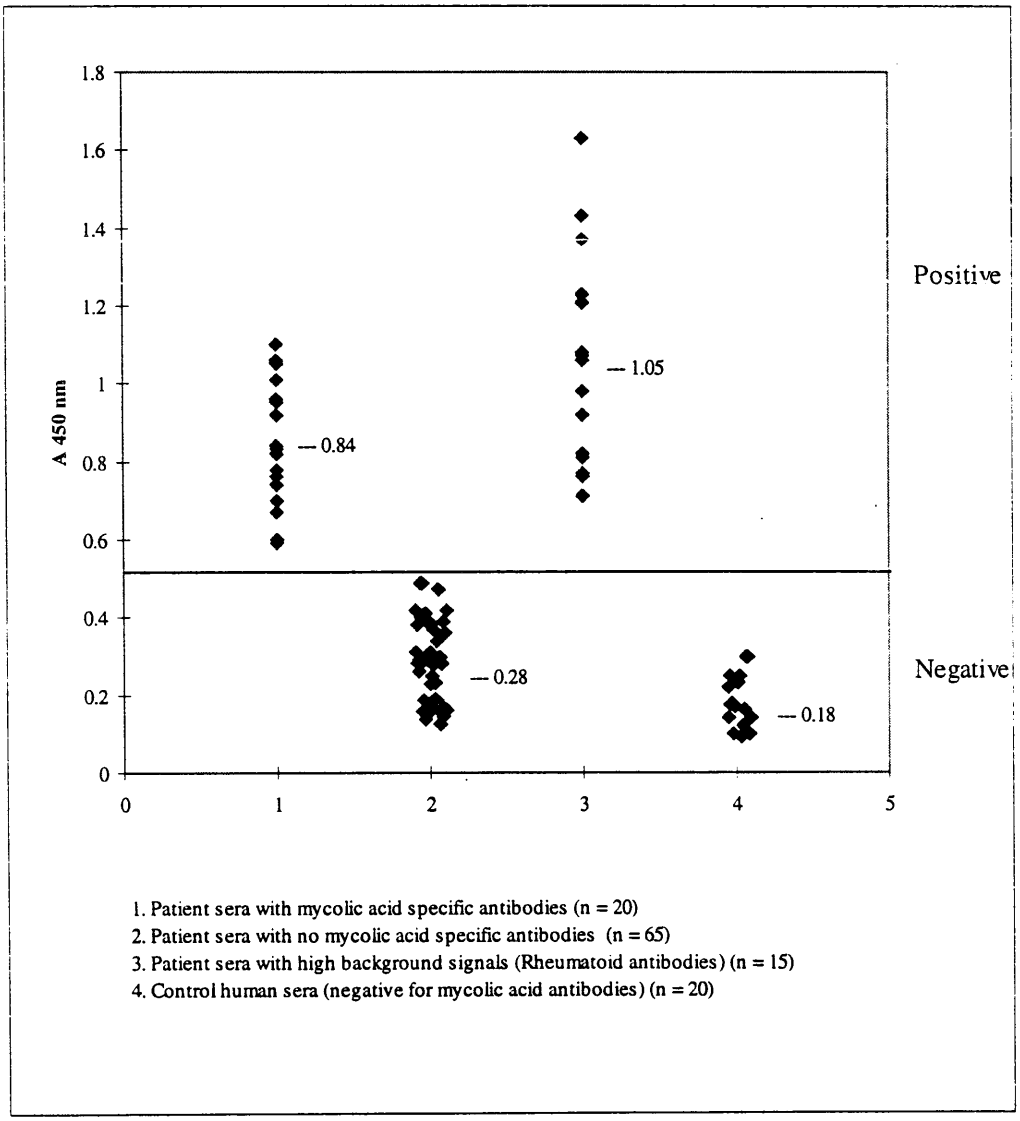


Figure 2.3. *Mycolic acids specific antibodies in M. tuberculosis-infected and control human sera as determined by ELISA. The average OD 450 of each group is indicated. Each point represents the mean value of triplicate measurements on different wells of the ELISA plates.*

3.3. Analysis of sera obtained from *Mycobacterium tuberculosis*-infected mice.

To determine whether anti-MA antibodies can also be induced by *M. tuberculosis* infection in mice, Balb/c mice were infected with 5 x 10^4 cells of virulent *M. tuberculosis*. Mice were bled 110 days after the infection was initiated. The sera, diluted 1:20 times, were analysed on MA-coated and uncoated ELISA plates. No signals were obtained with IgG conjugate (results not shown), but IgM antibodies were detected in this experiment.

From the ten individual mice analysed, only mouse No. 1 (M1) and mouse No. 8 (M8) gave signals that were two times higher than that of the negative control (C) at $OD_{450\ nm}$ (Figure 2.5). Mouse No. 4 and Mouse No. 6 showed high readings, but as the background reading was high as well, this could have been due to the presence rheumatoid antibodies, which non-specifically interact with one another and stick to the ELISA plate.

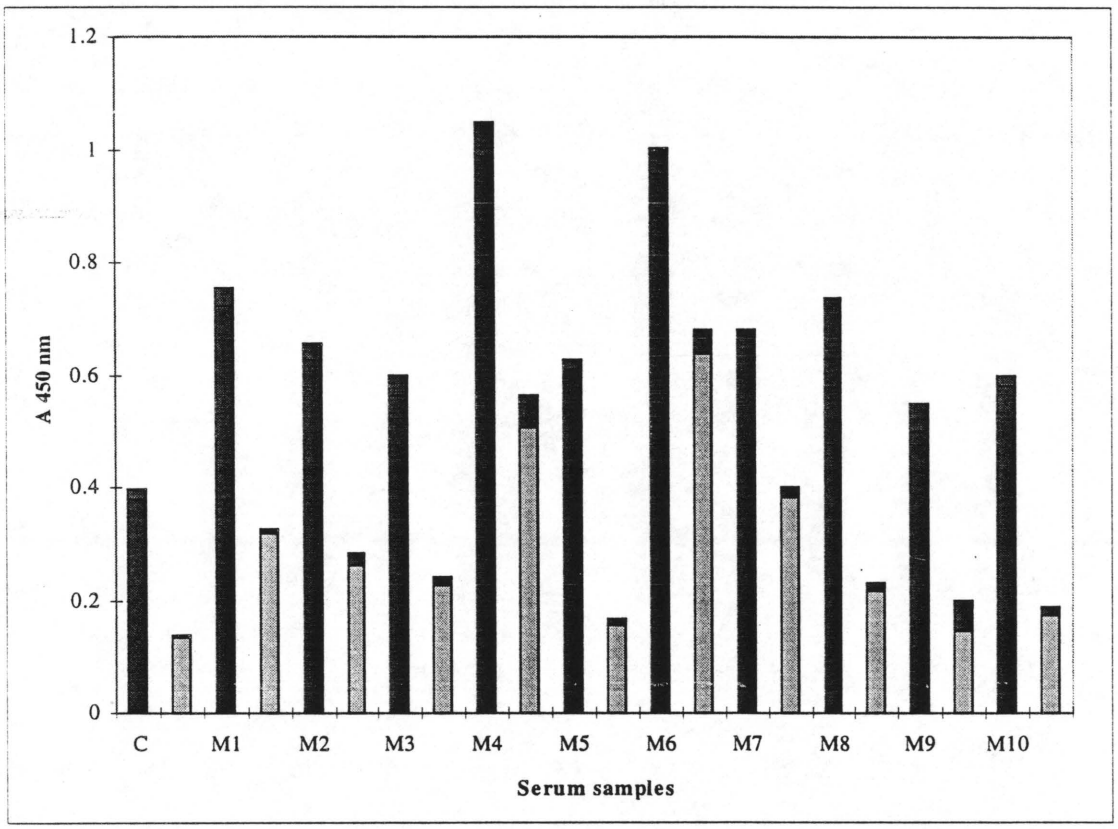


Figure 2.5. *Detection of MA-specific antibodies in M. tuberculosis-infected mouse sera on* MA *coated ELISA plates (■). All sera were diluted 1:20 times. Each mouse was allocated a specific number. The experiment was repeated on uncoated plates to indicate non-specific binding. (□) C= control, M= infected mouse (n = 10).*

These results seem to correlate with those obtained with the human sera. As in humans, 20 % of the mice infected with *M. tuberculosis* showed MA-specific antibodies and the presence of rheumatoid antibodies was also observed in some cases. Interestingly, in mouse sera, only IgM antibodies could be found, whereas in human sera the anti-MA antibodies appeared to be of the IgG class, by the way they were inhibited in ELISA by soluble ligand. This difference could be due to different isotypes of CD1 present in humans and mice, which are now known to be expressed on APC to present MA and other hydrophobic antigens to T cells.

4. Discussion

Antigen presenting cells (APC) isolated from the sera of *M. tuberculosis*-infected humans, have been shown to present MA on CD1, a MHC-like molecule (Bendelac, 1993; Porcelli *et al.*, 1995; Yoshimoto *et al.*, 1995). This has lead to the hypothesis that the presentation of MA on CD1 and the subsequent activation of T cells can induce antibodies directed to MA. The present study demonstrated that MA-specific antibodies were indeed detected in *M. tuberculosis*-infected human and mouse sera.

Western blot analysis of a gel of mouse MA-serum conjugate and mouse serum obtained under non-reducing conditions on SDS-PAGE, was initially performed to indicate the presence of MA-specific antibodies. The observed band at ± 90 kDa on the Wersten blot, not only suggested that anti-MA antibodies existed in *M. tuberculosis* patient serum, but also that MA could be carried on a preferred serum protein that rendered the MA molecule antigenic. This carrier protein seemed to be a minor protein, because no clearly visible Coomassie stained serum protein band corresponded to the MA-protein band.

Antibodies directed to the lipid fraction of the cell-wall of *M. tuberculosis* have been reported previously. These included antibodies directed to a phenolic glycolipid and cord factor (Cho *et al.*, 1992; He *et al.*, 1991; Kashima *et al.*, 1995; Simmoney *et al.*, 1995). Cord factor consists of a saccharine (trehalose, mannose or glucose) covalently linked to one or more MA residues that can vary in the carbon chain length (Orbach-Arbouys *et al.*, 1983; Guillermard *et al.*, 1993).

Protein-lipid complexes have been shown to have different mobilities under reducing and non-reducing SDS-PAGE conditions (Nakano *et al.*, 1996). The band observed at 90 kDa is therefore not an indication of the molecular weight of the carrier protein alone, but is rather an indication of the molecular weight of the protein-lipid complex.

As the lipid:protein ratio of the 90 kDa MA-protein complex is not known, the probable protein mass can not be determined. Protein such as albumin (a known lipid carrier protein in serum) cannot be excluded as a possible MA carrier protein. Other serum proteins can also be considered to be likely candidates.

In studies where cord factor was considered as an antigen for possible sero-diagnosis, 70-90 % of the *M. tuberculosis*-infected patients tested positive for anti-cord factor antibodies (He *et al.*, 1991; Simmoney *et al.*, 1995). In this study, only 20 % of human patients were shown to be positive for anti-MA antibodies. This difference may be explained by the fact that the saccharine moiety of cord factor was shown to be the dominant epitope. MA as the weaker epitope, would not be detected in typical immuno-assays such as ELISA in the presence of the stronger epitope (Kato *et al.*, 1972 and 1974). Furthermore, the presence of anti MA antibodies was also confirmed in an experiment, using a murine model. Two out of ten mouse sera tested positive for anti-MA antibodies.

The serum of one of the patients tested (Patient 38) was used to determine the specificity of the anti-MA antibodies, using an inhibition ELISA (Goodrum, 1998). MA are very hydrophobic molecules and therefore it cannot be dissolved in serum. MA was first conjugated to mouse serum prior to mixing with human serum. Patient 38 serum was incubated with either MA-mouse serum conjugate (1:1 v/v) or mouse serum (1:1 v/v) for 30 min at room temperature. The ELISA was repeated as described earlier. Mixing human patient serum with mouse MA-serum conjugate significantly reduced the ELISA signal (OD 450 ± 0.2), whereas the serum alone did not have any effect on the signal (OD 450 ± 0.8). Because inhibition of the signal was observed, the MA-antibodies may be considered specific for MA and were most likely of the IgG class.

In these experiments 15 % of the patient sera and 20 % of the mouse sera tested, gave very high background values on the ELISA. These high background values can be attributed to the presence of rheumatoid antibodies in the sera. In some of the Western blot experiments high background staining of the PVDF membranes was also observed. It was reported that the presence of *M. tuberculosis* in complete Freund's adjuvant can elicit anti-collagen auto-immune arthritis in mice (Mauri *et al.*, 1996; Kasama *et al.*, 1994). The presence of rheumatoid antibodies in 15 % of the *M. tuberculosis*-infected patients and in the *M. tuberculosis*-infected mice could be an early indication of the development of collagen-induced arthritis in these individuals. In the Western blot analysis a band of ±80 kDa was observed irrespective of the antigen used (mouse serum or MA-serum conjugate). This band

could be an indication of the development of auto-immune antibodies directed to serum protein molecules in *M. tuberculosis*-infected patients.

Human patients can be exposed to many pathogens in their lifetime. During *M. tuberculosis* infection or vaccination, an immune memory can develop, which can recall the specific immune response to *M. tuberculosis* after re-challenge with the pathogen, thereby launching a protective response without showing any detectable symptoms of the disease (DiPiro, 1997). This response could be accompanied by the production of IgG antibodies. In the mouse experiments, the mice were not vaccinated, therefore no previous exposure to *M. tuberculosis* occurred. It is possible that class switching from IgM to IgG did not occur in the mice, due to the lack of participation of T cells responsible for memory in adaptive immunity.

The fact that the patient sera contained anti-MA antibodies that differed from those in mice could be explained by means of the different isotypes of CD1 present in humans and mice. In humans, CD1 can be divided into two distinct groups according to their sequence homology, type of cells that express the molecules and the type of T cells that become activated. Group one consists of CD1 a, -b and -c whereas group two consists of CD1d that shows homology to murine CD1 (Beckman *et al.*, 1995; Porcelli *et al.*, 1995). CD1b is known to present MA to specific double negative αβ TCR T cells (Beckman *et al.*, 1994). There is no CD1b or its equivalent in mice. It has not yet been reported that CD1d or the murine homologue can present MA on APC, but according to the crystalline structure of the murine CD1, lipid presentation seems very likely (Zeng *et al.*, 1997). The presentation of galacto-ceramide and glycolipds on murine CD1d was however indicated (Brossay *et al.*, 1998; Kawano *et al.*, 1997).

In experiments on *M. tuberculosis*-infected human patient sera, an increased frequency of IL-4 producing cells have been reported in some tuberculosis patients. The investigators speculated that Th2 response mechanisms were involved in some cases of defective host resistance (DiPiro, 1997; Surcel *et al.*, 1994). This could explain the presence of MA-specific antibodies in the sera of some patients infected with *M. tuberculosis*.

Recently it has been observed that group 2 CD1d, which shows homology to murine CD1, presents antigen to a specific subgroup of T cells that express NK cell surface markers known as $NK1.1^+$ $CD4^+$ T cells (Park *et al.,* 1998; Couedel *et al.,* 1998; Bendelac, 1993). In a murine model it was shown that the primary stimulation of these T cells resulted in the secretion of IL-4 (Bendelac, 1993; Porcelli *et al.,* 1995; Yoshimoto *et al.*, 1995). IL-4 is known to direct the immune response to the Th2 mode that involves antibody production.

During initiation of *M. tuberculosis* infection, IL-4 is secreted by these $NK1.1^+$ $CD4^+$ T cells after stimulation with antigen, but normally it is down-regulated by cytokines such as IL-12 in the spleen (Dougelat and Kaufmann, 1996). One could, however, speculate that patients with an increased IL-4 secretion profile, a predominant Th2 response could therefore be a direct result of the inability of the host to down-regulate the early IL-4 secretion by $NK1.1^+$ T cells. The direct effect of this secreted IL-4 is therefore not observed. To date, most of these experiments were done on murine models as mice have only the CD1d homologue. Recently, a human NKT cell homologue has been identified, also recognising glycolipids presented on CD1d (Couedel *et al.,* 1998; Park *et al.,* 1998; Brossay *et al.,* 1998).

In contrast, studies using *Mycobacterium leprae* and human monocytes as a model, have shown an increase in the expression of IFN-γ after recognition of CD1b antigen presentation by T cells. There was also a correlation between the level of CD1b proteins expressed and the degree of effective cellular immunity. Expression of MA on CD1b is therefore implicated in inducing the Th1 mode of protection (Porcelli *et al.,* 1995).

Presentation of MA on the different isotypes of CD1 could therefore have an influence on the outcome of the disease and the level of antibodies that are present in host sera. Although antibody production is generally associated with the Th2 immune response, a functional consequence of Th1 activation is the expression of complement-fixing and opsonizing antibodies such as IgG2a. In contrast, Th2 cells promote the development of IgG1 and IgE expression (Bliss *et al,* 1996).

It would therefore be worthwhile to investigate the mechanism of MA antibody induction further by means of determining the specific isotype of IgG present in the MA antibody

positive human patients. This would give a good indication of the predominant T helper mode in the anti-MA positive patients.

Another factor that has to be taken into consideration is the recent observation that patients infected with multi drug resistant tuberculosis (MDR *M. tuberculosis*) show impaired Th1 responses (De Jong *et al.*, 1997). Some patients infected with MDR *M. tuberculosis* have shown a decrease in the number of CD4 cells, and have shown deficient production of IL-2 and IFN-γ by peripheral blood monocytes. These individuals could therefore be expected to express high levels of IL-4, influencing the antibody isotype that is produced. Further investigation into the medical history of each patient tested for anti-MA antibodies could reveal the mechanism whereby these antibodies came about.

Chapter 3
The Influence of Mycolic acids on Macrophage-derived Cytokines

3.1. Introduction

Mycobacterium tuberculosis is known to commonly infect human and animal hosts. Immunohistochemistry experiments done on the lungs of Balb/c mice have indicated that the progression of tuberculosis (TB) can be divided in two phases. The acute phase (0-35 days) is characterized by a Th1 type immune response. This is followed by a chronic phase (36 days-4 months). In the chronic phase a Th0 balance was observed, indicating that the Th1 and the Th2 cytokines are present at the same levels (Rook and Hernandez-Pando, 1996).

Inhalation of tubercle bacilli into the deep lungs ultimately results in the engulfment of the bacteria by alveolar macrophages. This leads to the initial activation of these alveolar macrophages. Activated macrophages perform three major functions: i) the production of proteolytic enzymes and other metabolites that exhibit mycobacteriocidal effects, ii) the processing and presenting of mycobacterial antigens to T cells and iii) the production of cytokines (Barnes and Modlin, 1996). Cytokines secreted by macrophages include pro-inflammatory cytokines (IL-12, IL-1, IL-6, GM-CSF and TNF-α) and inhibitory cytokines such as TGF-β and IL-10 (Fulton *et al.*, 1996 and 1998; Boom, 1996).

IL-12 is a well defined key factor in the induction of a protective immune response to *M. tuberculosis* infection. This cytokine is also produced by B cells, dendritic cells and neutrophils (Trinchieri, 1993; Trinchieri and Scott, 1994; Snijders *et al.*, 1996). IL-12 is known to activate T lymphocytes and NK cells to produce cytokines such as IFN-γ and the subsequent secretion of TNF-α. IL-12 is therefore a key factor in inducing a protective cell-mediated immune response (Trinchieri and Scott, 1994; Flynn *et al.*, 1995; Cooper *et al.*, 1997; Emoto *et al.*, 1997).

TNF-α plays an important role in the formation of granuloma in *M. tuberculosis* infection. *In vivo* neutralization of TNF-α, in a *M. tuberculosis*-infected murine model, resulted in uncontrolled bacterial multiplication (Andersen, 1997). IFN-γ, on the other hand, is crucial for

macrophage activation and can therefore promote cell-mediated immunity (DiPirio, 1997; Flynn *et al.,* 1993). The pro-inflammatory effects of these cytokines can be down-regulated by immuno-suppressive cytokines such as IL-10 and TGF-β.

It is known that *M. tuberculosis* and its antigens can induce cytokine production *in vitro*. Purified Protein Derivative (PPD) can induce IL-1, IL-10, TNF-α and TGF-β expression in mononuclear phagocytes. Induction of IL-12 expression apparently requires phagocytosis of particulate antigens, such as live or dead *M. tuberculosis* bacilli, rather than low molecular weight antigens such as PPD (Fulton *et al.,* 1996).

In the search for anti-mycobacterial vaccines and therapies, the emphasis has been put on protein antigens and antibiotics. The persistence of tuberculosis warrants research into possible involvement of compounds other than proteins as vaccine or antibiotic targets. Recently it has been shown that a MHC-like protein family, CD1, can present lipid antigens to specific T cell subsets (Porcelli *et al.,* 1995; Beckman and Brenner, 1995). The mechanism whereby CD1-lipid presentation exerts its effects on the immune system is still unclear. The very existence of these lipid-presenting molecules leads to the assumption that lipid antigens can also play an important role in the induction or inhibition of a protective immune response to infections caused by *M. tuberculosis*.

The mycobacterial cell-wall consists of an abundance of potential lipid antigens including lipoarabinomannan (LAM), glycolipids and mycolic acids (Fenton *et al.,* 1996; Brennan and Nikaido, 1995). Mycolic acids (MA) are high molecular weight α-alkyl β-hydroxy fatty acids, with the β-carbon chain varying in length. *M. tuberculosis*-derived MA have a β-carbon chain with 50 to 60 carbon residues (Almong, 1996). In the cell-wall, some MA are associated with trehalose molecules, and are then known as cord factor.

Cord factor (trehalose dimycolate) was the first *M. tuberculosis*-derived molecule that was shown to induce IL-12 stimulation *in vivo*. IL-12 production was found to coincide with IFN-γ secretion. Both these cytokines play an important role in the induction of the protective cellular immune response to tuberculosis infections (Oswald *et al.,* 1997). This finding places

the emphasis on glycolipid and or lipid molecules as possible inducers of IL-12 expression rather than on protein antigens.

The effects of cord factor on bacterial infections have been studied in great detail. It has been shown that cord factor administered 14 days before *M. tuberculosis* infection was initiated, had a protective effect on the development of the disease (Parant *et al.*, 1977). Cord factor was also found to enhance NK cell activity and γδ T cell migration to the lungs (Tabata *et al.*, 1996), which resulted in the enhancement of granuloma formation (Asano *et al.*, 1993; Matsunga *et al.*, 1996). To date, the involvement of either the carbohydrate or the MA moieties of TDM in the protective immune response to tuberculosis has not been established (Matsunga *et al.*, 1996).

Immunity to intracellular pathogens in murine models has been shown to be unique to each mouse strain tested. Experimental models using *Leishmania major* as intracellular pathogen, resulted in a disease with a dominant Th1 response in the more resistant C57BL/6J mice. The disease in the susceptible Balb/c strain was associated with increased levels of IL-4 in the spleen, a Th2 type cytokine (Kaufmann and Douglat, 1995). Furthermore, this difference in resistance could not be correlated to differences in major histocompatibility (MHC) gene (Medina and North, 1998).

Besides the MHC gene complex, the Bcg (Nramp1) gene, is also known as a resistance gene, and is located on chromosome 1 of the murine genome (Medina and North, 1996 and 1998). There is conflicting evidence regarding the role of Bcg during infection with virulent *M. tuberculosis*, but it is believed that the Nramp1 gene controls macrophage resistance or susceptibility to several microorganisms. Recently, it was indicated that Nramp1 could also regulate nitric oxide (NO) secretion, a potent anti-microbial agent (Arias *et al.*, 1997). Macrophages with the Bcg r (resistant) gene secrete more NO in the presence of IFN-γ than cells with the Bcg s (susceptible) gene. It is this regulation of NO secretion in different cells that might play a role in the regulation of macrophage resistance or susceptibility towards *M. tuberculosis* infection (Arias *et al.*, 1997; Bonecini-Almeida *et al.*, 1998).

In experiments done by Stanford and Stanford (1996a and b) and Rook and Herdnandez-Pando (1996), heat-killed *Mycobacterium vaccae* bacteria were injected into mice one week before initiation of *M. tuberculosis* infection. This resulted in an enhancement of the Th1 response and ultimately in protection of the experimental animals. A protective effect was also observed when heat-killed *M. vaccae* were administered after the infection was initiated (Stanford and Stanford, 1996; Rook and Hernandez-Pando, 1996).

The protocol of Stanford and Stanford (1996a and b) and Rook and Herdnandez-Pando (1996), served as a reference in this study to determine the immuno-regulatory properties of pure *M. tuberculosis*-derived MA during *M. tuberculosis* infection in a murine model. The immunological effects of MA were determined in two inbred mice strains: C57BL/6J mice that are relatively resistant to *M. tuberculosis* infection and Balb/c i.e. mice relatively susceptible to *M. tuberculosis* infection (Flynn *et al.*, 1995). By means of profiling the cytokines secreted by macrophages, the role of MA in the induction or inhibition of a protective immune response was determined. Cytokine profiling was done on the lungs, being the normal infection site of *M. tuberculosis*, although infection was introduced by intravenous injection to enable better control over the dose administered.

Cytokine profiles of macrophage-derived IL-12, TGF-β and T cell or NK cell-derived IFN-γ were determined. The IL-12 heterodimer (p70) is composed of two covalently linked glycosylated chains, p40 and p35. These chains are encoded on two separate genes on chromosome 1, in humans. The expression of both subunits is required for a biologically active protein. IL-12 production is regulated trough the inducible expression of p40, whereas p35 is expressed constitutively, or regulated minimally. This lead to the assumption that the level of expression of IL-12 p40 is representative of p70 production (Trinchieri, 1993; Trinchieri and Scott, 1994; Schoenhaut *et al.*, 1992; Snijders *et al.*, 1996).

The aim of the project was to determine the potential of MA to prevent or cure *M. tuberculosis* infection by correlating the macrophage-derived cytokine profiles to the survival provided by MA treatment either before or after *M. tuberculosis* infection in mice.

3.2. Materials and Methods

3.2.1. Materials

Inbred female and male C57BL/6J and Balb/c mice were obtained from the South African Institute for Medical Research (SAIMR), Johannesburg, RSA.

Mycolic Acids (MA) were purified according to the protocol described in patent no. SA 95/1464(PCT/GB 96/00416).

The *M. tuberculosis* H37 Rv ATCC 27294 stain was used as the source of MA and for animal infection. This was a virulent strain, originally isolated from infected human lungs, purchased in lyophilized form, from the American Type Culture Collection (ATCC), Maryland USA.

Materials used for MA purification include Methanol (AR), Chlorophorm (AR), Acetone (AR) and sodium chloride purchased from Merck, Darmstadt, Germany.

Sodium chloride, potassium hydroxide, hydrogen chloride, Tris (hydroxymethyl)-aminomethane, EDTA, sodium acetate, magnesium chloride, ethanol, boric acid, formaldehyde and potassium chloride were purchased from Merck, Darmstadt, Germany.

Methanol (AR), chloroform (AR) and methylene chloride (AR) were products obtained from BDH Laboratory Supplies, Dorset England.

Nitrogen was obtained from Afrox, Johannesburg, RSA.

Yeast extract, Tryptone and Agar was purchased from Difco Laboratories, Michigan, USA.

Qiagen Mini Prep column kit was obtained from Qiagen Inc., Chatsworth, USA.

AmplitaqGold was purchased from Perkin Elmer, Roche, New Jersey, USA.

Superscript RNase H Reverse Transcriptase, Oligo (dT) primers and Formamide (AR) were purchased from Gibco-BRL, supplied by Life Technologies Inc., Gaithersburg, USA.

Tri-reagent was obtained from Molecular Research Centre (MRC) Inc., Cincinnati, USA.

Promega Corporation, Madison, USA supplied Rnasin, dNTP and Agarose.

MOPS, diethyl pyrocarbonate and ethidium bromide were purchased from Sigma Co., St. Louis, USA

DMEM (Dulbecco's modified Eagle's Medium), streptomycin, pyruvate and ampicillin were purchased from Pharmagen Biotechnology, Pistacaway, New Jersey, USA.

SURE Epicurean *E. coli* competent cells were obtained from Stratagene, New York USA.

3.2.2 Methods

3.2.2.1 Infection of mice with *Mycobacterium tuberculosis*

Bacteria: *M. tuberculosis* H37 Rv (ATCC 27294) was cultured on Löwenstein-Jensen (LJ)-slants at 37°C for 3 to 6 weeks. For each experiment, bacteria of a total count varying between 10^4 - 10^6 per ml were suspended in 0.89 % sterile saline, washed twice by centrifugation (2000 x g for 15 min), and re-suspended in 0.89 % saline. Bacterial viable counts of the samples were determined by counting colony forming units (CFU) in 1:10 to 1:1 000 000 dilutions on Middlebrook 7H10 agar after incubation at 37 °C for 4 to 6 weeks. Total count was determined by means of a direct count using a Neubauer counting chamber and various dilutions of bacterial suspensions.

Infection of mice: Inbred female C57BL/6J and Balb/c mice (SAIMR) were used at 6-8 weeks of age. Animals were infected intravenously with *M. tuberculosis* H37 Rv (10^4-, 10^5- and 10^6 CFU in 100 µl sterile 0.89 % saline) *via* a lateral tail vein.

3.2.2.2 Treatment of mice with mycolic acids-serum conjugate

Female C57BL/6J and Balb/c mice were either pre-treated with control serum or with mycolic acids (MA)-serum conjugate one week before the infection with *Mycobacterium tuberculosis*, or were treated with control or MA-serum conjugate three weeks after the *M. tuberculosis* infection was initiated. Different MA concentrations were used in the different experiments.

MA Purification: *M. tuberculosis*-derived MA were extracted, purified and derivatised as described by Butler *et al.* (1991), and purified according to Goodrum (1998). Briefly, bacteria were scraped from LJ slants and resuspended in 25 % potassium hydroxide in methanol-water (1:1). The suspension was vortexed in the presence of glass beads and homogenized. The cells were subsequently saponified by autoclaving at 120 °C for 30 min. This was followed by the addition of 1.5 ml 50 % (v/v) HCl per 2 ml sample to adjust the pH to pH 1. The lower phase was transferred to a new vial, after chloroform-water (1:1) extractions. The chloroform was evaporated at 85 °C in a heat-block evaporator, under a flow of nitrogen. A volume of 100 µl of 2 % K_2CO_3 dissolved in methanol-water (1:1) was added and the sample was evaporated to dryness.

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

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

The samples were vortexed and heated for 20 min at 85 °C, followed by the addition of concentrated HCl-methanol-water (1:2:1). The bottom layer was removed and evaporated to dryness at 85 °C under a stream of nitrogen. The dried residues were re-suspended in methylene chloride and quantified by HPLC in comparison to an internal standard (Ribi *et al.*, 1977).

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

Preparation of MA-serum conjugate and control serum: Blood was obtained by bleeding male Balb/c and C57BL/6J mice from the dorsal tail vein. Serum was obtained after the blood was allowed to clot at 4 °C for 60 min, followed by centrifugation (12000 x g for 10 min). The sera of each strain were pooled and were stored at -20 °C until used.

Conjugates of MA and serum were obtained by adsorption. MA were analytically weighed and dissolved in chloroform. The volume of chloroform that was added never exceeded 2 % of the final volume of serum into which the MA were absorbed. The chloroform/MA were added to the serum and were sonicated on a Branson sonifier B-30 for 50 pulses at an output control of 2 and a 20 % duty cycle at room temperature. Control serum was prepared by adding 2 % pure chloroform (v/v) to the serum before the mixture was sonified as described for MA-serum conjugate. The chloroform was removed by vigorously bubbling nitrogen over

the surface. The serum was left to stand at room temperature for at least one hour to allow air bubbles to escape before it was injected in the mice.

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

3.2.2.3. Preparation of the organs used for cytokine profiling

The organs originating from both infected and uninfected mice, used for the semi-quantitative RT-PCR were the lungs, spleen, kidney, liver and the heart. Mice were sacrificed by rapid cervical dislocation. The organs were removed from each mouse aseptically, and kept at -72 °C after snap freezing in liquid nitrogen.

Histopathology: The organs of mice were also removed for histopathology. Sections of paraffin blocks containing lungs, liver and spleen were stained by the Ziehl-Nielsen method for acid-fast bacilli. The amount of granuloma per 10 x magnification field was determined by means of light microscopy.

3.2.2.4. Cytokine profiling

Optimization of the different Cytokine PCRs and the β-actin PCR

Cytokine PCRs were optimized by using recombinant plasmids containing deletion or insertion mutants of the genes of the various cytokines to be tested. Both the mutation and the wild type cDNA could therefore be amplified by using the same primers. Three different plasmids were used for this purpose: i) a plasmid obtained from K Bost (University of Tulane, USA) was used for IL-12 optimization, (ii) a plasmid obtained form Tarleton (University of Georgia, USA) was used for the optimization for TNF-α, and iii) a plasmid obtained from Locksley (University of California, USA) was used for the optimization of TGF-β.

To ensure that there was enough material to work with, the plasmid was amplified by transformation into SURE *Escherichia coli.* This host is rec$^-$ (the *E. coli.* K restriction system

is absent) and no chromosomal recombination can occur. The bacteria were transformed with 5·µl plasmid / 100 µl competent SURE *E. coli* by the heat shock method. The SURE *E. coli* cells and the plasmid DNA were incubated on ice, in a 50 mM calcium chloride solution (ice-cold) for 30 min. This was followed by a heat shock at 42 °C for two minutes.

The transformed bacteria were plated out on LB agar plates containing ampicillin (amp) at a concentration of 50 µg/ml. The cells were incubated in LB broth (10 % triptone, 5 % yeast extract and 5 % sodium chloride), to allow the phenotypic properties conferred by the plasmid (amp resistance) to be expressed. The plasmid-containing bacteria were then transferred to LB agar plates, containing amp. After 16 h incubation at 37 °C, clones were observed. Four independent clones were picked and were grown in LB broth, with amp, for 17-18 h at 37 °C. Plasmid was then isolated with the Qiagen Mini Prep Column Kit. The resulting plasmid was re-suspended in 100 µl TE buffer (10 mM Tris pH 8, 1 mM EDTA) and quantified with the fluorimeter.

The PCR conditions for the different cytokines were optimized by using different $MgCl_2$, primer and enzyme concentrations. The annealing temperature and the amount of cycles used were the same as described in various articles (Chong *et al.*, 1996; Reiner *et al.*, 1994; Benavides *et al.*, 1995). The β-actin PCR was optimized by using cDNA as no plasmid was available. The primer sequences and the annealing temperature used in the optimization of the PCR are listed in Table 3.1.

Table 3.1: *A list of the sequence of the sense and the antisense primers, their annealing temperatures and the wild type fragments size.*

Type of PCR	Sequence of the primers (S-sense; AS-antisense)	Fragment Size	Annealing Temperature
1. β-actin [1]	S: $^{5'}$CTC CAT CGT GGG CCG CTC TAG$^{3'}$ AS: $^{5'}$GTA ACA ATG CCA TGT TCA AT$^{3'}$	133 bp	59 °C
2. IL-12 [2]	S: ^{5}CCA CTC ACA TCT GCT GCT CCA CAA G$^{3'}$ AS:^{5}ACT TCT CAT AGT CCC TTT GGT CCAG$^{3'}$	266 bp	60 °C
3. TNF-α[3]	S: $^{5'}$ GTC TAC TTT AGA GTC ATT GC $^{3'}$ AS: $^{5'}$ GAC ATT CGA GGC TCC AGT G $^{3'}$	275 bp	48 °C
4. TGF-β[4]	S: $^{5'}$ ACA GGG CTT TCG ATT CAG CGC $^{3'}$ AS: $^{5'}$ CAC CTA GGT GCT CGG CTT CCC $^{3'}$	306 bp	60 °C
5. IFN-γ[4]	S: $^{5'}$ CAT TGA AAG CCT AGA AAG TCT G $^{3'}$ AS: $^{5'}$ GCT TTT TCC TAC GTA AGT ACT C $^{3'}$	267 bp	60 °C

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

Semi-quantitative RT-PCR

RNA extraction from control and infected organs

RNA was isolated from all the organs using the TRI-Reagent protocol. TRI-Reagent is based on the Chomeynski and Sacchi (1987) RNA isolation method - an acid guanidium thiocyanate-phenol-chloroform extraction. The isolated RNA was quantified by a spectrophotometer at wavelengths of 260 nm and 280 nm. For pure RNA the A260/280 ratio should be between 1.8-2.0.

The integrity of the isolated RNA was determined with a denaturing formaldehyde gel. The gel contained 1 % Agarose in MOPS (3-(*N*-morpholino) propanesulphonic acid) buffer (0.1 M MOPS, 40 mM sodium acetate and 5 mM EDTA, pH 8.0) formamide and formaldehyde. The water used was diethyl pyrocarbonate (DEPC)-treated. Ethidium bromide was added to the RNA sample, before it was loaded on the gel at a concentration of 0,5 ng/ml (Sambrook *et al.*, 1989).

These denaturing formaldehyde gels were also used to quantify the intact RNA further by means of densitometry profiling of the 18S rRNA band. The density of each sample was either compared to that of a standard RNA sample of known concentration or to the mean density of all the samples on the gel. The spectrophotometrically determined concentrations were adjusted according to the sample density - reference density ratio.

The reverse transcriptase reaction

Two different protocols were used for the preparation of first strand cDNA as described in the different experiments performed.

In the first protocol 3 µg intact total RNA was used. The first complementary DNA strand of mRNA was primed by using oligo (dT). From all the types of RNA isolated only the mRNA contains a poly-A tail. The reaction was done by using the enzyme Superscript RNase H Reverse Transcriptase. The Superscript manufacture's protocol was used to complete the reaction.

The second protocol involved precipitation of the mRNA prior to the reverse transcriptase reaction. Total RNA (6 µg) was hybridized with 3 pmol Oligo $(dT)_{12\text{-}18}$ primer and was incubated in the presence of 0.1 M NaOAc pH 5.5 and ethanol to a ratio of 1:2 (v/v) reaction mixture to ethanol. This mixture was incubated overnight at -20 °C.

The mRNA precipitate was obtained by means of centrifugation in a Sorval RMC 14 centrifuge (12000 x g for 15 min at 4 °C). The pellet was washed with 70 % ethanol (diluted with DEPC-treated water) and was again centrifuged (12 000 x g for 15 min at 4 °C). The ethanol was discarded and the pellet was left to dry for 10 to 15 minutes.

The RNA was fully denatured in a buffer containing 80 mM Tris-HCl pH 8.3, 80 mM KCl and 40 U Rnasin in a 21 µl reaction mixture. The samples were heated to 70 °C for 10 min, followed by a 3 hour incubation step at 37 °C.

First strand cDNA was then synthesized for 60 min at 37 °C in a 40 µl reaction mixture containing 60 pmol Oligo $(dT)_{12-18}$ primer, 20 mM DTT, 0.5 mM dNTP and 200 U of Moloney murine leukemia virus Rnase⁻ reverse transcriptase (Superscript RNase⁻: Gibco-BRL). The reaction was terminated by heat inactivation of the enzyme at 95 °C for 3 min.

The reaction mixture was stored at -20 °C and used within three weeks.

The PCR reactions

The first strand cDNA was amplified by PCR in a thermal cycler (MJ Research Peltier Thermal Cycler-PTC-200). Different dilutions of the RT-mixture were used in a 20 µl reaction mixture containing 1.5 mM $MgCl_2$, 1x buffer, 0.1 M dNTPs, 0,75 U of AmplitaqGold polymerase (Perkin Elmer), and 250 ng of sense and anti-sense primers. AmplitaqGold polymerase is a thermostable enzyme and is heat activated at 94 °C.

All samples were denatured at 94 °C for 10 min and then cycled with the following log-linear parameters:

i) for β-actin: 3 cycles of denaturation (94 °C for 45 seconds) annealing (59 °C for 75 seconds) and extension (72 °C for 105 seconds), followed by 24 cycles of denaturation (94 °C for 35 seconds) annealing (59 °C for 45 seconds) and extension (72 °C for 75 seconds).

ii) for IL-12 p40, IFN-γ, TGF-β and IL-4: 3 cycles of denaturation (94 °C for 45 seconds) annealing (60 °C for 75 seconds) and extension (72 °C for 105 seconds), followed by 26 to 32 cycles (depending on the type of cytokine tested) of denaturation (94 °C for 35 seconds) annealing (60 °C for 45 seconds) and extension (72 °C for 75 seconds).

iii) for TNF-α the same protocol as that of IL-12 p40, IFN-γ, TGF-β and IL-4 was used but with an annealing temperature of 48 °C. Primer sequences used in these experiments are indicated in Table 3.1.

The amplified PCR product mixture was electrophoresed (65 V for 1 hour) through a 2 % agarose gel (in 1x Tris-boric acid-EDTA) containing 0.5 µg/ml ethidium bromide. The PCR product was visualized with 360 nm UV (Spectroline TC-312A transilluminator) and was scanned into an AppleMac for densitometry by means of a program called NIH-image.

3.3. Results.

3.3.1 Optimisation of the dose of *M. tuberculosis* infection

Intravenous *M. tuberculosis* infections with different doses of live tubercle bacilli result in different survival times inversely proportional to dose in murine models (Flynn *et al.*, 1995). Here, the aim was to determine if mycolic acids (MA), a *M. tuberculosis* cell-wall component, would have any effect on the development of the immune response in mice against intravenous *M. tuberculosis* infection.

In the experimental protocol that was followed, MA conjugated to mouse serum were injected into the mice at two different time points:
i) MA-serum conjugate at different concentrations administered one week prior to *M. tuberculosis* infection initiation - this is referred to as pre-treatment.

ii) MA-serum conjugate administered two to three weeks after initiation of the *M. tuberculosis* infection - this is referred to as treatment.

Initial experiments were designed to optimise the dose of bacteria administered to each mouse. The chosen dose of *M. tuberculosis* bacteria had to allow for the mice to remain in a healthy condition without showing any macroscopic tuberculosis symptoms for at least four weeks. Weight loss of more than two grams in one week is one of the early detectable macroscopic symptoms of *M. tuberculosis* infection in mice. Female Balb/c mice were infected intravenously *via* a lateral tail vein with three doses of *M. tuberculosis*: 1×10^6, 1×10^5 or 1×10^4 bacteria in 100 µl 0.89 % saline. The mice were closely monitored for any signs of weight loss. The results obtained can be seen in Figure 3.1.

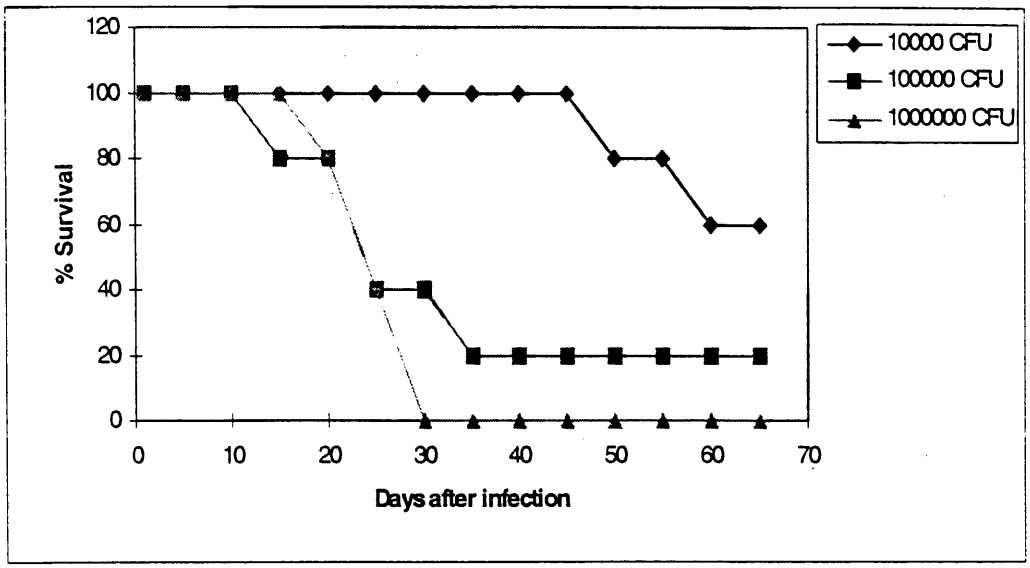


Figure 3.1. *Optimisation of the dose of M. tuberculosis infection. Mice were infected with three different doses: 1 x 10^4, 1 x 10^5 and 1 x 10^6 bacteria per mouse as indicated.*

Mice infected with 1 x 10^4 viable *M. tuberculosis* bacteria survived for at least 45 days after the initiation of infection. The survival of mice infected with 1 x 10^5 and 1 x 10^6 viable *M. tuberculosis* bacteria seem to correlate. At these doses mice did not survive more than 30 days. Mice infected with 1 x 10^5 started to die seven days after infection was initiated, leaving insufficient time to treat these mice with MA-serum conjugate. A dose of 1 to 5 x 10^4 viable *M. tuberculosis* bacteria was used as the optimum dose in all subsequent experiments done.

Flynn *et al.* (1995) indicated that Balb/c mice are more susceptible to *M. tuberculosis* infection than C57BL/6J mice. To compare the survival of these two mouse strains, the experiment described above was repeated to determine the survival differences between Balb/c and C57BL/6J mice. The results obtained are presented in Table 3.2.

Table 3.2. *The results indicating the differences in survival between the susceptible Balb/c and the more resistant C57BL/6J strain.*

	Survival of Balb/c mice (weeks)	Survival of C57BL/6J mice (weeks)
10^6	3 ✝	3 ✝
	3 ✝	3 ✝
	3 ✝	3 ✝
	3 ✝	3 ✝
	3 ✝	10 ✝
10^5	3 ✝	15 ✝
	3 ✝	18 ✝
	11 ✝	19 ✝
	11 ✝	20 ✝
	11 ✝	20 ✝
10^4	6 ✝	Alive
	7 ✝	Alive
	8 ✝	Alive
	19 ✝	Alive
	21 ✝	22 ✝

The results obtained by Flynn *et al.* (1995) were confirmed in this experiment. A dose of 10^6 CFU was too high to see any significant difference in survival between the two strains. At a dose of 10^4 CFU bacteria the difference was the most pronounced, and C57BL/6J mice survived much longer than Balb/c mice.

3.3.2. Cytokine profiling of macrophage-derived cytokines

3.3.2.1. Optimisation of cytokine expression by means of reverse transcriptase polymerase chain reaction (RT-PCR)

The expression of cytokines in mice infected with *M. tuberculosis* after exposure to mycolic acids (MA) was determined by means of semi-quantitative RT-PCR. The expression of the different cytokines that was determined in these experiments was optimised by using recombinant plasmid DNA. Each plasmid contained a deletion or insertion fragment of the wild type cytokine gene, which can be amplified using the same primers as those required for the wild type.

IL-12 p40 was optimised using a plasmid obtained from K.L. Bost (University of Tulane, USA). The plasmid was digested with XbaI to linearise the DNA, before experiments were done, as this increased the detection limit of the PCR (Figure 3.2). Different concentrations of primer, $MgCl_2$ and template were tested. The results (not shown) indicated that the optimum conditions for IL-12 expression were 70 pM of the sense and antisense primer, 0.01 M dNTPs, $MgCl_2$ concentration of 1.5 mM at an annealing temperature of 60 °C. The detection limit of the PCR, determined with digested plasmid under the described conditions, was found to be as low as 2 femtograms (fg) of plasmid DNA (Figure 3.3).

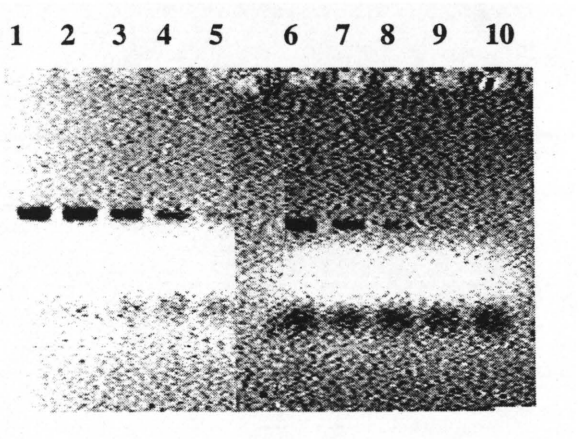


Figure 3.2. *IL-12p40 amplification using the Bost plasmid. In lanes 1 to 5, a dilution range of digested plasmid was loaded and in lanes 6 to 10 the same dilution of non-digested plasmid was loaded. Template concentrations were in lanes 1 and 6: 1 pg, in lanes 2 and 7: 0.5 pg; in lanes 3 and 8: 0.25 pg; in lanes 4 and 9: 0.125 pg and in lanes 5 and 10: 0.0625 pg.*

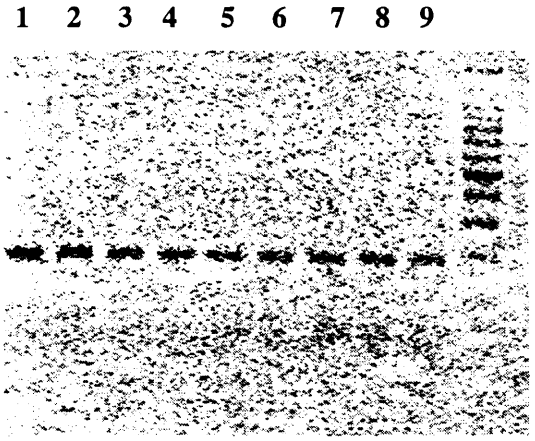


Figure 3.3. *Detection limit of IL-12 p40 RT-PCR with digested plasmid as template with lane 1 containing 500 fg, lane 2, containing 250 fg; lane 3, containing 125 fg; lane 4, containing 62.5 fg, lane 5, containing 31 fg; lane 6, containing 16 fg; lane 7, containing 8 fg; lane 8, containing 4 fg and lane 9, containing 2 fg.*

TGF-β expression was optimised by using a plasmid obtained from Locksley (Reiner *et al.*, 1994). The optimum conditions were determined at 1.5 mM $MgCl_2$, 10 pg or lower template, and 70 pmol of sense and antisense primer at an annealing temperature of 60 °C. TNF-α expression was optimised by using a plasmid obtained from Tarleton (Benavidez *et al., 1995*). The optimum conditions were found to be the same as for TGF-β, but for an annealing temperature of 48 °C.

The optimum conditions for β-actin were the same as for IL-12, but required fewer PCR cycles to obtain a clearly visible band on the gel, without reaching the PCR amplification curve plato.

3.3.2.2. Determination of IL-12 expression in different organs of *Mycobacterium tuberculosis*-infected C57BL/6J and Balb/c mice

To investigate if the difference in susceptibility to intravenous *M. tuberculosis* infection in Balb/c and C57BL/6J mice was due to the degree to which infection could induce IL-12 production in these mouse strains, the expression of this cytokine was measured in various organs. Macrophages are activated to produce cytokines during the first two weeks after initiation of infection. During this time, IFN-γ levels were shown to increase (Andersen, 1997). One would therefore expect IL-12 levels to increase during the first two weeks as well.

IL-12 p40 expression was determined, which is an indication of the amount of biologically active IL-12 p70 expressed in the organs tested. Mice were infected with 10^5 bacteria and organs were extracted two weeks after the infection was initiated. From each of the Balb/c and the C57BL/6J groups of mice, three infected and two control mice were used to complete the experiment. Organs used for RNA extraction were the spleen, liver, kidney, lungs and the heart. The RNA concentration was determined spectrophotometrically, and total RNA to an amount of 3 µg was loaded on a denaturing RNA gel. The concentrations of these samples were verified by densitometry and were adjusted according to the density of an RNA sample of known concentration (Figure 3.4).

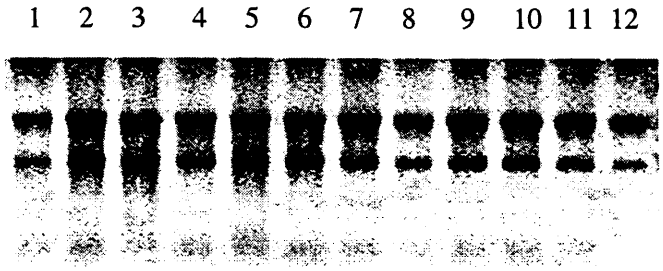


Figure 3.4. *An example of a denaturing RNA gel. RNA was isolated from lungs of M. tuberculosis-infected (lanes 2-4) and control (lanes 5 and 6) Balb/c mice. In lanes 7 to 9 RNA from M. tuberculosis-infected lung from C57BL/6 mice was loaded and the control RNA in lanes10 and 11. Reference RNA samples were loaded in lanes 1 and 12, each containing 3 µg of total RNA. The concentrations of the experimental RNA were adjusted accordingly.*

PCR of IL-12 p40 and β-actin genes were done from the same reverse transcriptase reaction mixture of each sample. The amplification products were loaded on one gel (Figure 3.5a, b and c). Densitometry profiles for both IL-12 and β-actin bands were determined. Using the β-actin expression as a reference for the amount of the initial quantity of cDNA, the IL-12 expression was adjusted accordingly (Table 3.3). The corrected IL-12p40 expression values are represented in Figure 3.6.

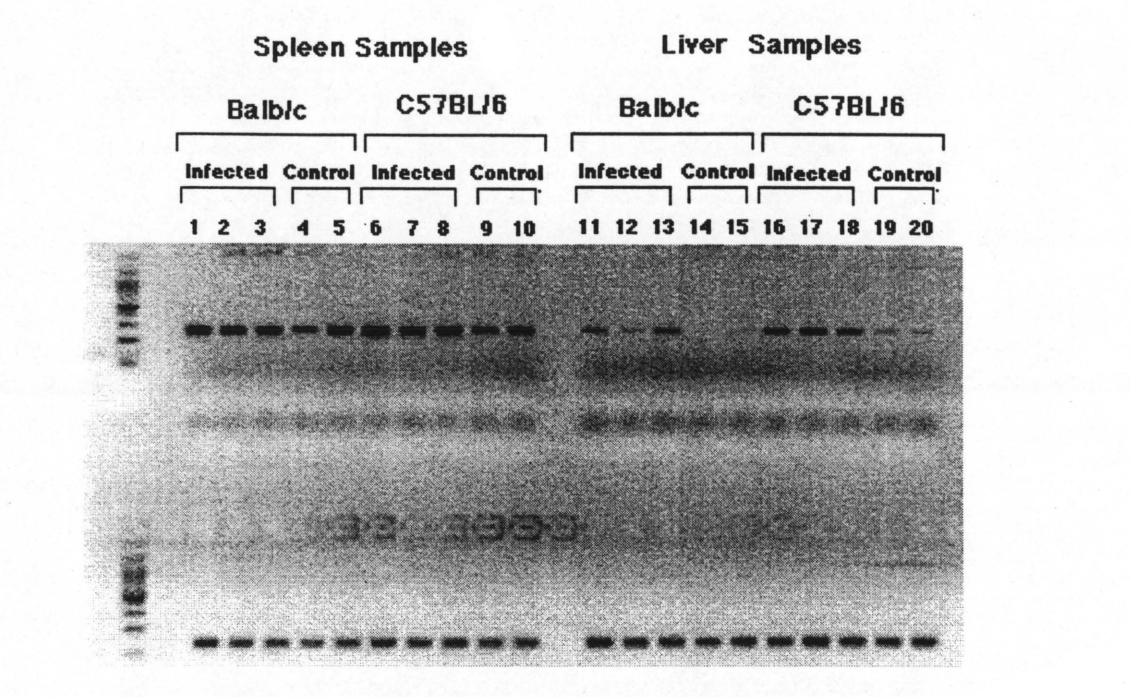


Figure 3.5a. *IL-12 expression (top row) compared to B-actin expression (bottom row) in spleen and liver of Balb/c and C57BL/6J mice infected with M. tuberculosis compared to control mice.*

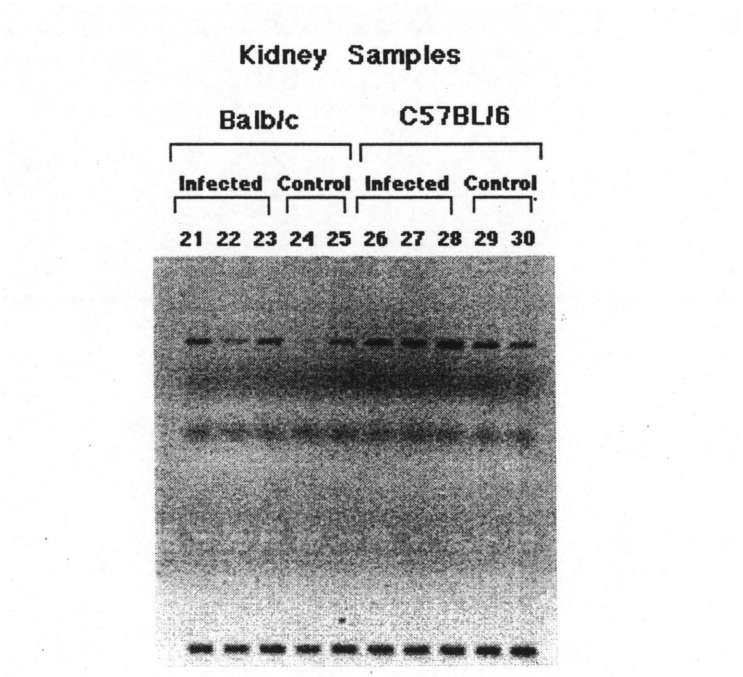


Figure 3.5b. *IL-12 Expression (top row) compared to B-actin expression (bottom row) in kidneys of Balb/c and C57BL/6J mice infected with M. tuberculosis compared to control mice.*

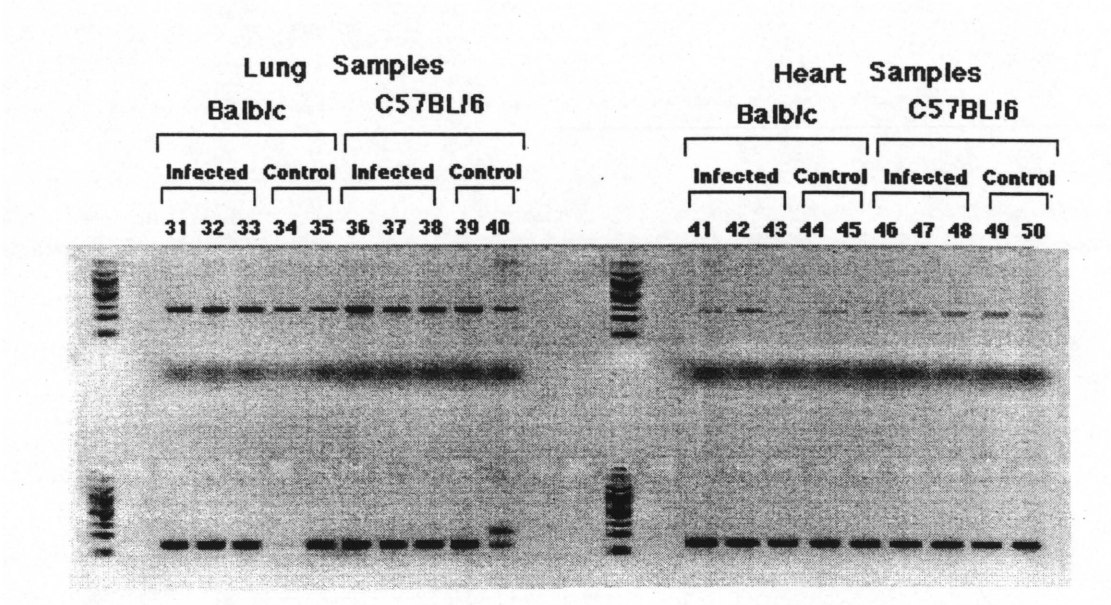


Figure 3.5c *IL-12 expression (top row) compared to B-actin expression (bottom row) in lung and heart of Balb/c and C57BL/6J mice infected with M. tuberculosis compared to control mice.*

Table 3.3. *The results obtained in the determinations of IL-12 expression by densitometry by using β-actin as reference of the quantity of input cDNA. Two weeks after M. tuberculosis-infection.*

1. Spleen:

	Balb/c *M. tuberculosis* infected			Balb/c control		C57BL/6J *M. tuberculosis* infected			C57BL/6J control	
	Mouse 1	Mouse 2	Mouse 3	Mouse 1	Mouse 2	Mouse 1	Mouse 2	Mouse 3	Mouse 1	Mouse 2
β-actin density	612	511	507	379	476	574	496	558	438	443
Factor Y[1]	0.82	0.98	0.99	1.32	1.05	0.87	1.01	0.89	1.14	1.13
IL-12 density	680	631	693	444	646	817	695	705	464	540
IL-12 corrected density[2]	554.89	616.68	682.61	585.05	677.76	710.82	699.76	630.96	529.04	608.75
AVE[3]	618.06			631.40		680.51			568.90	
STDEV[4]	63.87			65.55		43.27			56.36	

2. Liver:

	Balb/c *M. tuberculosis* infected			Balb/c control		C57BL/6 *M. tuberculosis* infected			C57BL/6J control	
	Mouse 1	Mouse 2	Mouse 3	Mouse 1	Mouse 2	Mouse 1	Mouse 2	Mouse 3	Mouse 1	Mouse 2
β-actin density	619	460	514	421	536	504	713	549	513	570
Factor Y[1]	0.87	1.17	1.05	1.28	1.01	1.07	.076	0.98	1.05	0.95
IL-12 density	198	110	240	49	60	273	348	279	117	95
IL-12 corrected density[2]	172.7	129.11	252.09	62.84	60.44	292.45	263.51	274.38	123.14	89.98
AVE[3]	184.63			61.64		276.78			106.56	
STDEV[4]	62.36			1.7		14.62			23.44	

3. Kidney:

	Balb/c *M. tuberculosis* infected			Balb/c control		C57BL/6 *M. tuberculosis* infected			C57BL/6J control	
	Mouse 1	Mouse 2	Mouse 3	Mouse 1	Mouse 2	Mouse 1	Mouse 2	Mouse 3	Mouse 1	Mouse 2
β-actin density	571	556	623	500	596	632	544	619	606	678
Factor Y[1]	1.04	1.07	0.95	1.19	0.99	0.94	1.09	0.96	0.98	0.87
IL-12 density	268	155	259	81	197	392	333	501	301	238
IL-12 corrected density[2]	278.09	165.18	246.32	95.99	195.84	367.5	362.69	479.55	294.29	207.99
AVE[3]	229.86			145.91		403.25			251.14	
STDEV[4]	58.23			70.61		66.13			61.03	

1. Average of all the measured β-actin densities divided by the density of each sample.
2. Product of the IL-12 density and Factor Y.
3. Average of the corrected IL-12 densities in each group i.e. in each of the infected or control groups.
4. Standard deviation of the IL-12 density in each group.

4. Lung:

	Balb/c *M. tuberculosis* infected			Balb/c control		C57BL/6J *M. tuberculosis* infected			C57BL/6J control	
	Mouse 1	Mouse 2	Mouse 3	Mouse 1	Mouse 2	Mouse 1	Mouse 2	Mouse 3	Mouse 1	Mouse 2
β-actin density	647	697	705	703	703	677	656	679	656	340
Factor Y[1]	1.00	0.93	0.92	0.92	0.92	0.95	0.99	0.95	0.99	1.9
IL-12 density	463	487	422	275	561	563	432	497	486	208
IL-12 corrected density[2]	462.5	451.58	386.86	252.82	239.95	537.47	425.61	473.06	478.81	395.38
AVE[3]	433.65			246.38		478.72			437.10	
STDEV[4]	40.88			9.10		56.14			58.99	

5. Heart:

	Balb/c *M. tuberculosis* infected			Balb/c control		C57BL/6J *M. tuberculosis* infected			C57BL/6J control	
	Mouse 1	Mouse 2	Mouse 3	Mouse 1	Mouse 2	Mouse 1	Mouse 2	Mouse 3	Mouse 1	Mouse 2
β-actin density	612	900	507	379	476	574	496	558	438	443
Factor Y[1]	0.88	0.60	1.06	1.42	1.13	0.94	1.09	0.96	1.23	1.22
IL-12 density	113	157	46	77	83	111	129	133	83	0
IL-12 corrected density[2]	99.39	93.90	48.84	109.36	93.86	104.10	140.00	128.3	102.01	0
AVE[3]	80.71			101.61		124.13			51.00	
STDEV[4]	27.74			10.96		18.31			72.00	

1. Average of all the measured β-actin densities divided by the density of each sample.
2. Product of the IL-12 density and Factor Y.
3. Average of the corrected IL-12 densities in each group i.e. in each of the infected or control groups.
4. Standard deviation of the IL-12 density in each group.

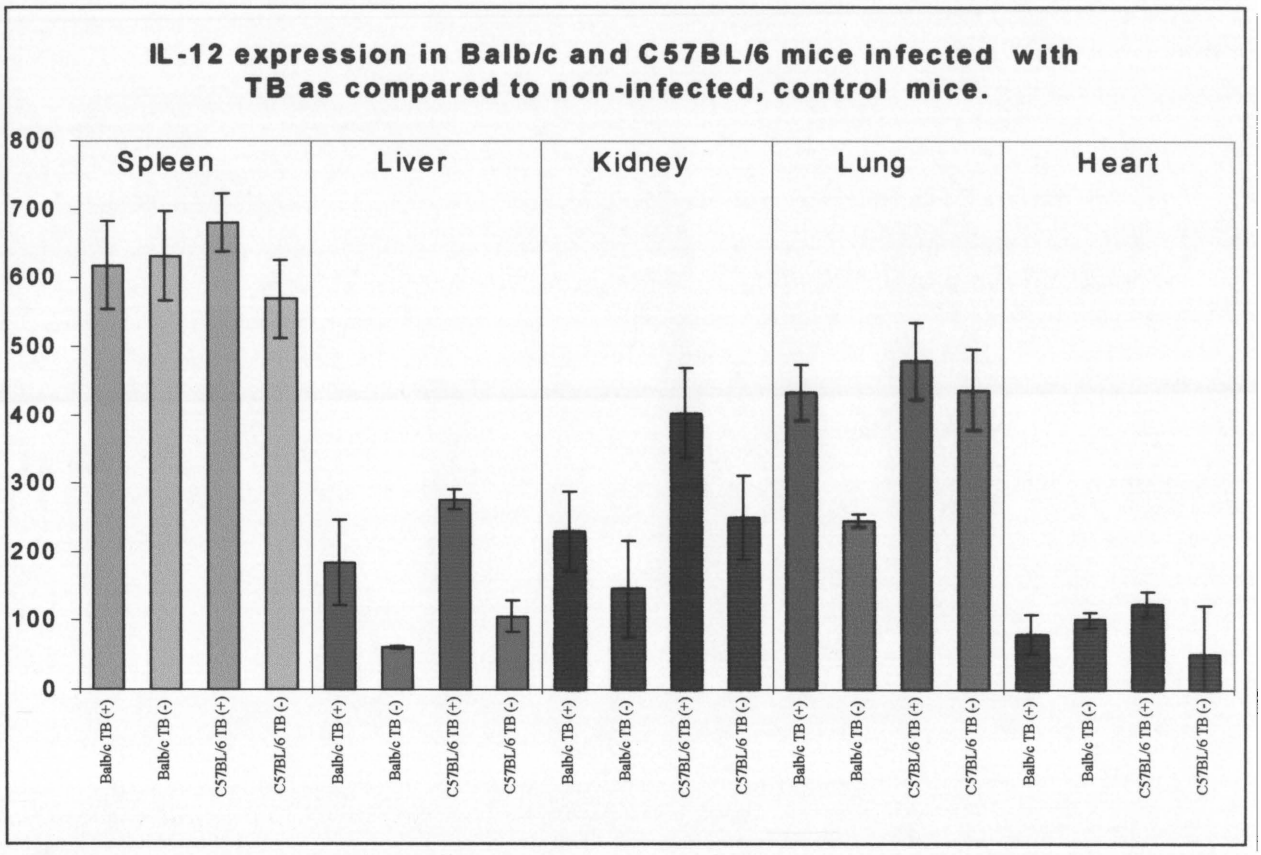


Figure 3.6. *IL-12 expression in Balb/c mice and C57BL/6 mice infected with M. tuberculosis in the spleen, liver, kidney, lung and heart. Organs were removed two weeks after infection. TB^+ indicates that animals were experimentally infected with M. tuberculosis, while TB^- indicates the non-infected control animals.*

Two weeks after the initiation of infection there was no difference in IL-12 expression observed in the spleen and heart of Balb/c and C57BL/6J mice, as compared to their negative controls. In these organs therefore, IL-12 expression can be regarded as base level expression where IL-12 induction was not influenced by *M. tuberculosis* infection, at least not two weeks after infection was initiated. The absence of the induction of IL-12 after infection could be an indication that the splenic and heart macrophages remain unaffected during intravenous *M. tuberculosis* infection.

The liver and kidney in both mouse strains responded to the *M. tuberculosis* infection with regard to IL-12 expression. A significant increase in IL-12 production was observed two weeks after the infection in these infected organs. The only clear difference between the two strains in respect of their IL-12 response to tuberculosis infection occurred in the lungs. It appeared that IL-12 production in the control C57BL/6J mice was almost as high as in the infected organ, whereas a much lower level of IL-12 expression was measured in the lungs of the more susceptible Balb/c mice, which were not infected (Figure 3.6). Tuberculosis

infection induced expression of IL-12 in Balb/c mice, whereas the levels of expression of IL-12 in the more resistant C57BL/6J mice were already high and remained unaffected by the tuberculosis infection. Higher base levels of IL-12 in the lungs of C57BL/6J mice could be one of the factors, if not the most important factor, that contributed to the observed resistance to *M. tuberculosis* infection in C57BL/6J mice.

The base level IL-12 expression in the lungs was high for this experiment. In all the subsequent experiments the base level of IL-12 expression in the control animals, was zero irrespective of the number of PCR cycles used. This might be attributed to the fact that in the later experiments the control mice were kept in a temperature- and humidity- controlled environment in a filtered air isolator. In the experiment for which the results are presented in Figure 3.6, only the infected mice were kept in the isolator, while the control mice were kept outside the isolator in another room.

The results presented in this experiment can be regarded as an example of how all the results were analysed. Only the final results are presented as from here.

3.3.2.3 The effect of mycolic acids on healthy Balb/c and C57BL/6J mice

Cord factor, a mixture of the 6-6' diester of α D trehalose with mycolic acids (MA), has been reported as the first *M. tuberculosis* substance to induce IL-12 production *in vivo* (Oswald *et al.*, 1997). The ability of *M. tuberculosis*-derived MA (an important element of cord factor) to induce IL-12 expression, was investigated. The effect of MA-serum conjugate on the induction of a biased Th1 or Th2 immune response in the spleen of C57BL/6J and Balb/c mice, was also determined by means of cytokine profiling.

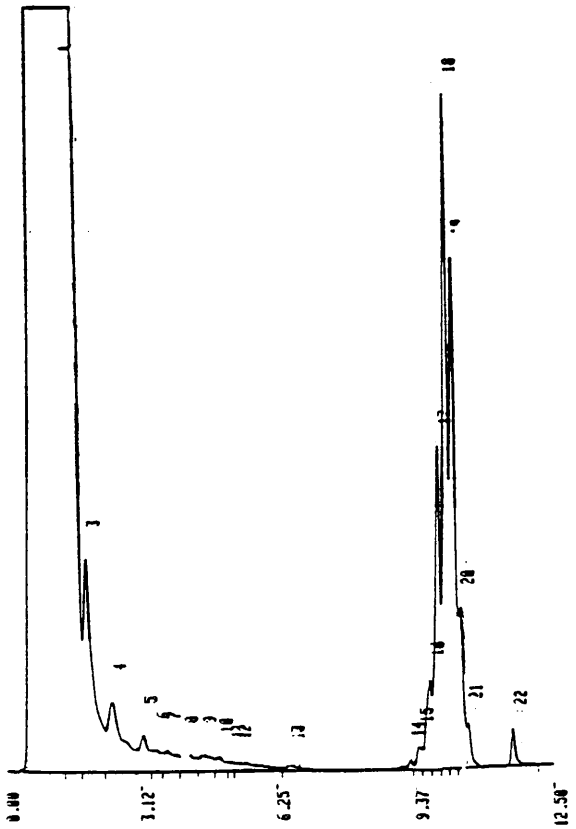
Cytokine profiling was done on the spleens of MA primed mice at different time periods after a single injection of MA. Purified MA were conjugated to mouse serum at a concentration of 25 µg/100 µl mouse serum. The presence of MA in the samples was verified by means of HPLC analysis. In the MA-mouse serum conjugate samples the characteristic MA fatty acid chains, with a retention time of between 9 and 10 min were observed (Figure 3.7b). These peaks were absent in the control mouse serum samples which were treated similarly, but with MA omitted (Figure 3.7a).

The experimental animals were pre-treated intravenously with 25 μg of MA conjugated to 100 μl of mouse serum, and were sacrificed in groups of three, at different time points, i.e. after 4, 8, 17, 41 hours after treatment was initiated. The MA were either resaponified (MA with free carboxylate) or were left non-saponified (MA with the carboxylate in the methyl ester form) to determine whether the carboxylate influences the biological activity of MA.

The spleens were dissected aseptically and were snap-frozen in liquid nitrogen. The RNA was extracted and reverse-transcriptase-reactions were done after the RNA was quantified. IL-12, TNF-α, TGF-β and β-actin PCR was performed on the same RT-reaction mixture of each sample.

The results for IL-12, TNF-α, TGF-β expression in Balb/c mice can be seen in Figures 3.8, 3.9 and 3.10 respectively and the IL-12 expression in C57BL/6J mice can be seen in Figure 3.11.

A:



B:

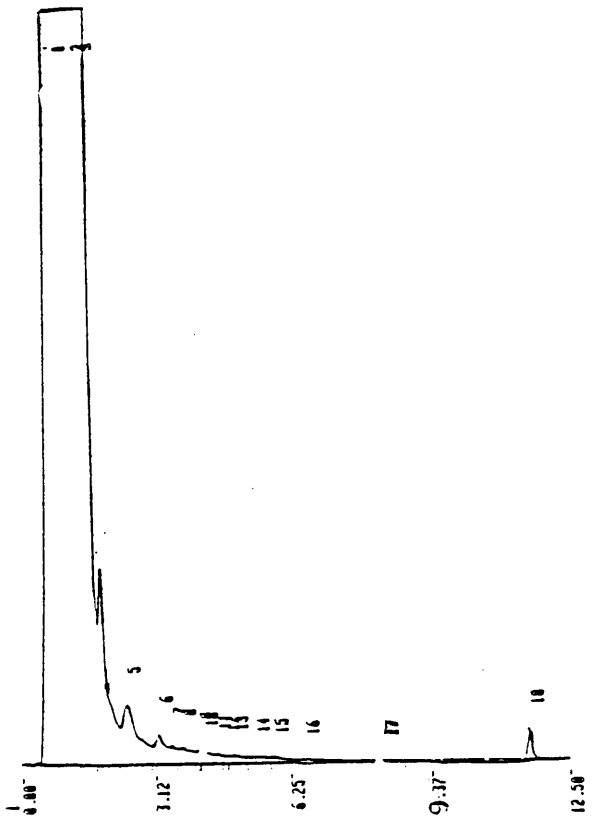


Fig 3.7. *A typical HPLC analysis profile of mouse serum conjugated to MA (a) compared to that of mouse serum treated similarly, but omitting the MA (b).*

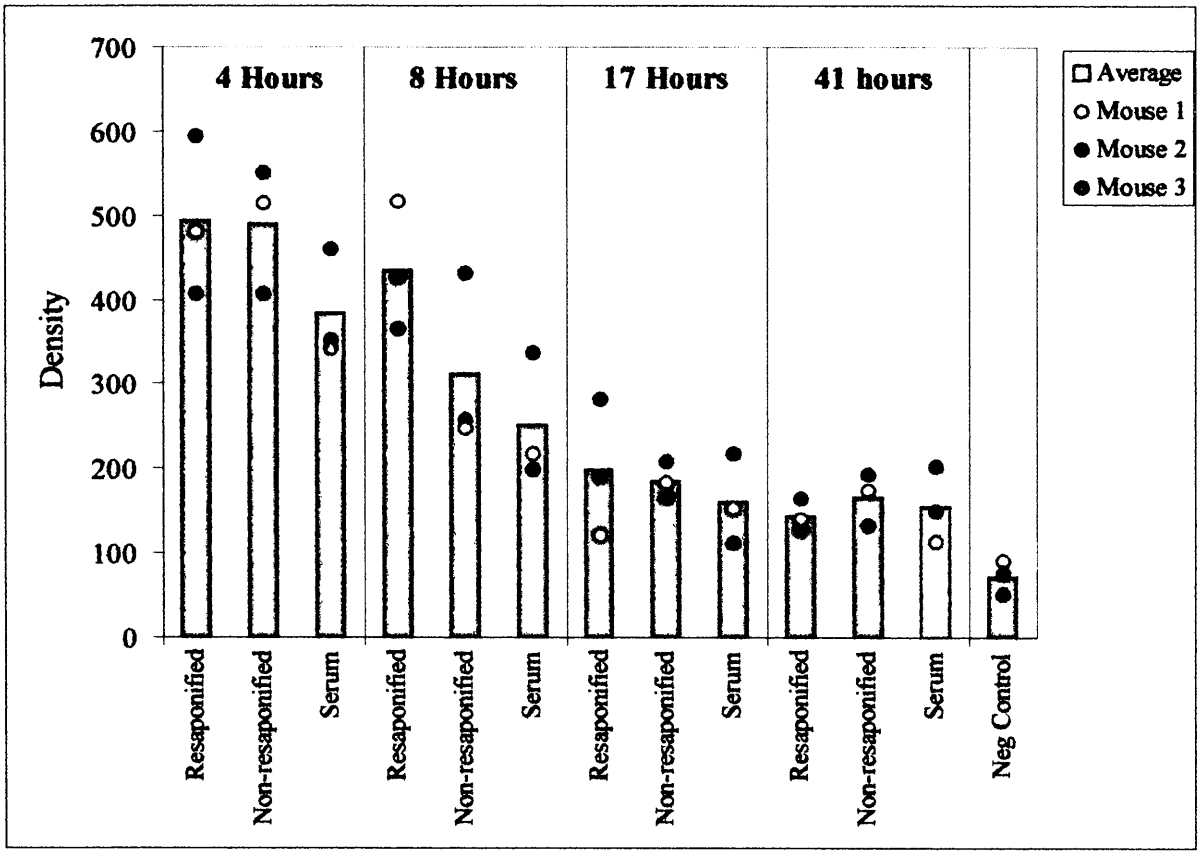


Figure 3.8. *IL-12 expression in the spleen of Balb/c mice treated with saponified or non-saponified MA as compared to serum controls.*

There was no difference in the expression of IL-12 in the spleen of Balb/c mice treated with serum conjugates of saponified or non-resaponified MA as compared to mice treated with serum only, observed at different time intervals over 41 hours (Figure 3.8). The level of IL-12 expression declined, especially after 17 hours, indicating that the serum as such could have induced an IL-12 response that peaked between 0 and 4 hours after administration.

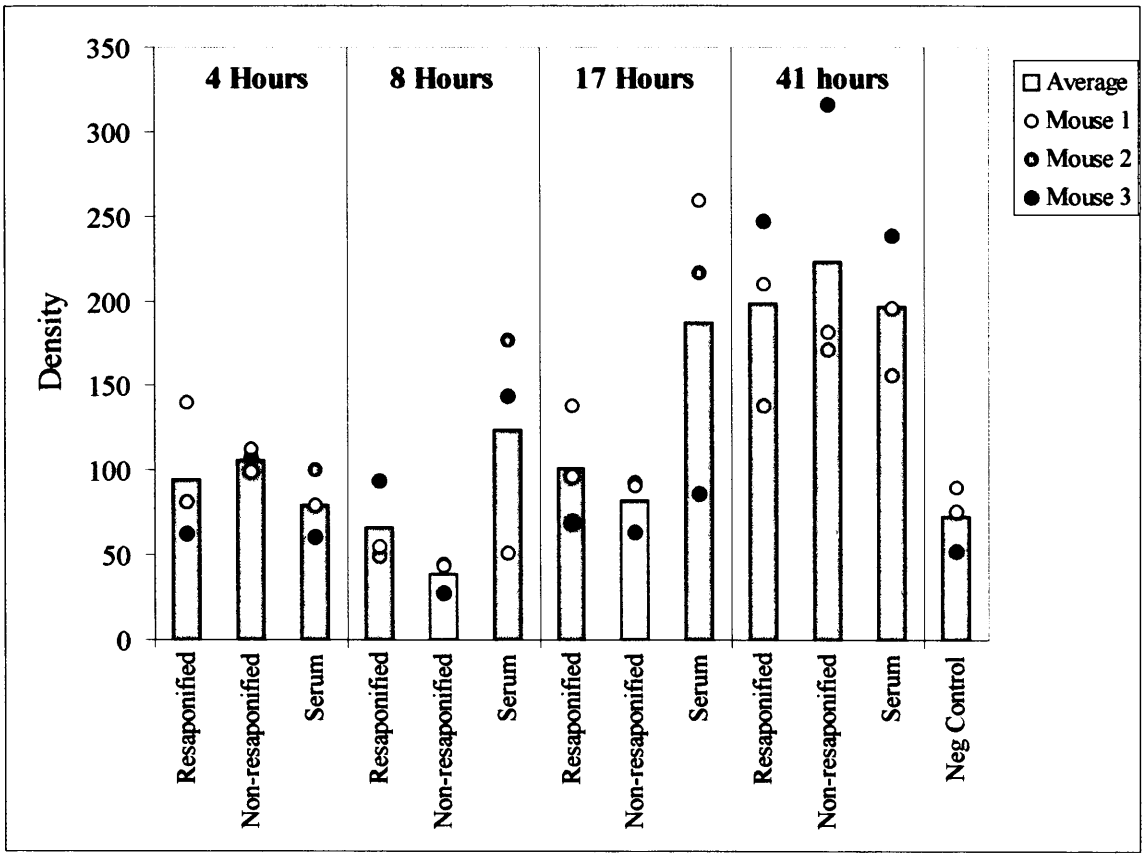


Figure 3.9. *TNF-α expression in the spleen of Balb/c mice treated with saponified or non-saponified MA as compared to serum controls.*

There was again no difference in the expression of TNF-α in the spleens of mice treated with either resaponified or non-resaponified MA-serum conjugate, when compared to mice treated with serum only (Figure 3.9) The over-all expression of TNF-α did however increase at 17 hours after MA were administered. This increase in TNF-α was probably due to the serum effect, as it appeared the same for both the MA-serum conjugates as well as the serum-only control groups.

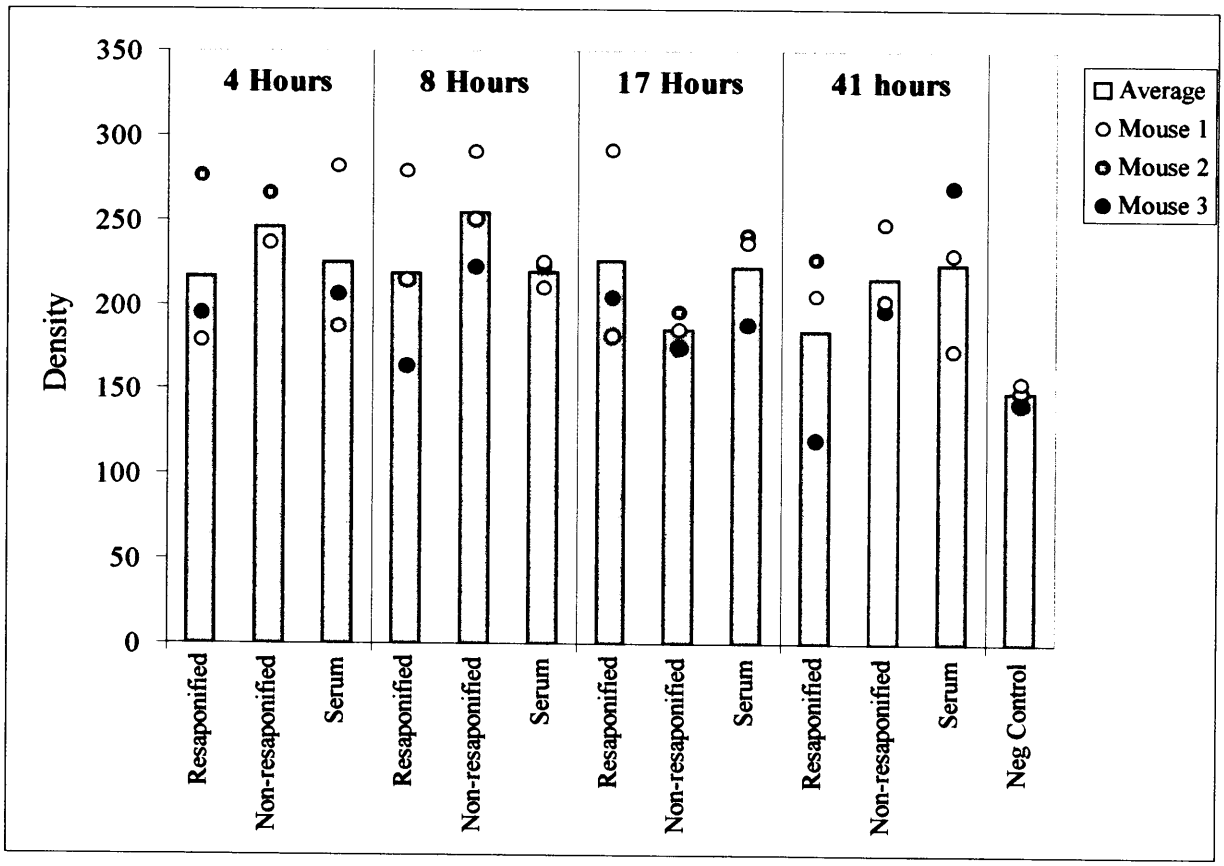


Figure 3.10. *TGF-β expression in the spleen of Balb/c mice treated with saponified or non-saponified MA as compared to serum controls.*

There was no difference observed in TGF-β expression in the spleens of mice treated with the MA-serum conjugates as compared to the mice treated with serum only (Figure 3.10). Furthermore no overall increase or decrease in the TGF-β mRNA levels was observed in the spleen. When the standard deviation is taken in account, the expression level correlated with the expression in the negative, non-treated control group.

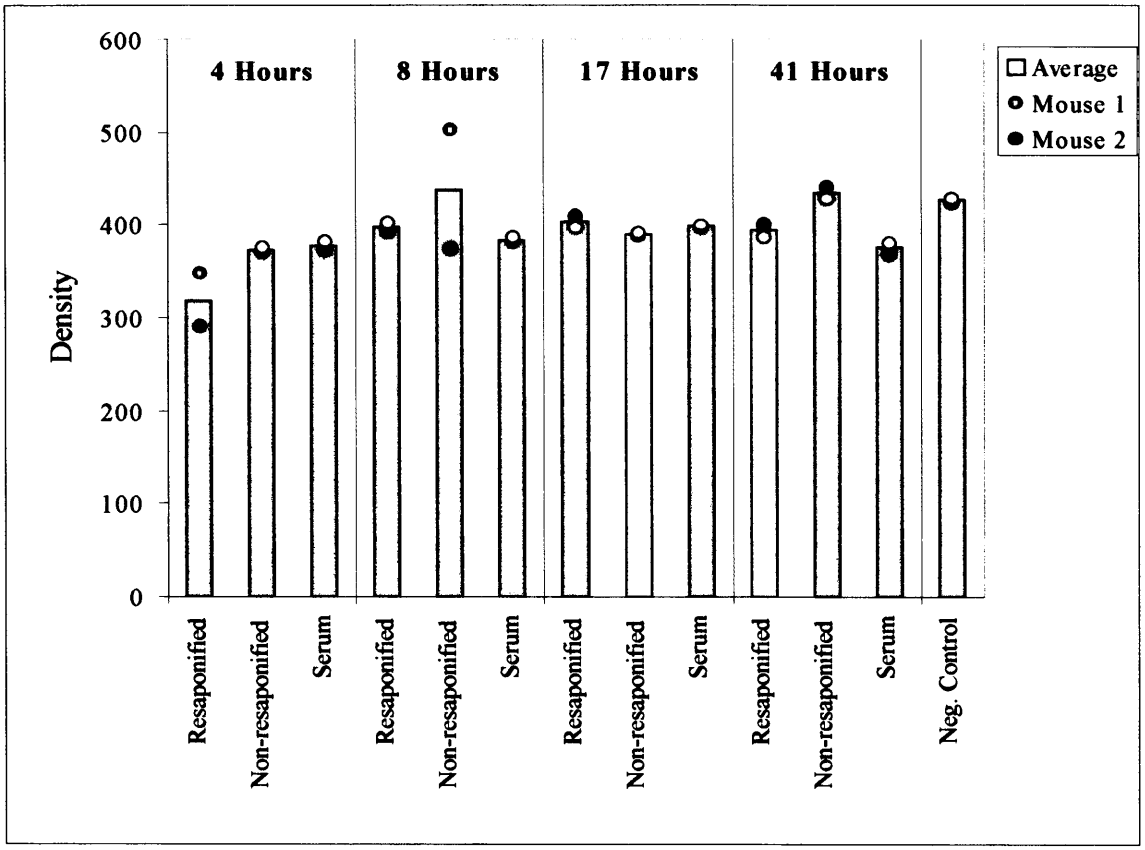


Figure 3.11 *IL-12 expression in the spleen of C57BL/6J mice treated with saponified or non-saponified MA as compared to serum controls.*

In C57BL/6J mice only the expression of IL-12 was measured. In Figure 3.11 no difference in IL-12 expression was observed in the spleens of these animals over 41 hours. In conclusion, a single administration of MA does not seem to have any effect on the induction of macrophage-derived cytokines in the spleen of both Balb/c and C57BL/6J mice other than the effect induced by the administration of the serum carrier itself.

Alternatively, the response induced by MA may only be observed after a booster injection of MA, if memory T and B-lymphocytes were to play a role. One would already have expected some indication of this after the primary MA-challenge. The effects of serum administration as such prevent obtaining an unequivocal answer from the experiments done.

In a follow-up experiment, the hypothesis that MA as such could influence the cytokine response in an animal was tested by adjusting several parameters according to what was learned from the experiment described and discussed above. First, lungs rather than spleen

were selected for cytokine profiling as Figure 3.6 indicated this to be the responsive organ to *M. tuberculosis* infection. Secondly, a higher initial dose of MA (250 µg) was administered followed by two boosters (25 µg). Cytokine profiling of IL-12, TGF-β and IFN-γ in the lungs of Balb/c mice was done after the last MA booster. Mice were injected with 250 µg saponified MA in 100 µl serum, at first. The mice were then injected with 25 µg /100 µl serum four and six weeks after the initial MA treatment. The mice were sacrificed at two different time points: 24 h and 48 h after the third MA administration. Serum controls were included as before.

Cytokine profiles of IL-12, IFN-γ, and TGF-β were determined on the individual RT-reactions obtained after RNA extraction (Figure 3.12).

An increase in the secretion of IL-12 was observed after 24 h and 48 h. The data variation did not allow interpretation as to whether the peak of activity occurred before or after 24 h. No visible induction of IL-12 was indicated in the serum control mice. The increase in the expression of IFN-γ coincided with that of IL-12 expression but showed that the peak of activity occurred before or at 24 hours. The secretion of TGF-β was high in both MA-treated and serum control mice.

These results suggest that the fatty acid fraction of cord factor (MA) induce the production of IL-12 and subsequent expression of IFN-γ at the infection site in the lungs. This finding supports the hypothesis that MA can be administered to increase the resistance against tuberculosis infection in mice, at least in Balb/c mice.

3.3.2.4. Determination of the survival rate of *M. tuberculosis* infected mice pre-treated and treated with mycolic acids.

To determine if the administration of mycolic acids (MA) can increase the survival of *M. tuberculosis*-infected mice, Balb/c mice (8-10 weeks of age) were treated with MA-serum conjugate either before or after *M. tuberculosis* infection. This strain was chosen because Balb/c mice are known to be more susceptible to *M. tuberculosis* infection than C57BL/6J mice due to the difference of expression of IL-12 (Flynn *et al.*, 1995).

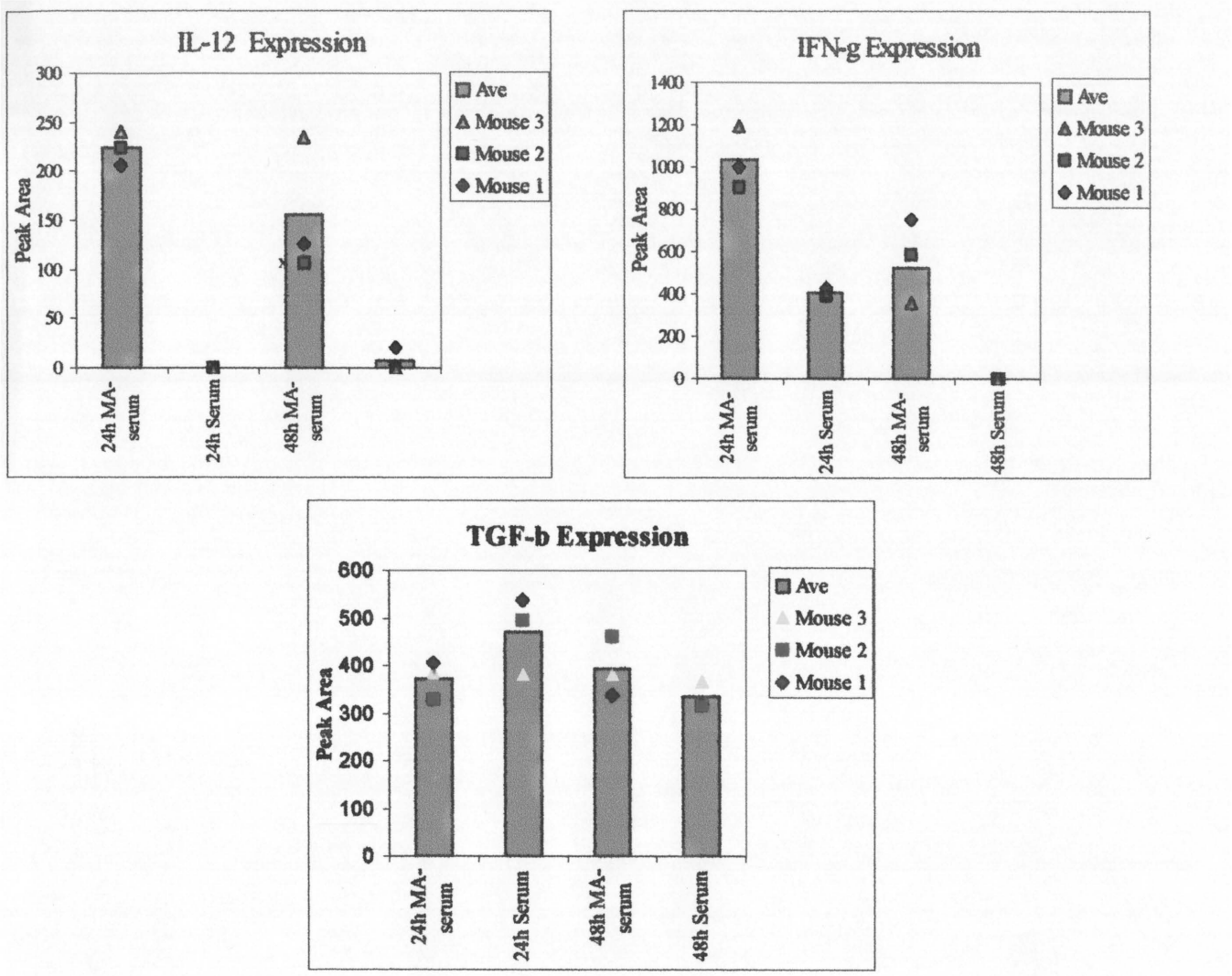


Figure 3.12. *Expression of* macrophage-derived *cytokines in the lungs. Mice were immunised with 250 µg MA/ 100 µl mouse serum. After 25 µg MA serum boosters at week 4 and 6, mice were sacrificed 24 and 48 hours after the final booster injection. The lungs were removed and RT-PCR was done. A represents the IL-12 profile, B the profile of IFN-γ C the profile of TGF-β.*

In a preliminary survival experiment using Balb/c mice, the animals were infected with a dose of 4 x 10^4 CFU *M. tuberculosis via* the lateral tail vein. The mice were treated with MA either one week before *M. tuberculosis* infection or during the third week (day 11, 13, 15) after *M. tuberculosis* infection was initiated. For the pre-treatment of mice a single dose of either 5 µg or 25 µg MA-serum conjugate was administered intravenously one week before the *M. tuberculosis* infection was initiated (Table 3.4). Mice were treated with MA in a single dose of either 5 µg or 25 µg MA or the mice were treated in triplicate doses of 1 µg or 5 µg MA as MA-serum conjugates with a two-day interval between each injection during the third week after infection.

Table 3.4 *A summary of the method used to treat and infect different groups of Balb/c mice in survival studies to determine the protective role of MA in treatment or pre-treatment of M. tuberculosis-infected mice.*

Group . Number	Type of treatment	Repeated or single treatment	Infection and treatment times		
			Week -1	Week 0	Week +3
1	Negative control-no infection				
2	Positive control - *M. tuberculosis* infection only			MTB	
3	*M. tuberculosis* infection and serum treatment	Single	Serum	MTB	
4	*M. tuberculosis* infection and MA-serum pre-treatment (5µg)	Single	MA/Serum	MTB	
5	*M. tuberculosis* infection and MA-serum pre-treatment (25µg)	Single	MA/Serum	MTB	
6	*M. tuberculosis* infection and serum treatment	Single		MTB	Serum
7	*M. tuberculosis* infection and MA-serum treatment (25µg)	Repeated (3x)		MTB	MA/Serum
8	*M. tuberculosis* infection and MA-serum treatment (1µg)	Repeated (3x)		MTB	MA/Serum
9	*M. tuberculosis* infection and MA-serum treatment (5µg)	Repeated (3x)		MTB	MA/Serum

The survival curve of the Balb/c mice (pre-treatment) can be seen in Figure 3.13 (p. 78). Pre-treatment of the Balb/c mice with 25 µg MA-serum gave significant protection, when compared to the mice treated with serum only, 50 days after *M. tuberculosis* infection was initiated. No difference in the survival between the mice treated with 5 µg MA-serum conjugate and the mice treated with serum was observed. The Balb/c mice seemed to be rendered more resistant by pre-treatment with 25 µg MA-serum conjugate. After 50 days, these mice became ill and eventually died.

No difference was observed between the single or repeated post-infection treatment with MA-serum conjugate in Balb/c mice as compared to the serum controls (data not shown).

These results indicate that MA pre-treatment may increase the resistance towards *M. tuberculosis* infection, possibly in a manner similar to what is genetically achieved in the more resistant C57BL/6J mice strain (Flynn *et al.*, 1995). The underlying cytokine mechanism for this partial protection will be investigated further, in a repeat of the experiment using both Balb/c and C57BL/6J mice.

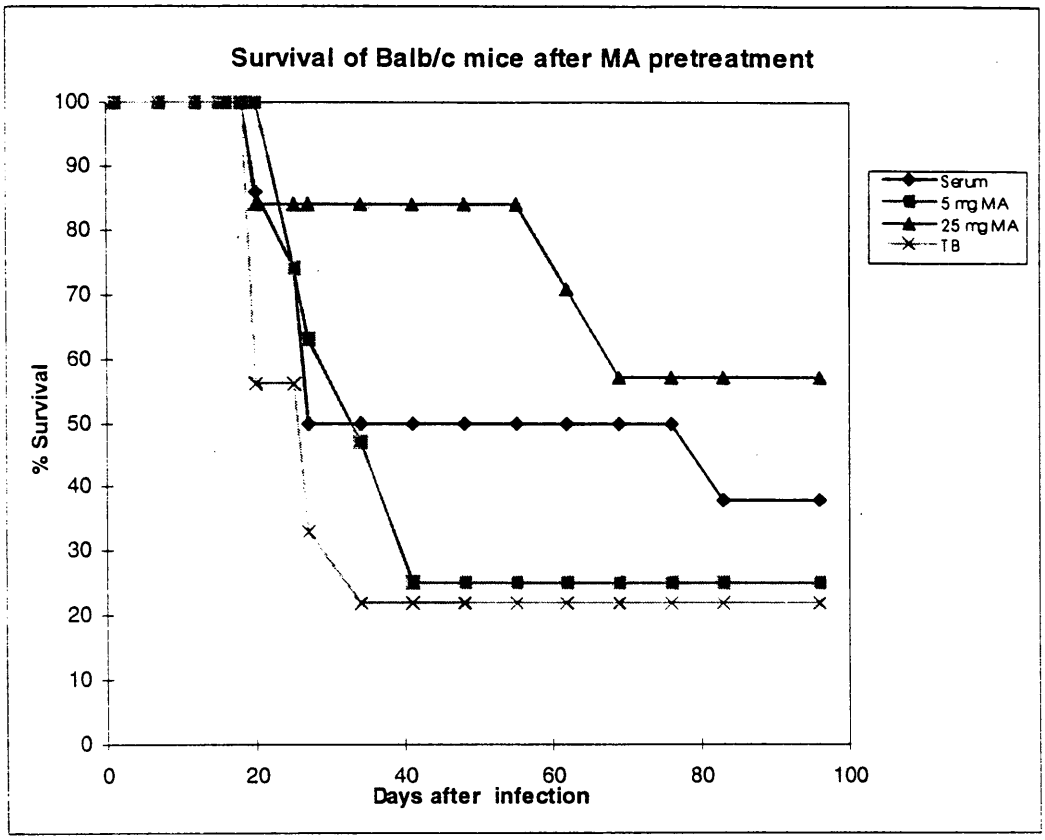


Figure 3.13. *Survival of M. tuberculosis -infected Balb/c mice after pre-treatment with MA.(Serum: n = 6; 5 mg MA: n = 8; 25 μg MA: n = 7; TB: n = 8)*

3.3.2.5. Survival determination and cytokine profiling of *Mycobacterium tuberculosis*-infected mice treated and pre-treated with mycolic acids.

In the previous experiment an indication of partial protection provided by pre-treatment with 25 μg mycolic acid (MA) was observed in Balb/c mice. To determine whether this short-term protection can be linked to increased or decreased expression of IL-12, IFN-γ and TGF-β , the experiment was repeated. The same protocol was used as previously described, but different doses of MA were administered to the both Balb/c and C57BL/6J mice as indicated in Table 3.5.

Table 3.5. *A summary of the method used to treat and infect different groups of Balb/c and C57BL/6 mice for survival studies to determine the protective role of MA in treatment or pre-treatment of M. tuberculosis-infected mice.*

Group . number	Type of treatment	Repeated or single treatment	Infection and treatment times		
			Week -1	Week 0	Week +3
1	Negative control-no infection				
2	Positive control - *M. tuberculosis* infection only			MTB	
3	*M. tuberculosis* infection and serum treatment	Single	Serum	MTB	
4	*M. tuberculosis* infection and MA-serum pre-treatment (12.5 µg)	Single	MA/Serum	MTB	
5	*M. tuberculosis* infection and MA-serum pre-treatment (25 µg)	Single	MA/Serum	MTB	
6	*M. tuberculosis* infection and MA-serum pre-treatment (50 µg)	Single	MA/Serum	MTB	
7	*M. tuberculosis* infection and serum treatment	Repeated (3x)		MTB	Serum
8	*M. tuberculosis* infection and MA-serum treatment (3 x 8 µg)	Repeated (3x)		MTB	MA/Serum
9	*M. tuberculosis* infection and MA-serum treatment (3 x 16 µg)	Repeated (3x)		MTB	MA/Serum

Mice were divided in groups of 12 mice each. Three mice were sacrificed five weeks after *M. tuberculosis* infection was introduced. The remaining two to six mice per group were retained to determine survival. Only the results obtained from mice that were infected and treated successfully were interpreted. The survival of the mice in each group can be seen in Figures 3.14-15. Compared to the survival of Balb/c mice, these results indicate that C57BL/6J mice were more resistant to *M. tuberculosis* infection than Balb/c mice when serum without MA was injected prior to infection (Figure 3.16a). There was also an indication of an absence of significant response towards MA pre-treatment in the C57BL/6J mice.

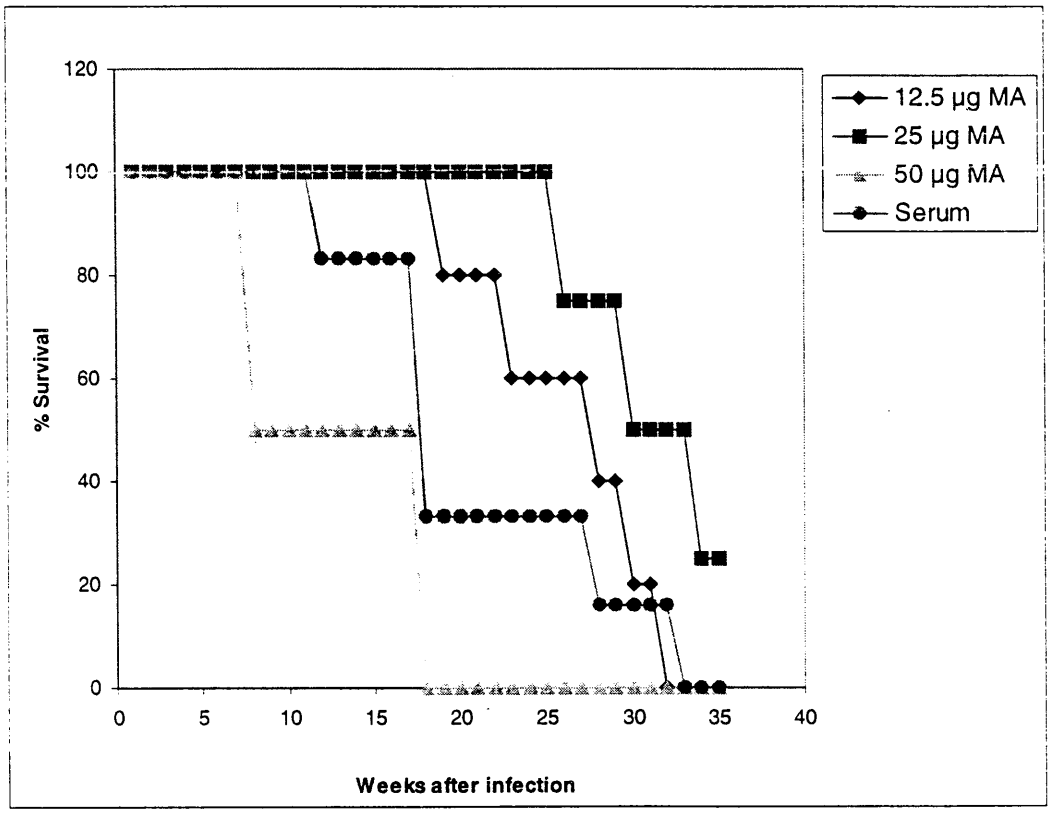


Figure 3.14. *The survival curves of Balb/c mice(5-6 mice per group) pre-treated with different doses of MA. Only two mice were included in the 50 µg group.*

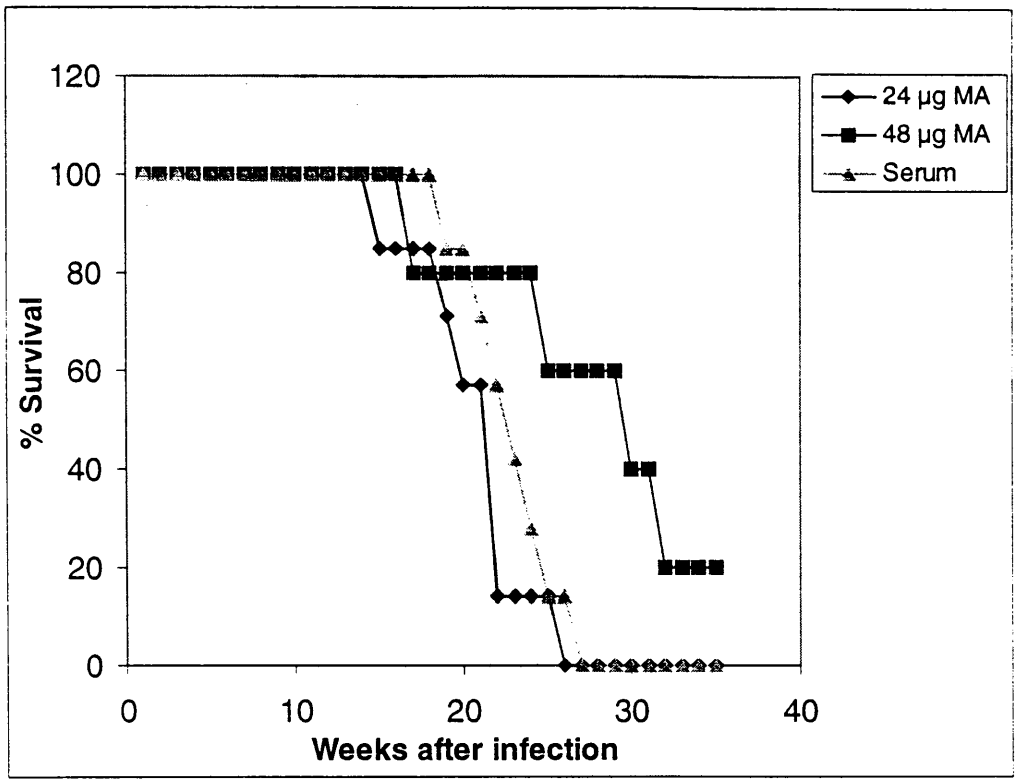


Figure 3.15. *The survival curves of Balb/c mice (5 to 6 mice per group) treated with different doses of MA.*

The Balb/c mice pre-treated with 25 µg and 12.5 µg MA-serum conjugates showed an increased resistance to the onset of disease when compared to the mice treated with serum only. The mice treated with 50 µg MA-serum conjugates appeared to have increased susceptibility to the *M. tuberculosis* challenge. This could imply a narrow dose range of MA to be used effectively in MA pre-treatment, although the results may not be accurate as only two mice remained for the survival study of those that were successfully treated. The mice treated with serum only started to die 10 weeks after infection was initiated, and all the mice were dead at week 18 (Figure 3.14). For mice pre-treated with MA-serum conjugates the protection provided by MA lasted only for ± 30 weeks, after which the mice started to get sick and died. This increased resistance corroborates the results observed previously.

The C57BL/6J mice did not show any significant increase in survival after MA-serum conjugate pre- or post-treatment. When the survival of Balb/c mice was correlated to the survival of C57BL/6J mice, it was observed that the 25 µg MA-serum conjugate pre-treatment rendered the mice as resistant to *M. tuberculosis* as the C57BL/6J mice (Figure 3.16a).

In the experiment where Balb/c mice were treated three weeks after the infection, the survival rate of the mice treated with 3 x 8 µg MA-serum conjugate did not differ from that of the mice treated with serum only (Figure 3.15, p. 81). Mice treated with 3 x 16 µg MA-serum conjugate seemed to be more effective. As was found for MA pre-treatment, the protection by MA-treatment rendered the Balb/c mice resistant to the same level as that observed for C57BL/6J mice (Figure 3.16b). As with the pre-treatment, MA had no significant effect on the survival of the C57BL/6J mice.

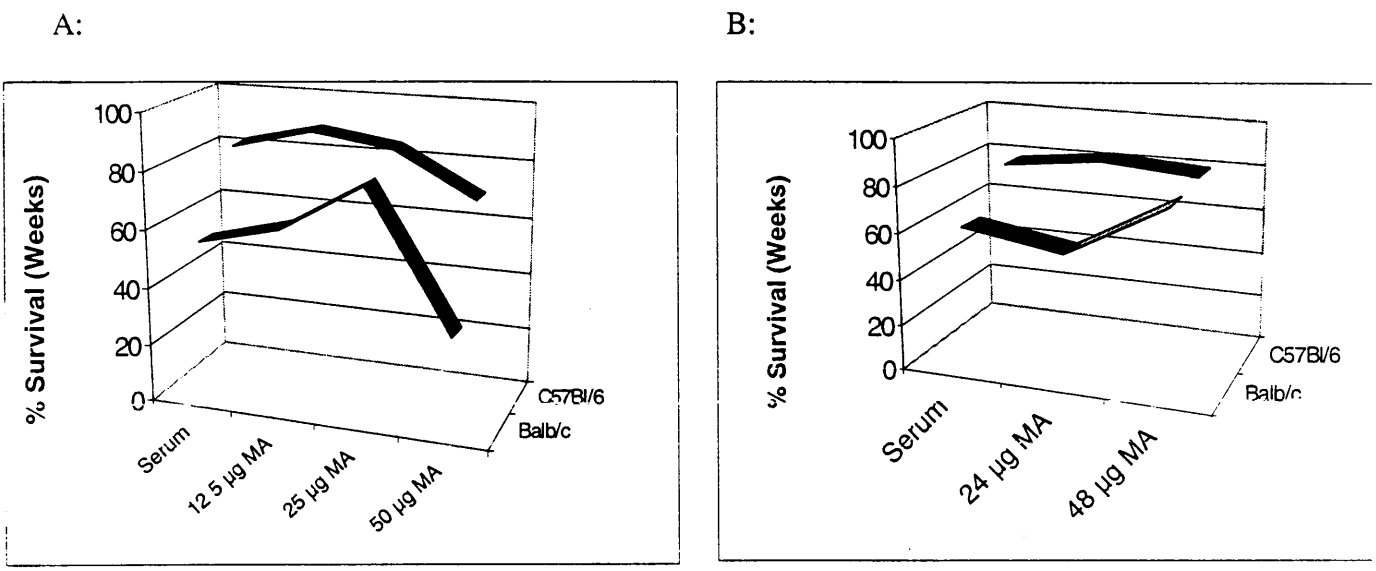


Figure 3.16 *A schematic representation of the survival of both C57BL/6 and Balb/c infected with tuberculosis. A) Represents mice pre-treated with MA, B) represents mice post treated with MA.*

Cytokine profiling:

Cytokine profiling done on untreated mice infected with *M. tuberculosis*, indicated the lungs, kidney and liver to be the responsive organs in terms of IL-12 expression (Figure 3.6, p. 66). In mice that were treated with MA-serum conjugate in the absence of *M. tuberculosis* infection, the lungs responded to MA-serum administration with IL-12 and IFN-γ secretion (Figure 3.12, p. 75). Expression of IL-12 appears to be the key factor in inducing resistance in C57BL/6J mice. Furthermore, the survival of Balb/c mice pre-treated with 25 µg MA and those treated with 3 x 16 µg MA rendered the mice resistant to *M. tuberculosis* to the same levels as those observed for C57BL/6J mice (Figure 3.14–16). To determine if the increase in resistance in Balb/c mice can be linked to IL-12 expression, cytokine profiling on lung tissue was done. The expression of IFN-γ, a cytokine normally expressed in synergism with IL-12,

and of TGF-β a cytokine normally expressed antagonistic to IL-12 expression, were also determined.

The lungs of Balb/c mice were aseptically removed five weeks after initiation of *M. tuberculosis* infection. RNA was extracted from individual lung samples and mRNA precipitated before the reverse transcriptase reaction. Semi-quantitative RT-PCR was done with each individual RT-reaction to profile macrophage-derived IL-12, TGF-β, with β-actin as constitutively expressed reference gene. The expression of IFN-γ, a T and NK cell cytokine, was also determined to corroborate IL-12 bio-reactivity.

The cytokine PCR reactions were first optimised for each individual cytokine to be tested. Five randomly chosen RT-samples from each of the different pre- or post-treated groups of Balb/c mice were used for optimisation reactions. First, a dilution range PCR was performed. Different dilutions of RT-reaction mix were set up, ranging from 0.1-1 µl in a total reaction mixture of 20 µl. The lowest concentration to give detectable bands on ethidium bromide gels in all 5 the samples tested in each group, were regarded as the optimum dilution for a specific cytokine.

As the amount of cytokine mRNA in each sample is different, three PCR cycles were done to ensure that the PCR amplification plato was not reached for each sample tested. To have an indication of the range of cycles to use, 3 different PCR experiments were done, differing in the number of PCR cycles in a range from 24-30 cycles. This was initially done on five randomly chosen samples from the pre-treatment and post-treatment groups, at the optimum dilution, as determined in the previous experiment.

The densitometry profile for the amplified DNA in each sample was determined after gel electrophoresis. In the log linear amplification range the density of a sample should increase fourfold from 24 to 26 PCR cycles used. If the density increased less than fourfold, it could indicate that the amplification plato was reached. An optimum cycle range was deduced that would ensure that the amplification plato in all five samples tested was not reached. The final PCR reactions were then done for each individual cytokine tested. Experiments using three different numbers of PCR cycles were subsequently done on each lungs RT-sample at the

optimum dilution and cycle range. All of the samples tested were done with fresh RT-reaction mixtures. Optimisation and final experiments were completed within two weeks from initiation of RT-reactions.

Figure 3.17 represents the cytokine profiles of Balb/c mice five weeks after infection was initiated. The expression of IL-12 increased significantly with 12.5 μg MA pre-treatment ($p < 0.0054$), whereas mice treated with 25 μg of MA-serum conjugate showed a 9 fold increase ($p < 0.00015$) as compared to the serum control mice (Figure 3.17). IFN-γ expression at 12.5 μg of MA pre-treatment showed a significant increase with a density average of 180 ($p < 0.0047$) and 25 μg MA pre-treatment increased with an average density of 90 ($p < 0.001$) as compared to the serum control (Figure 3.17). In both the IL-12 and IFN-γ experiments the serum control seemed to have a negative regulatory effect, as the cytokine expression was much lower than that of the *M. tuberculosis* only control. Serum was therefore not the ideal MA carrier. Investigation into other MA carriers seems worthwhile.

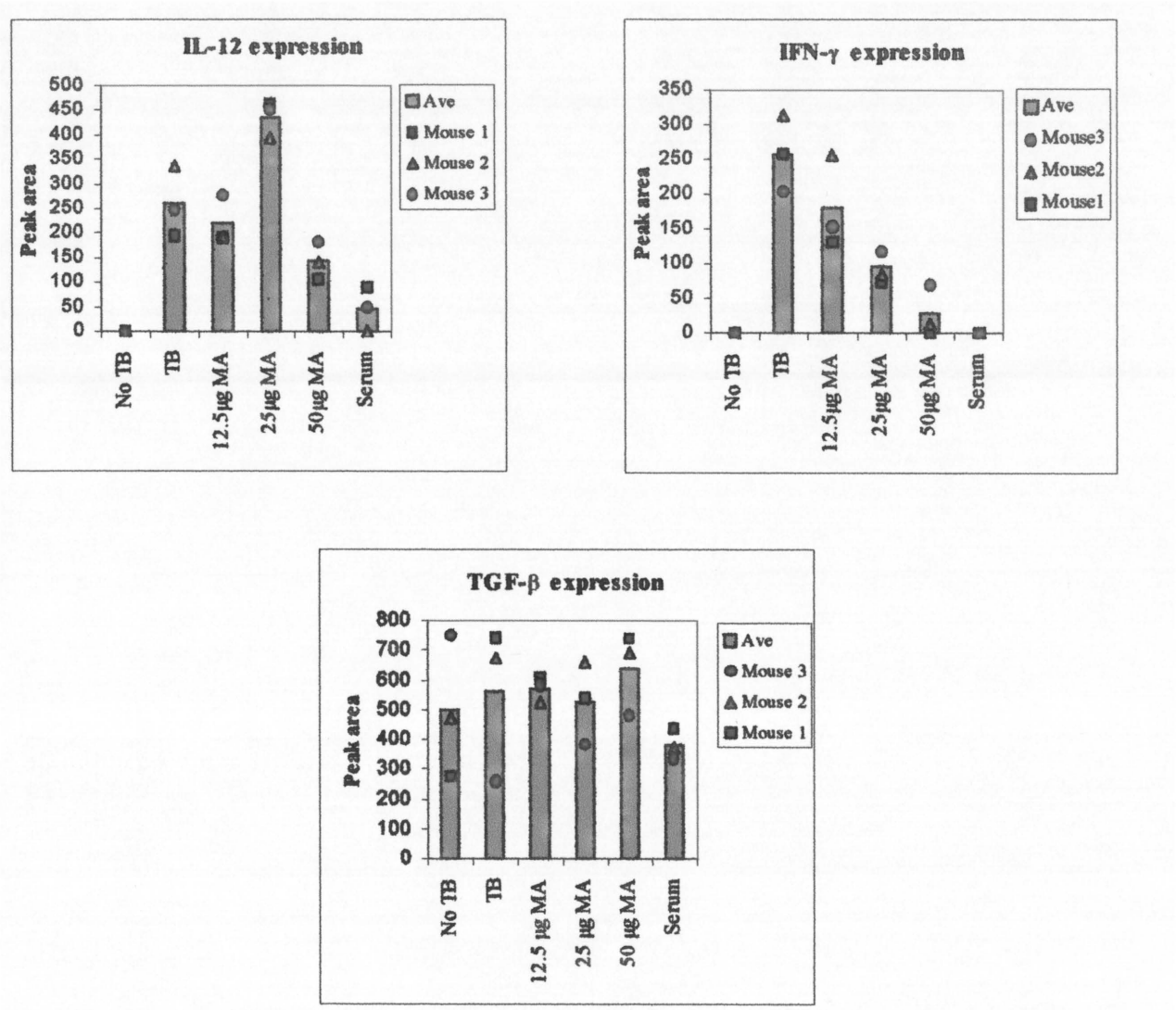


Figure 3.17 *Cytokine profiles of lungs of Balb/c mice pre-treated with MA in different doses as indicated, 5 weeks after infection was initiated.*

Expression of TGF-β did not seem to be influenced by the pre-treatment with MA or *M. tuberculosis* infection (Figure 3.17). The same levels of TGF-β mRNA expression were measured in both the controls and experimental groups with fluctuations varying between 30 - 40 %. This was the only cytokine that was measured that did not register an increase in the expression of cytokines for the *M. tuberculosis*-infected groups, as compared to the non-infected controls. Expression of IL-12, and IFN-γ did not occur at detectable levels in the negative control groups, while high values were detected for TGF-β.

The improved survival in the 12.5 μg MA and 25 μg MA pre-treatment groups of the Balb/c mice seems to correlate with the expression of IL-12 mRNA within the constraints imposed by the small number of animals per group. A concomitant increase in the expression of IFN-γ mRNA was also observed in these groups.

These findings support the hypothesis that the observed prolonged protection provided by MA-serum conjugate pre-treatment correlated with an increased expression of IL-12 and IFN-γ. It could well be that MA stimulate IL-12 expression in the lungs of Balb/c mice to the same levels as those observed in the more resistant C57BL/6J mice.

The cytokine profiles of the lungs of Balb/c mice treated with MA-serum conjugate after *M. tuberculosis* infection are presented in Figure 3.17. Mice were treated with different doses of MA-serum conjugate during the third week after the infection was initiated and the organs were extracted at week 5. PCR reactions were optimised as described above.

In contrast to the results obtained in the pre-treated groups, IL-12 expression seemed to be down-regulated as compared to the serum control group. Mice treated with 24 μg of MA-serum conjugate expressed 9 fold less IL-12 ($p < 0.0056$), and mice treated with 48 μg of MA-serum conjugate expressed 4 fold less IL-12 than the serum control group ($p < 0.0067$). Expression of IFN-γ again correlated with the IL-12 expression in the same groups. The reduced expression in IL-12 seemed to be MA specific, as IL-12 expression was not reduced in the serum control groups (Figure 3.18).

TGF-β is known to down-regulate IL-12 expression (Toossi *et al.*, 1997). If IL-12 expression were down-regulated by TGF-β, one would expect to see increased levels of the latter cytokine in the lungs. However, the expression of TGF-β remained unchanged when compared to the serum control group. This indicates that IL-12 expression could have been down-regulated by other cytokines, such as IL-10.

Survival studies indicated increased survival of Balb/c mice treated with 3 x 16 μg MA-serum conjugate after *M. tuberculosis* infection. This observed increased resistance did not correlate with IL-12 and IFN-γ expression. A different cytokine expression mechanism seems to be

mechanism seems to be involved in the 3 x 16 µg MA treatment group. The role of T-cell cytokines was not investigated in this experiment and the role of cytokine such as IL-10, known to down regulate IL-12 (Fulton *et al.*, 1998), might be worthwhile.

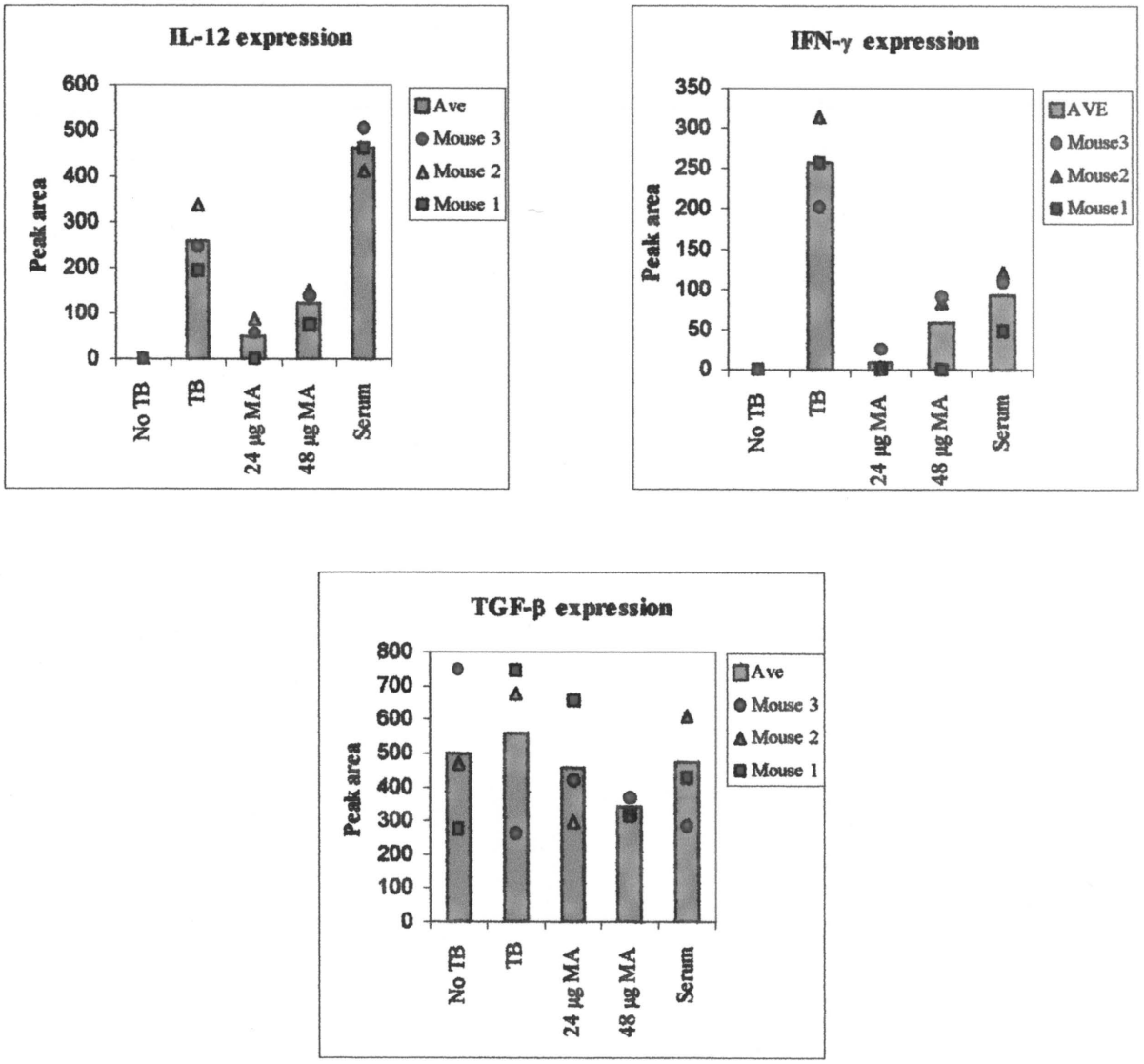


Figure 3.18 *Cytokine profiles of the lungs of Balb/c mice treated with MA in different doses as indicated. Organs were extracted 5 weeks after initiation of infection.*

These results indicate that increased IL-12 and IFN-γ expression could not explain the improved survival observed by MA administration after disease was initiated.

4. Discussion

Mycobacterium tuberculosis-derived peptide and lipid antigens have been shown to activate macrophages to secrete cytokines such as IFN-γ, IL-12 and TNF-α (DiPirio, 1997; Toossi, 1996). In fact, cord factor (trehalose dimycolate) was shown to be the first *M. tuberculosis*-derived glycolipid molecule to induce IL-12 secretion by macrophages (Oswald *et al.*, 1997). The recent discovery of the presentation of lipids on CD1 molecules (Porcelli *et al.*, 1992; Beckman *et al.*, 1994) provided a possible mechanism explaining how lipids could play an important role in the induction or inhibition of a protective immune response to intracellular pathogens.

In the lungs, alveolar macrophages are the first line of defense against infection with *M. tuberculosis* (Thompsen-Snipes *et al.*, 1998; Keane *et al.*, 1997). Phagocytosis of the bacilli by these mononuclear phagocytes leads to the generation of cytokines for the induction of the cell-mediated immune response. Failure to irradicate an initial tuberculosis challenge may lead to disease (Keane *et al.*, 1997; Bermudez and Goodman 1996; Barker *et al.*, 1997; McDonough *et al.*, 1993). In fact, macrophage-derived IL-12 is one of the key factors in directing and inducing a protective Th1 immune response. IL-12 is known to induce IFN-γ secretion by NK cells and T cells (Andersen, 1997; DiPirio 1997; Flynn *et al.*, 1995). IL-12 together with TNF-α secretion facilitates maximal IFN-γ secretion, which in turn promotes granuloma formation (Cooper *et al.*, 1995, Cooper *et al.*, 1997 and Gazzinelli *et al.*, 1993). In this study we wanted to determine whether MA, the wax-like molecule from the cell-walls of *M. tuberculosis*, can induce or inhibit a protective immune response during tuberculosis infection in a murine model. This was further elucidated by means of macrophage-derived cytokine profiling.

Strains of mice have been shown to respond differently to the same intracellular pathogen. Using murine models with *Leishmania major* as an intracellular pathogen, it was indicated that Balb/c mice (more susceptible) responded with a dominant Th2 response, compared to C57BL/6J mice which responded with a dominant Th1 response (Dauglat and Kaufmann, 1995). In an experiment done on DBA/2 and Balb/c mice infected with *Mycobacterium avium* it was shown that the Balb/c mice have decreased expression of IL-12 compared to C57BL/6J mice (Kobayashi *et al.*, 1997). In murine *M. tuberculosis* models using Balb/c and C57BL/6J

mouse strains, Flynn *et al.* (1995) could indicate that C57BL/6J mice were more resistant to intravenous *M. tuberculosis* infection than Balb/c mice. This was confirmed in this investigations, in an experiment showing that the difference between the two strains was especially pronounced when lower doses of live *M. tuberculosis* was used for infection.

In experiments done to determine if the major histocompatibility (MHC)-gene complex could confer resistance, it was evident that resistance more likely involved a non-MHC gene (Medina and North, 1998). Flynn *et al.* (1995) indicated that the more susceptible Balb/c mouse strain could be rendered more resistant by exogenous administration of IL-12 one day before induction of *M. tuberculosis* infection.

In the course of this work, it was shown that the base level expression of IL-12 in the lungs of non-infected negative control Balb/c mice, was lower than that of C57BL/6J mice. This observation was confirmed in two separate experiments for lung tissues. One could postulate that the increased level of IL-12 present in the lungs of C57BL/6J mice, was one of the factors that contributed to the increased resistance observed in this mouse strain.

It has been shown that exogenous administration of IL-12 increased the resistance to pathogens such as *Leishmania major* (Heinzel *et al.*, 1993a; Dauglat and Kaufmann, 1995), *Listeria monocytogenes* (Tripp *et al.*, 1994), *M. tuberculosis* (Cooper *et al.*, 1995; Flynn *et al.*, 1995), *Toxoplasma gondii* (Gazzinelli *et al.*, 1993) and *Schistosoma mansoni* (Wynn *et al.*, 1995). Moreover, the administration of exogenous anti-IL-12 antibodies rendered the mice more susceptible to tuberculosis infection as illustrated by increased numbers of bacteria in infected organs (Flynn *et al.*, 1995). In IL-12 p 40 gene knock-out mice the lack of bioactive IL-12 p70 molecules induced uncontrollable *M. tuberculosis* bacterial growth (Cooper *et al.*, 1997). Using *M. bovis* as an experimental model, it was demonstrated that the addition of anti-IL-12 to BCG-susceptible animals, induced a two- to threefold increase in spleen *M. bovis* colony forming units (CFU). In contrast, the addition of anti-IL-12 antibodies had no effect on the spleen *M. bovis* CFU in the BCG-resistant mice (Thompson-Snipes *et al.*, 1998). IL-12 seems to be of primary importance in the induction of a protective immune response against intracellular pathogens.

In C57BL/6J mice it seems as if the higher initial IL-12 p40 mRNA levels played a crucial role in the initial pre-activation of alveolar macrophages. The enhanced activation of a protective immune response could therefore enhance survival. In fact, macrophages pre-activated by cytokines have been shown to be more effective in killing internalized microorganisms (Barker *et al.*, 1997; McDonough *et al.*, 1993). IL-12, as mentioned before, activates IFN-γ secretion (Flynn *et al.*, 1993). IFN-γ is known to induce high levels of nitric oxide in cells that have the resistance gene Bcg, also known as Nramp1 (Aries *et al.*, 1997). However, it was indicated that resistance to *M. tuberculosis* was not determined by the resistance allele, Bcg (Nramp1), found on chromosome 1 in murine models (Medina and North, 1996).

M. tuberculosis-derived PPD could not induce IL-12 mRNA expression *in vitro* (Fulton *et al.*, 1996). Molecules known to induce IL-12 include LPS originating from Gram-negative bacteria, lipoteicohic acid from Gram-positive bacteria and a recombinant leishmanial protein antigen (Heinzel *et al.*, 1994; Cleveland *et al.*, 1996; Skeen *et al.*, 1996; Skeiky *et al.*, 1995). Recently, cord factor (trehalose dimycolate) was claimed to be the first *M. tuberculosis*-derived molecule shown to induce IL-12 mRNA expression in macrophages (Oswald *et al.*, 1997). In this chapter it is illustrated that *M. tuberculosis*-derived MA-serum conjugate can also induce IL-12 expression that coincides with an increase in IFN-γ expression in the lungs of Balb/c mice. This observation corroborates the results of Oswald *et al.* (1997) and indicates that it is in fact the MA part of the cord factor (CF) molecule that activates IL-12 expression in the lungs. The spleen remained unresponsive to MA administration.

The mechanism of the activation of IL-12 expression in macrophages is still unknown. Peptide antigens from the purified protein derivative failed to induce IL-12 expression in alveolar macrophages *in vitro* (Toossi *et al.*, 1995). Only particulate antigens such as polystyrene beads and bacteria have been shown to induce IL-12 expression in macrophages (Fulton *et al.*, 1996). This indicates that phagocytosis might be the determining factor to induce macrophages to secrete IL-12. On the transcriptional level the induction of IL-12 can be mediated by specific transcriptional factors such as STATs or NF kappa β (Yoshimoto *et al.*, 1997). Yoshimoto *et al.* (1997) postulated that phagocytosis induced conformational

changes in cytoskeletal proteins associated with tyrosine kinases. Thus kinases, once activated, phosphorylate the transcriptional factor and induce IL-12 mRNA transcription.

Bacterial lipopolysacharrides (LPS) have been shown to induce IL-12 mRNA expression in murine monocytes or macrophages (Snijders *et al.*, 1996). Recently a LPS-inducible DNA-protein complex was shown to be composed of NF kappa β components (Deng *et al.*, 1996). Yoshimoto *et al.* (1997) could indicate with electrophoretic mobility shift assays that the NF kappa β binding sequence is located 120 bp upstream of the transcription initiation site in murine IL-12 p 40 genes. The mechanism of lipid activation of NF kappa β is still unclear, but lipids such as ceramide and linoleic acid have been implicated as second messengers in the activation the p50 subunit of NF-kappa β (Kim and Kim, 1998; Boland and O'Neil, 1998; Young *et al.*, 1998). Whether cord factor and MA induce IL-12 mRNA expression according to a similar mechanism, remains to be determined

The role of molecules such as CD1 in the induction of a protective immune response still has to be elucidated. Recently it has been shown that murine CD1, which is homologous to human CD1d, also presents glycolipids to and activates $NK1.1^+$ T cells (Brossay *et al.*, 1998; Couedel *et al.*, 1998; Kawanu *et al.*, 1997). Whether the presentation of MA on CD1d would directly or indirectly activate macrophages to produce IL-12, remains to be determined.

In this chapter it was shown that the exogenous administration of MA can be used to elevate IL-12 mRNA expression levels in the lungs of Balb/c mice. Here, it was indicated that pre-treatment of Balb/c mice with a concentration of 12.5 μg and 25 μg MA adsorbed on homologous serum induced short-term protection to the onset of *M. tuberculosis* disease of mice as compared to mice treated with serum only. This observation was reproducible in two statistically important experiments. The short-term protection of these mice in the pre-treatment groups coincided with elevated levels of IL-12 and IFN-γ in the lungs of Balb/c mice, five weeks after the infection with *M. tuberculosis* was introduced. These results corroborate the finding that exogenous administration of recombinant IL-12 in murine models protects against tuberculosis. This exogenous administration of IL-12 also increased the survival, but could not prevent the onset of *M. tuberculosis* infection in experimental animals (Flynn *et al.*, 1995; Cooper *et al.*, 1997).

The results obtained show that expression of IL-12 could be induced within 48h after non-infected healthy mice were pre-treated with MA adsorbed on homologous serum. Upon initiation of infection, resident levels of IL-12 would increase further due to phagocytosis of the bacteria by macrophages (Fulton *et al.*, 1996). Indeed, higher levels of IL-12 expression were observed in the MA-serum conjugate treated experimental groups receiving 12.5 μg and 25 μg MA conjugated to mouse serum. This coincided with elevated IFN-γ expression when compared to the mice treated with serum only. The expression of IFN-γ in the MA pre-treated mice was never higher than that of the mice infected with *M. tuberculosis* only. A serum effect was also observed as treatment with serum seemed to down-regulate IL-12 expression when compared to the mice infected with *M. tuberculosis.* Serum is therefore not the ideal MA-carrier and further investigation into more suitable carriers seems worthwhile.

Mice treated with MA-serum conjugate during the third week after infection also showed enhancement in survival. This did not coincide with up-regulation of IL-12 mRNA expression five weeks after the infection was initiated. Partial protection provided by MA administered after infection appears to be induced by a different mechanism than administration of MA as a prophylactic treatment. Two to three weeks after the initiation of intravenous *M. tuberculosis* infection, T cells, NKT cells and natural killer cells start to migrate to the infection site (Andersen, 1997; Orme, 1996). In this study the role of other cytokines such as T cell-derived IL-10 was not investigated. IL-10 is known as an inhibitor of IL-12 expression and Th1 responses (Fulton *et al.*, 1998), and could be the cytokine responsible for IL-12 down-regulation observed here.

As mentioned before, IL-12 is essential for the induction of a protective immune response to intracellular pathogens including *M. tuberculosis* (Flynn *et al.*, 1995 and Cooper *et al.*, 1997). This is due to the fact that IL-12 is a known inducer of IFN-γ by NK and T cells (Bliss *et al.*, 1996 and Gazzinelli *et al.*, 1993). IFN-γ is necessary for the recruitment and activation of macrophages, and plays an essential role in granuloma formation (Zhang *et al.*, 1994; De Jong *et al.*, 1997; Sypec *et al.*, 1993 Flynn *et al.*, 1995 and Andersen 1997).

In all the experiments reported here, IL-12 up- or down-regulation coincided with the expression of IFN-γ as compared to serum control. Flynn *et al.* (1993) and Cooper *et al.*

(1993) found that administration of exogenous IFN-γ led to the migration of large numbers of Th1 cells to the infection site. In IFN-γ gene knock-out mice an increase in the amount of viable bacteria was observed at the infection site. These authors provided evidence that the induction of IL-12 and the concomitant activation of IFN-γ appeared to be the key factors that induce short term protection in Balb/c mice. In this study, only the mRNA expression of cytokines was determined, but the fact that IFN-γ and IL-12 expressions correlated is indicative of biological p70 IL-12 protein expression.

IFN-γ mRNA expression was recently shown to be induced by the expression of another cytokine, IL-18. In experiments done on *M. tuberculosis*-infected IL-12 gene knock-out mice however, the expression levels of IFN-γ did not increase. Exogenous administration of IL-12 did however restore the IFN-γ levels (Cooper *et al.*, 1997). Interleukin 12 appears to be one of the more important cytokines in determining the degree of resistance to *M. tuberculosis* in mice.

In the experiments carried out on Balb/c mice the resident levels of TGF-β mRNA in the lungs seem to remain unchanged. This was true for all the MA-serum conjugate treatments done in non–infected and *M. tuberculosis*-infected mice. TGF-β is known to inhibit both IFN-γ and IL-12 expression (Toossi *et al.*, 1995). Previously it has been shown that *M. tuberculosis*-derived PPD and LAM induced TGF-β production in peripheral blood monocytes (PBMC) *in vitro* (Dahl *et al.*, 1996; Ellner, 1997). It was therefore surprising that no increase in TGF-β mRNA production was detected in the lungs of *M. tuberculosis*-infected experimental animals as PBMC are known to be recruited to the infection site. This could be explained by the fact that alveolar macrophages do not express TGF-β mRNA and protein at the same levels as recruited monocytes (Toossi *et al.*, 1996).

The fact that in our experiments, TGF-β mRNA expression remained more or less unresponsive implies that another cytokine was responsible for the observed down-regulation of IL-12, in mice treated with MA-serum conjugate during the third week after infection. One likely candidate is IL-10, a cytokine also known for the down regulation of Th1 immune responses (Andersen, 1997).

It is interesting that the expression of IL-12 was not affected in the spleen of both C57BL/6J and Balb/c mice (Siko, 1998). In previous experiments, exogenous administration of IL-12 did not increase the IFN-γ expression in the spleens of infected animals (Flynn *et al.*, 1995). This occurred in spite of the intravenous introduction of *M. tuberculosis* bacteria. The fact that the spleen remained inactive, could be an indication that the innate-immune response in the lungs was enhanced rather than a systemic immune response governed by the spleen.

The cell types that express IL-12 in the lungs were not determined in this study. Macrophages, B cells and neutrophils have the ability to express the biologically active p70 IL-12 protein. The immune response to *M. tuberculosis* infection can be divided into different stages where the cellular response is concerned. The first innate stage is characterised by the activation of alveolar macrophages leading to the secretion of cytokines such as IL-12. This is followed by the recruitment of various T cells, including $CD8^+$, DN and $CD4^+$ T cells to the infection site. Activation of these T cells lead to secretion of IFN-γ that would in turn activate additional macrophages and monocytes. This feedback regulation is very important for the formation of granuloma, i.e. in the final stage of activation (Andersen, 1997; Kaufmann and Andersen, 1998; Vanham *et al.*, 1997).

The protective effect of MA could be dependent on the type of cells activated in the immune response. To elucidate the protective role of MA in these experiments, it would be important to determine the cell types involved in the secretion and activation of IL-12. As 95% of the leukocytes in lungs, obtained after lung-lavage experiments are mononuclear phagocytes, the source of IL-12 is most likely alveolar macrophages and recruited monocytes (Denis *et al.*, 1994).

To determine the cell source of IFN-γ would be more complex. There are various cell types that can secrete IFN-γ, especially in the infected lungs. This includes NK cells, DN T cells, CD4- and CD8 positive T cells and γδ T cells (Silva *et al.*, 1998; Andersen, 1997; Dougelat and Kaufmann, 1996; Sypec *et al.*, 1993). In experimental models using *T. gondii* as model, T cell-depleted mice induced NK cell-derived IFN-γ in the lungs in the presence of IL-12 (Gazzinelli *et al.*, 1993). It is also known that IL-12 enhances proliferation of NK cells (Silva *et al.*, 1998; Sypec *et al.*, 1993). In experiments using cord factor as model, IFN-γ was still

produced in the absence of T cells (CD3 knock-out mice) by NK cells (Orbach-Arbouys *et al.*, 1993). As no effect on the Th1/Th2 cytokine production was observed in the spleen (work done by G Siko and A Lenaerts) we argue that the most likely source of IFN-γ in the lungs are NK cells. This has to be confirmed by further investigations.

In conclusion, we were able to indicate that C57BL/6J mice have high IL-12 base level expression in the lungs as compared to Balb/c mice. Treatment of Balb/c mice with MA-serum conjugate, in the absence of *M. tuberculosis* infection did, however, induce IL-12 expression in the lungs. Furthermore, pre-treatment of the Balb/c mice with MA-serum conjugate, showed increased resistance to *M. tuberculosis* at the same level as seen in C57BL/6J mice after *M. tuberculosis* infection. This resistance coincided with increased expression of IL-12 and IFN-γ mRNA. Interleukin–12 appeared to be one of the determining factors that could explain the observed increased resistance to *M. tuberculosis* infection in C57BL/6J mice as compared to that of Balb/c mice. In the case of MA post-infection treatment, improved survival was also observed, but the mechanism of protection appears to be different, i.e. not by stimulation of IL-12 and IFN-γ.

Chapter 4
Concluding Discussion

In the search for therapies and vaccines to prevent *Mycobacterium tuberculosis* infection, a lot of attention was placed on antibiotics and protein antigens as possible candidates to control disease. Until recently the knowledge of what was known of the mechanism of *M. tuberculosis* infection and the subsequent host immune response, was based on *M. tuberculosis*-derived protein, lipo-polysaccharides and saccharide antigens (Andersen, 1997; DiPirio, 1997). The recent discovery of the presentation of *M. tuberculosis*-derived mycolic acids (MA) on CD1, a MHC-like molecule, has shifted the emphasis to include lipid antigens as possible mediators of the immune response (Beckman and Brenner, 1994).

A protective immune response to *M. tuberculosis* infection is dependent on the development of a T helper 1 (Th 1) immune response (Orme *et al.*, 1993; Spargo *et al.*, 1991). This involves the secretion of cytokines such as IL-12, IFN-γ and TNF-α (Reviewed by Andersen, 1997). In this study it was attempted to prove that *M. tuberculosis*-derived MA have an influence on the development of the immune response to *M. tuberculosis* infection. To determine the immunogenicity of MA in its native state the antibody response in human *M. tuberculosis* patient sera and *M. tuberculosis*-infected mice were determined. Western blot analysis was initially performed to indicate the presence of MA-specific antibodies. Mouse serum proteins were conjugated with MA and separated under non-reducing conditions on SDS-PAGE. Not only could it be indicated that MA-specific antibodies exist, but it was indicated that patient antibodies recognised MA preferentially on a preferred serum protein of ± 90 kDa. Conjugation of MA onto the mouse serum proteins most likely resulted in a homogenous distribution of MA on the serum proteins. This would indicate that the association of MA with the 90 kDa protein renders it the preferred immunogen for eliciting antibodies against MA.

Protein-lipid complexes have been shown to have different mobilities under reducing and non-reducing SDS-PAGE conditions (Nakano *et al.*, 1996). The band observed at 90 kDa is therefore not an indication of the molecular mass of the transporter protein alone, but is rather

an indication of the molecular mass of the protein-lipid complex. As the lipid:protein ratio of this complex is not known, the probable protein mass cannot be determined. Proteins such as albumin, a known lipid carrier protein in serum, cannot be excluded as a probable mycolic acid carrier in serum. Other serum proteins can also be likely candidates.

In order to facilitate the screening of large quantities of patient sera, the presence of MA-specific antibodies was determined by means of ELISA. Sixty five percent of one hundred human patients' sera tested, were negative for MA-specific antibodies. Twenty percent of the sera did indeed have antibodies directed to MA. The remaining 15 % of the patient sera tested gave high background values in the ELISA, which could possibly be attributed to the presence of rheumatoid antibodies.

Previously, cord factor (trehalose dimycolate) was one of the glycolipid antigens considered as an antigen for possible sero-diagnosis. In those experiments 70-90 % of the *M. tuberculosis*-infected patients tested positive for anti-cord factor antibodies (He *et al.*, 1991; Simonney *et al.*, 1995). This observed difference (20 % of tuberculosis patients positive for anti MA antibodies vs. 70-90 % of tuberculosis patients positive for anti-cord factor antibodies) could be explained by the fact that the saccharide moiety of cord factor appeared to be the dominant epitope. MA, as the weaker epitope, would not be detected in typical immuno assays such as ELISA in the presence of the stronger epitope (Kato *et al.*, 1972 and 1974).

In an attempt to indicate that MA-specific antibodies also exist in a murine model, Balb/c mice were intravenously infected with *M. tuberculosis*. In the course of this investigation, two out of ten mice showed an increased expression of MA-specific antibodies, but of the IgM class. Again sera with high background values were present.

It was reported that the presence of *M. tuberculosis* in complete Freund's adjuvant can result in collagen-induced auto-immune arthritis in rats (Mauri *et al*, 1996 and Kasarma *et al,* 1994). The presence of rheumatoid antibodies in *M. tuberculosis*-infected patients and -mice could be an early indication of the development of collagen-induced arthritis in these individuals. In the Western blot analysis, a band of ±80 kDa was observed irrespective of the antigen used (mouse serum or MA-serum conjugate). This band could be an indication of the development

of auto-immune antibodies directed to serum protein molecules in *M. tuberculosis*-infected patients.

Antibody production to MA in humans could be dependent on the isotype of CD1 on which MA is presented. Presentation of MA on CD1 would activate different subsets of T cells depending on the CD1 isotype. In humans, different subsets of CD1 exist which can be divided into two groups: Group one consisting of CD1a, -b and -c and Group 2 consisting of CD1d (Powrie and Coffmen, 1993, Simmoney *et al.*, 1995). It has been shown in humans that MA associated with CD1b are presented to a subset of T cells known as $CD4^-$ $CD8^-$ T cells or double negative (DN) T cells (Bendelac, 1993). Furthermore, the expression of glycolipids on CD1a and c activates DN T to secrete high levels of IFN-γ. Whereas the presentation of lipids on CD1d has been shown to activate a subset of T cells known as $NK1.1^+$ $CD4^+$ T cells (NKT cells) to secrete IL-4 (Park *et al.*, 1998; Bendelac, 1993; Yoshimoto *et al.*, 1995). NKT cells are CD4(+) T cells that also express a marker naturally associated with NK cells. To date, most of these experiments were carried out in murine models as mice have only the CD1d homologue. Recently, a human NKT cell homologue have been identified, also recognising glycolipids presented on CD1d (Brossay *et al.*, 1998; Couedel *et al.*, 1998).

If DN T cells were involved in the induction of antibodies to MA, this would be very useful in the diagnosis of *M. tuberculosis* in patients that are HIV positive, because activation of antibodies *via* this pathway evades CD4(+) T cell mediation and may involve non-specific polyclonal B cell activation (Sousa *et al.*, 1998). In this study, however, the presence of MA-specific antibodies was distributed between HIV positive and HIV negative patients (results not shown). In the murine model, antibody production was mediated by NKT cells, as only CD1d homologues are present in mice. To understand the mechanism of antibody production in the human system, which is much more complex than the murine system, it would be very useful to investigate the role of DN T cells, NKT cells and the possible presentation of MA on CD1d further.

The presentation of MA on CD1 molecules and the existence of antibodies to MA relate to the fact that MA is immunogenic and could play a role in the progression of *M. tuberculosis* infection. MA as such could play a very important role in the induction of a protective

immune response to tuberculosis. This molecule could also orientate the immune system to increase the vaccination properties of BCG .

In an attempt to determine the underlying mechanism of cytokine regulation by MA in a murine model, survival studies and subsequent cytokine profiling were carried out. In the experiments done to compare the IL-12 expression in various organs, it became evident that the lungs were the responsive organs to *M. tuberculosis* infection as compared to the spleen and that the innate immune response might be mediated by IL-12 expression. Flynn *et al.* (1995) indicated that the exogenous addition of IL-12 one day after infection increased the survival in Balb/c mice. Furthermore it was indicated that IL-12 had no effect in the absence of IFN-γ (IFN-γ gene knock-out mice). In this study, low levels of IL-12 expression were observed in all the organs of Balb/c mice, except the spleen of the negative control group. After infection was introduced, the levels of IL-12 expression increased significantly. Furthermore, in C57BL/6 mice the base level IL-12 expression in the lungs of the negative control mice was almost as high as that of mice infected with *M. tuberculosis*. This experiment confirms the results obtained by Kobayashi *et al.* (1997), in which DBA/2 mice were shown to have increased levels of IL-12 mRNA in the lungs as compared to Balb/c mice when infected with *Mycobacterium avium*.

Could the elevated levels of IL-12 mRNA in the lungs of C57BL/6 mice, irrespective of *M. tuberculosis* infection, be one of the factors that contribute to the observed resistance on the onset of disease in these mice? Up-regulation of IL-12 was found to be very important in the resistance to various intracellular pathogen infections. IL-12 activates macrophages, NK cells and T cells to secrete IFN-γ. Granuloma formation and the recruitment of other lymphocytes to the site of infections are just some of the important functions of IFN-γ (De Jong *et al.*, 1997; Sypec *et al.*, 1993 Flynn *et al.*, 1995 and Andersen 1997). It seems as if IL-12 and the subsequent secretion of IFNγ conferred protection against invading pathogens such as *M. tuberculosis* (Cooper *et al.*, 1995; Flynn *et al.*, 1993) *Listeria monocytogenes* (Tripp *et al.*, 1994), *Leishmania major* (Heinzel *et al.*, 1993b), *Toxoplasma gondii* (Gazzinelli *et al.*, 1993) and *Schistosoma mansoni* (Wynn *et al.*, 1995). The increased expression of IL-12 in the lungs of C57BL/6 can therefore be regarded as a protective mechanism to the onset of the disease.

The observation that the lung was the responsive organ was further corroborated in an experiment where mice were immunised with MA alone (i.e. in the absence of *M. tuberculosis* infection). The spleen again remained non-responsive and only the lungs showed increased expression levels of IL-12 in Balb/c mice. It has been shown that protein antigens derived from *M. tuberculosis* could not induce IL-12 expression in alveolar macrophages (Fulton *et al.*, 1996). This result confirms the previous observation by Oswald *et al.* (1997) who showed that cord factor (trehalose dimycolate) does indeed activate IL-12 expression in alveolar macrophages. In this work, it was shown that the mycolic acid moiety of the cord factor molecule is responsible for or contributes to the activation of IL-12.

In two separate survival studies it was confirmed that pre-treatment of Balb/c mice with 25 μg MA-serum conjugate increased the resistance to *M. tuberculosis* infection. Furthermore, this increased resistance seemed to correlate with the resistance normally seen in *M. tuberculosis*-infected C57BL/6 mice. The lungs seemed to be the responsive organ as it was shown in the two experiments mentioned previously. To determine the underlying mechanism of this short term protection, cytokine profiling on the macrophage-derived cytokines was done on the lung tissue. The partial protection seems to coincide with elevated levels of IL-12 and IFN-γ when compared to serum controls. Mice treated with 48 μg MA, during the third week after infection, also showed increased resistance to *M. tuberculosis* infection. It seemed as if the mode of protection differed in this case, as no correlation between IL-12 and IFN-γ expression and survival was detected.

In the lungs, there is a very fine balance between the expression of pro-inflammatory cytokines, such as IL-2, IL-12 and IFN-γ, and inhibitory cytokines such as TGF-β (DiPirio, 1996). Cytokines such as IFN-γ and TNF-α are very important in the containment and destruction of the bacteria in the lungs during infection. This involves the activation of RNI and ROI (Toossi *et al.*, 1997; Aliprantis, 1996). These radicals do, however, also affect the healthy lung tissue. In order to maintain the healthy lung tissue, the effects of IFN-γ and TNF-α are down-regulated by TGF-β. In this study, no difference in the expression of TGF-β was detected in the spleens and lungs of Balb/c mice. This could indicate that the immune response in these mice was not in a stage where down-regulation of cytokines such as IFN-γ and TNF-α was needed.

The protective effect of MA could be dependent on the type of cells activated in a specific stage of the immune response. To elucidate the protective role of MA in these experiments, it would be important to determine the cell types involved in the secretion and activation of IL-12. When mice were treated with MA-serum without *M. tuberculosis* infection, induction of IL-12 was observed. This would indicate that the macrophages, neutrophils or B cells in the innate stage were activated. This pre-activation seems sufficient to induce increased resistance in the mice when they were pre-treated with MA-serum, before initiation of infection. When the mice were post-treated with MA-serum during the third week after infection, resistance to infection was also observed. In this scenario, the MA could have an influence on totally different cell types at the infection site.

Three weeks after the induction of *M. tuberculosis* infection, the second phase of the immune response to *M. tuberculosis* is activated (Andersen, 1997; Kaufmann and Andersen, 1998). This involves the activation of T cells. A mycolic acids-T cell specific response would involve the presentation of MA on CD1 molecules. Murine CD1 is homologous to human CD1d and has been shown to present glycosylceramides to NK1.1 $CD4^+$ T cells (Kawano *et al.*, 1997). NK1.1 CD4 T cells have been shown to secrete Th2 type cytokines including IL-4. In this study the Th2 T cell cytokines were not determined at the infection site. The determination of inhibitory cytokines such as IL-4 and IL-10, known to down-regulate a Th1 immune response (Fulton *et al.*, 1998), might explain the absence of IL-12 and IFN-γ in the lungs of mice treated with MA.

In this study, homologous MA-serum conjugate was shown to induce IL-12 mRNA expression in healthy lungs of mice kept in a pathogen-free environment. Activation of IL-12 mRNA expression in antigen presenting cells can occur in one of two pathways: i) a T cell-specific pathway that requires activation of T cells and IL-12 expression *via* CD40 triggering, and ii) a pathway independent of T cell mediation requiring activation by bacterial products such as LPS or the phagocytosis of bacteria (DeKruyff et al., 1997). T cell dependent activation of IL-12 expression would be expected in the late phases of the immune response to *M. tuberculosis* infection. T cell independent activation of IL-12 would involve the activation of IL-12 mRNA synthesis *via* transcriptional factors independent of receptor-ligand activation.

It is very difficult to extrapolate the results obtained in murine models to a human model. Mice have only one type of CD1 that is homologous to human CD1d. The effect of the presentation of MA on human CD1b on the immune response to *M. tuberculosis* still needs to be determined. CD1b presentation of lipids seems to activate the Th1 immune response and involves presentation to DN T cells (Beckman and Brenner, 1994). The role of CD1d has not been determined in humans to date.

The cytokine profiling experiment of mice pre-treated and post-treated with MA could be regarded as a screening experiment. These results give an indication of the type of cytokine expression expected in the lungs of mice treated with MA after *M. tuberculosis* infection. It was determined that the dose of MA needed to induce a protective response occur over a narrow range. Additional experiments can also be done to establish the role of MA in the activation of IL-12 m RNA expression. Treating mice with MA in the presence of anti IL-12 antibodies or using IL-12 p 40 gene knockout mice can corroborate the crucial role that MA play in the induction of IL-12 mRNA expression. If IL-12 expression is essential for increased resistance in Balb/c mice, the p40 gene knock-out mice, treated with MA, should die at the same time as non-MA treated knockout mice. If the increased resistance is still observed, it would imply that other factors are responsible for the observed increased resistance in these mice.

In the course of this investigation, we detected differences in IL-12 expression 5 weeks after the induction of an immune response to *M. tuberculosis*. Flynn et al. (1995) could show an increase in IFN-γ expression four days after the initiation of *M. tuberculosis* infection. The response observed here might be at a stage where the effects of IL-12 are beginning to be down-regulated. A kinetic study to determine the expression of IL-12 over different time intervals would indicate at what stages of the development of an immune response peak levels of IL-12 are produced.

Semi-quantitative RT-PCR was used to determine the cytokine profiles induced by a range of MA-serum concentrations. In order to determine exact increases in the various groups, this experiment should be repeated at the determined optimum conditions with **quantitative** competitive RT-PCR (QC-RT-PCR). QC-RT-PCR is a technique developed to determine the

concentration of mRNA in the presence of a competitive plasmid, containing a deletion mutant of the gene. Both plasmid (dilution range of known concentration) and wild type cDNA are amplified in the same reaction mixture with the same primers. The exact concentration of the wild type cDNA can be determined by comparing the density of the wild type to that of the plasmid DNA of known concentration (Benavides *et al.,* 1995). To compare the expression of cytokines between individual mice, the concentration of the mRNA that is used should also be determined. This can be done by quantitation of a house-hold gene in each sample by using QC-RT-PCR.

In conclusion, we were able to indicate that anti-MA antibodies do exist in human and murine sera, therefore MA appears to be immunogenic. In murine models it seemed as if the innate immune response in the lungs, governed by IL-12 secretion, is important in the induction of a protective immune response to *M. tuberculosis* infection. Furthermore, exogenous administration of MA one week prior to the onset of disease seemed to increase the resistance of Balb/c mice to the same level as normally found in C57BL/6 infected with *M. tuberculosis*. This observed short-term protection might involve the induction of IL-12 and IFN-γ expression. Based on the research reported in this dissertation, we propose that MA may contribute to the priming of macrophages, enhancing the protective immune response when used together with known vaccines such as BCG, or may play a part in the shortening course of antibiotic treatment of tuberculosis patients.

References.

ALIPRANTIS A.O, DIEZ-ROUX G, MULDER L.C, ZYCHLINSKY A, and LANG R.A. (1996). Do macrophages kill through apoptosis?
***Immunol Today,* 17**:573-576

ALLENDOERFER R and DEEPE G.S. jr. (1998). Blockade of endogenous TNF-alpha exacerbates primary and secondary pulmonary histoplasmosis by differential mechanisms.
***J Immunol,* 160**: 6072-6082

ALMOG R and MANNELLA C.A. (1996). Molecular packing of cord factor and its interaction with phosphatidylinositol in mixed monolayers.
***Biophys J*, 71**:3311-9

ANDERSEN P, ASKGAART D, LJUNGQVIST L, BENTZON M.W and HERON I. (1991). T-cell proliferative response to antigens secreted by *Mycobacterium tuberculosis.*
***Infect Immun*,59:**1558-15563

ANDERSEN P, ANDERSEN A.B, SORENSEN A.L and NAGAI S. (1995). Recall of long-lived immunity to *Mycobacterium tuberculosis* infection in mice.
***J Immunol,* 154:** 3359-3372

ANDERSEN P. (1997). Host responses and antigens involved in protective immunity to *Mycobacterium tuberculosis.*
***Scand J Immunol*,45:** 115-131

ARIAS M, ROJAS M, ZABALETA J, RODRIGUEZ J.I, PARIS S.C, BARRERA L.F and GARCIA L.F. (1997). Inhibition of virulent *Mycobacterium tuberculosis* by Bcg(r) and Bcg(s) macrophages correlates with nitric oxide production.
***J Infect Dis,* 176:** 1552-1558

ASANO M, NAKANE A. and MINAGAWA T. (1993). Endogenous gamma interferon is essential in granuloma formation induced by glycolipid containing mycolic acid in mice.
***Infect Immun,* 61:** 2872-2878

AUNG H, TOOSSI Z, WISNIESKI J.J, WALLIS R.S, CULP L, PHILLIPS N.B, PHILLIPS M, AVERILL L.E, DANIEL T.M. and ELLNER J.J. (1996). Induction of monocyte expression of tumour necrosis factor α by the 30-kD antigen of *Mycobacterium tuberculosis* and synergism with fibronectin.
***J Clin Invest,* 98:**1261

AVERILL L, TOOSSI Z, AUNG H, BOOM W.H. and ELLNER J.J. (1995). Regulation of production of tumour necrosis factor alpha in monocytes stimulated by the 30-kilodalton antigen of *Mycobacterium tuberculosis.*
***Infect Immun,* 63**:3206

BALAJI K.N and BOOM W.H. (1998). Processing of *Mycobacterium tuberculosis* bacilli by human monocytes for CD4+ alpha-beta and gamma-delta T cells: role of particulate antigen.
***Infect Immun,* 66**: 98-106

BARBULESCU K, BECKER C, SCHLAAK J.F, SCHMITT E, MEYER Z.U.M, BUSCHENFELDE K.H and NEURATH M.F. (1998). IL-12 and IL-18 differentially regulate the transcriptional activity of the human IFN-γ promoter in primary CD4+ T lymphocytes.
***J Immunol* 160:** 3642-3647

BARKER L.P, GEORGE K.M, FALKOW S and SMALL P.L. (1997). Differential trafficking of live and dead Mycobacterium marinum organisms in macrophages.
***Infect Immun,* 65**: 1497-1504

BARNES P.F. and MODLIN R.L. (1996). Human cellular immune responses to *Mycobacterium tuberculosis.* ***Curr Op Microbiol Immunol,*** **215:** 197-219

BATONI G, ESIN S, HARRIS R.A, KALLENIUS G, SVENSON S.B, ANDERSEN R, CAMPA M and WIGZELL H. (1998). Gamma-delta+ and CD4+ alpha-beta+ human T cell subset responses upon stimulation with various *Mycobacterium tuberculosis* soluble extracts.
Clin Exp Immunol, **112**:52-62

BECKMAN E.M, PORCELLI S.A, MORITA G.T, BEHAR S.M, FURLONG S.T. and BRENNER M.B. (1994). Recognition of a lipid antigen by CD1-restricted ß$^+$ T cells.
Nature, **327:** 691-694

BECKMAN E.M. and BRENNER M.B. (1995). MHC class I like, class II-like and CD1 molecules: distinct roles in immunity.
Immunol Today, **16:** 349-352

BENAVIDES G.R, HUBBY B, GROSSE R.A, McGRAW R.A. and TARLETON R.L. (1995). Construction and use of a multi-competitor gene for RT-PCR using existing primer sets.
Infect Immun, **62:** 145-156

BENDELAC A. (1995). CD1: Presenting unusual antigens to unusual T lymphocytes.
Science, **269:** 185-186

BENDELAC A, LANTZ O, QUIMBY M.E, YEWDELL J.W, BENNINK J.R. and BRUTKIEWICZ R.R. (1995). CD1 Recognition by mouse NK1(+) T lymphocytes.
Science, **268:** 863-865

BENDENELLI M. and FRIEDMAN H. (1989). *Mycobacterium tuberculosis* Interactions with the immune system.
Plenum Press. **New York and London.**

BERMUDEZ L.E. and GOODMAN J. (1996). *Mycobacterium tuberculosis* invades and replicates within type II alveolar cells.
Infect Immun, **64:** 1400

BERMUDEZ L.E, WU M. and YOUNG L.S. (1995). Interleukin-12-stimulated natural killer cells can activate human macrophages to inhibit growth of *Mycobacterium avium.*
Infect Immun, **63**: 4099

BILYK N and HOLT P.G. (1995). Cytokine modulation of the immunosuppressive phenotype of pulmonary alveolar macrophage populations.
Immunology, **86**: 231-237

BLISS J, CLEAVE V, MURRY K, WIENCIS A, KETCHUM M, MAYLOR R, HAIRE T, RESMINI C, ABBAS A.K and WOLF S.F. (1996). IL-12 as an adjuvant promotes a T helper 1 cell but does not suppress a T helper 2 cell recall response.
J Immunol, **156:**887-894

BONECINI-ALMEIDA M.G, CHITALE S, BOUTSIKAKIS I, GENG J, DOO H, HE S and HO J.L. (1998). Induction of in vitro human macrophage anti-*Mycobacterium tuberculosis* activity: requirement for IFN-gamma and primed lymphocytes.
J Immunol, **160**: 4490-4499

BOOM W.H. (1996). The role of T cell subsets in *Mycobacterium tuberculosis* infection.
Infect Agents Dis, **5:** 73-81

BOLAND M.P and O'NEILL L.A. (1998). Ceramide activates NF-kappa B by inducing the processing of p105
J Biol Chem, **273**:15494-500

BOST K.L and CLEMENTS J.D. (1995). In Vivo Induction of Interleukin-12 mRNA Expression after Oral Immunization with *Salmonella dublin* or the B Subunit of *E.coli* Heat-Labile Enterotoxin.
***Infect Immun*, 63:** 1076-1083

BRENNAN P.J. and NIKAIDO H. (1995). The envelope of mycobacteria.
***Annu Rev Biochem*, 64:** 29-63

BROSSAY L, NAIDENKO O, BURDIN N, MATSUDA J, SAKAI T and KRONENBERG M. (1998). Structural requirements for galactosylceramide recognition by CD1-restricted NK T cells.
***J Immunol*, 161**: 5124-8

BRUNDA M.J. LUISTRO L and WARRIER R.R.(1993). Antitumor and antimetastatic activity of IL-12 against murine tumours.
***J Exp Med*, 178:** 1223

BUTLER W.R, JOST K.C jr and KILBURN J.O. (1991). Identification of mycobacteria by high-performance liquid chromatography
***J Clin Microbiol*, 29**: 2468-2472

CALABI F, JARVIS J.M, MARTIN L. and MILSTEIN C. (1989). Two classes of CD1 genes.
***Eur J Immunol*, 19:** 285-292

CAMPOS-NETO A, OVENDALE P, BEMENT T, KOPPI T.A, FANSLOW W.C, ROSSI M.A and ANDERSEN M.R. (1998). CD40 ligand is not essential for the development of cell-mediated immunity and resistance to *Mycobacterium tuberculosis*.
***J Immunol*, 160:** 2037-41

CARSWELL E.A, OLD L.J, KASSEL R.L, GREEN S, FIORE N, and WILLIAMSON B. (1975). An endotoxin-induced serum factor that causes necrosis of tumours.
***Proc Natl Acad Sci U S A*,72**:3666-3670

CHAN J. TANAKA K. CARROLL D. FLYNN J. and BLOOM B.R. (1995). Effects of Nitric Oxide Synthase Inhibitors on Murine Infection with *Mycobacterium tuberculosis.*
***Infect Immun*, 63:**736

CHO S, SHIN J, DAFFE M, CHONG Y, KIM S. and KIM J. (1992). Production of monoclonal antibody to a phenolic glycolipid of *Mycobacterium tuberculosis* and its use in detection of the antigen in clinical isolates.
***J Clin Microbiol*, 30:** 3065-3069

CHOMEYNSKI P and SACCHI N. (1987). Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction.
***Anal Biochem*, 162**, 156-159

CHONG C, BOST K.L and CLEMENTS J.D. (1996). Differential Production of Interleukin-12 mRNA by murine Macrophages in Response to Viable or Killed *Salmonella* spp.
***Infect Immun*, 64:**1154-1160

CLEVELAND M.G, GORHAM J.D, MURPHY T.L, TUOMANEN E and MURPHY K.M. (1996). Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway.
***Infect Immun*, 64**:1906-1912

COOPER A.M, ROBERTS A.D. RHOARDES E.R, CALLAHAN J.E, GETZY D.M. and ORME I.M (1995). The role of IL-12 in acquired immunity to *Mycobacterium tuberculosis* infection.
***Immunol*, 84:** 423-432

COOPER A.M, MAGRAM J, FERRANTE J. and ORME I.M. (1997). Interleukin-12 is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis.*
***J Exp Med*, 186:**39-45

COUEDEL C, PEYRAT M.A, BROSSAY L, KOEZUKA Y, PORCELLI S.A, DAVODEAU F and BONNEVILLE M. (1998). Diverse CD1d-restricted reactivity patterns of human T cells bearing "invariant" AV24BV11 TCR
***Eur J Immunol,* 28**: 4391-7

DAHL K.E, SHIRATSUCHI H, HAMILTON B.D, ELLNER J.J and TOOSSI Z. (1996). Selective induction of transforming growth factor beta in human monocytes by lipoarabinomannan of *Mycobacterium tuberculosis*
***Infect Immun,* 64**:399-405

DAUGLAT S, LADEL C.H and KAUFMANN S.H.E. (1995). Influence of mouse strain and vaccine viability on T-cell responses induced by *Mycobacterium bovis* bacillus Calmette-Guerin.
***Infect Immun,* 63**:2033-2040

DAUGLAT S and KAUFMANN S.H.E. (1996). Role of Th1 and Th2 cells in bacterial infections.
Romagnani S (ed): Th1 and Th2 cells in health and disease.
***Chem Immunol,* 63**:66-97

DEKRUYFF R.H, GIENI R.S and UMETSU D.T. (1997). Antigen-driven but not lipopolysaccharide-driven IL-12 production in macrophages requires triggering of CD40.
***J Immunol,* 158**:359-366

DE JONG R, ALTARE F, HAAGEN I.A, ELFERINK D.G, BOER T, VAN BREDA, VRIESMAN P.J, KABEL P.J, DRAAISMA J.M, VAN DISSEL J.T, KROON F.P, CASANOVA J.L and OTTENHOFF T.H. (1998) Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients.
***Science,* 280**: 1435-8

DE JONG R, JANSON A.A, FABER W.R, NAAFS B and OTTENHOFF T.H. (1997). IL-2 and IL-12 act in synergy to overcome antigen-specific T cell unresponsiveness in mycobacterial disease.
***J Immunol,* 159**: 786-793

DENG W, OHMORI Y and HAMILTON T.A. (1996). LPS does not directly induce STAT activity in mouse macrophages.
***Cell Immunol,* 170**: 20-24

DENIS M. (1991). Tumour necrosis factor and Granulocyte macrophage colony stimulating factor stimulate human macrophages to restrict growth of virulent *Mycobacterium avium* and to kill avirulent *M. avium.*: killing effector mechanisms depends on the generation of reactive nitrogen intermediates.
***J Leukocyte Biol,* 49**: 380

DENIS M. (1994). Interleukin 12 augments cytolytic activity of Natural Killer cells towards *Mycobacterium tuberculosis* infected human monocytes.
***Cell Immunol,* 156**: 529

DESMEDT M, ROTTIERS P, DOOMS H, FIERS W and GROOTEN J (1998) Macrophages induce cellular immunity by activating Th1 cell responses and suppressing Th2 cell responses.
***J Immunol,* 160**: 5300-5308

DICK A.D. McMENAMIN P.G. KORNER H. SCALLON B.J. GHRAYEB J. FORRESTER J.V. and SEDGWICK J.D. (1996). Inhibition of tumour necrosis factor activity minimises target organ damage in experimental autoimmune uveoretinitis despite quantitatively normal activated T cell traffic to the retina.
***Eur J Immunol,* 26**: 1018

DIPIRO J.T. (1997). Cytokine networks with infection: Mycobacterial infections, leishmaniasis, human immunodeficiency virus infection and sepsis.
***Pharmacotherapy*,17**:205-223

DUNN P.L and NORTH R.J. (1995). Virulence ranking of some *Mycobacterium tuberculosis* and *Mycobacterium bovis* strains according to their ability to multiply in the lungs, induce lung pathology, and cause mortality in mice.
***Infect Immun*, 63**: 3428-3437

EIGLER A, MOELLER J and ENDRES S. (1995). Exogenous and endogenous nitric oxide attenuates tumour necrosis factor synthesis in the murine macrophage cell line RAW 264.7.
***J Immunol*, 154:** 4048

ELHERS S, MIELKE M.E.A. and HAHN H. (1994). Progress in TB research: Robert Koch's dilemma revisited.
***Immunol Today*, 15:** 1-4

ELLNER J.J. (1994). Current issues in tuberculosis.
***J Lab Clin Med*, 123:** 478

ELLNER J.J. (1997). Regulation of the human immune response during tuberculosis.
***J Lab Clin Med*, 130**:469-475

EMOTO M, EMOTO Y and KAUFMANN S.H.E. (1997). Bacille Calmette Guérin and interleukin-12 downmodulate interleukin-4 producing $CD4^{+}NK1^{+}$ T lymphocytes.
***Eur J Immunol*, 27**:183-188

FENTON M.J. and VERMEULEN M.W. (1996). Immunopathology of tuberculosis: Roles of macrophages and monocytes. Minireview.
***Infect Immun*, 64:** 683-690

FINE P.E, STERNE J.A, PONNIGHAUS J.M and REES R.J. (1994). Delayed-type hypersensitivity, mycobacterial vaccines and protective immunity.
Lancet, **344**:1245-1249

FLYNN J.L, CHAN J, TRIEBOLD K.L, DALTON D.K, STEWART T.A. and BLOOM B.R. (1993). An essential role for interferon-gamma in resistance to *Mycobacterium tuberculosis* infection.
***J Exp Med*, 178**:2249-2254.

FLYNN J.L, GOLDSTEIN M.M, TRIEBOLD K.J, SYPEC J, WOLF S. and BLOOM B.R. (1995). IL-12 increases resistance of Balb/c mice to *Mycobacterium tuberculosis* infection.
***J Immunol*, 155**:2515-2524

FULTON S.A, CROSS J.V, TOOSSI Z.T, BOOM W.H. (1998) Regulation of Interleukin-12 by Interleukin-10, transforming growth factor-beta, tumour necrosis factor-alpha, and interferon-gamma in human monocytes infected with *Mycobacterium tuberculosis* H37Ra.
***J Infect Dis*, 178**: 1105-14

FULTON S.A, JOHNSEN J.M, WOLF S.F, SIEBURTH D.S. and BOOM W.H. (1996). Interluekin-12 production by human monocytes infected with *Mycobacterium tuberculosis*: Role of Phagocytosis.
***Infect Immun*, 64:** 2523-2531

GARCIA I, MIYAZAKI Y, MARCHAL G, LESSLAUER W and VASSALLI .P. (1997). High sensitivity of transgenic mice expressing soluble TNFR1 fusion protein to mycobacterial infections: synergistic action of TNF and IFN-gamma in the differentiation of protective granulomas.
***Eur J Immunol*, 27**:3182-3190

GAZZINELLI R.T, HIENY S, WYNN T.A, WOLF S. and SHER A. (1993). Interleukin-12 is required for the T-lymphocyte-independent induction of interferon-γ by an intracellular parasite and induces resistance in T-cell-deficient hosts.
***Proc Natl Acad Sci USA*, 90,** 6115

GILLILAND G, PERRIN S and BUNN H. (1990). Competitive PCR for Quantitation of mRNA.
Academic Press. Inc. New York.

GOODRUM, M. (1998) M Sc. dissertation.
Department of Biochemistry, University of Pretoria.

GOTOH K, MITSUYAMA M, IMAIZUMI S, KAWAMURA I and YANO I.. (1991). Mycolic acid-containing glycolipid as a possible virulence factor of Rhodococcus equi for mice.
***Microbiol Immunol,* 35**:175-185

GUILLAMARD E, GENITEAU-LEGENDRE M, BOUX M.B, POILANE I, KERGOT R, LEMAIRE G, PETIT J.F, LABARRE and QUERRO A.M. (1993). Antiviral action of trehalose dimycolate against EMC virus: role of macrophages and interferon α/β
***Antiviral Res,* 22:** 201-213

HAMBLIN A.S. (1993). Cytokines and cytokine receptors.
***IRL Press.* London.**

HANSCH H.C, SMITH D.A, MIELKE M.E, HAHN H, BANCROFT G.J and EHLERS S. (1996). Mechanisms of granuloma formation in murine *Mycobacterium avium* infection: the contribution of CD4+ T cells.
***Int Immunol,* 8**: 1299-1310

HE H, OKA S, HAN Y, YAMAMURA Y, KUSUNOSE E, KUSUNOSE M. and YANO K. (1991). Rapis serodiagnosis of human mycobacteriosis by ELISA using cord factor purified from *Mycobcterium tuberculsosis* as antigen.
***FEMS Microbiol Immunol,* 76:** 2011-204

HEINZEL F.P, RERKO R.M, HATAM F and LOCKSLEY R.M (1993a). IL-2 is necessary for the progression of leishmaniasis in susceptible murine hosts.
***J Immunol,* 150:** 3924-3931

HEINZEL F.P, SCHOENHAUT D.S, RERKO R.M, ROSSER L.E and GATELY M.K (1993b). Recombinant interleukin 12 cures mice infected with Leishmania major.
***J Exp Med,* 177**:1505-1509

HEINZEL F.P, RERKO R.M, LING P, HAKIMI J and SCHOENHAUT D.S. (1994). Interleukin 12 is produced in vivo during endotoxemia and stimulates synthesis of gamma interferon.

***Infect Immun,* 62**:4244-4249

HENDERSON R.A, WATKINS S.C and FLYNN J.L (1997). Activation of human dendritic cells following infection with *Mycobacterium tuberculosis*.
***J Immunol,* 159:**635-643

HIBBS J, HIBBS J.B, TAINTOR R.R and VAVRIN Z (1987). Macrophage cytotoxicity: role of L-ariginine deaminase and imino nitrogen oxidation to nitrate.
***Science,* 235**: 473

HIRSCH C.S, HUSSAIN R, TOOSSI Z, DAWOOD G, SHAHID F and ELLNER J.J. (1996). Cross-modulation by transforming growth factor βin human tuberculosis: suppression of antigen-driven blastogenesis and interferon gamma production.
***Proc Natl Acad Sci USA,* 93,** 3193

HSIEH C, MACATONIA S.E, TRIPP C.S, WOLF S.F, O'GARRA A and MURPHY K.M. (1993). Development of Th1 $CD4^+$ T cells through IL-12 Produced by *Listeria*-induced Macrophages.
***Science,* 260:** 547

JANEWAY C.A and TRAVERS P. (1996). Immuno biology: the immune system in health and disease.
***Blackwell Scientific Publications,* Oxford.** Chapter 7, p 7:42

JANIS E.M, KAUFMANN S.H.E., SCHWARTZ R.H and PARDOLL D.M (1990). Activation of gamma delta T cells in the preliminary immune response to *Mycobacterium tuberculosis*.
***Science*, 171:** 667

KASAMA T, STREITER R.M, LUKACS N.W, LINCOLN P.M. and BURDICK M.D. (1995). Interleukin 10 expression and chemokine regulation during the evolution of murine type II collagen induced arthritis.
***J Clin Invest*, 95:** 2868-2876

KASHIMA K, OKA S, TABATA A, YASUDA D, KITANO A, KOBAYASHI K. and YANO I. (1995). Detection of anti-cord factor antibodies in intestinal tuberculosis for its differential diagnosis from Crohn's disease and ulcerative colitis.
***Dig Dis Sci*, 40:**2630-2634

KASINRERK W, BAUMRUKER T, MAJDIC O, KNAPPP W. and STOCKINGER H.(1993). CD1 molecule expression on human monocytes induced by Granulocyte-Macrophage Colony-Stimulating Factor.
***J Immunol*, 150:** 579-584

KATO M. (1992). Antibody formation to trehalose-6,6'-dimycolate (cord factor) of *Mycobacterium tuberculosis*.
***Infect Immun*, 5**: 203-212

KATO M. (1974). Further study on neutralization of biochemical activity of cord factor by anti-cord factor antibody.
***Infect Immun*, 10**: 277-279

KAUFMANN S.H.E and ANDERSEN P. (1998) Immunity to mycobacteria with emphasis on tuberculosis: implications for rational design of an effective tuberculosis vaccine.
***Chem Immunol*, 70**: 21-59

KAUFMANN S.H.E. (1997). Antibacterial vaccines: impact of antigen handling and immune response.
***J Mol Med*, 75**:360-3

KAWANU T, CUI J, KOEZUKA Y, TOURA I, KANEKU Y, MOTOKI K, UENO H, NAKAGAWA R, SATO H, KONDO E, KOSEKI H and TANIGUCHI M. (1997). CD1d-Restricted and TCR-Mediated Activation of V-alpha NKT cells by Glycosylceramides.
***Science*, 278:** 1626-1629

KEANE J, BALCEWICZ-SABLINSKA M.K, REMOLD H.G, CHUPP G.L, MEEK B.B, FENTON M.J and KORNFELD H. (1997). Infection by *Mycobacterium tuberculosis* promotes human alveolar macrophage apoptosis.
***Infect Immun*, 65**: 298-304

KEMP K, HVIID L, KHARAZMI A and KEMP M. (1997). Interferon-gamma production by human T cells and natural killer cells in vitro in response to antigens from the two intracellular pathogens *Mycobacterium tuberculosis* and *Leishmania major*.
***Scand J Immunol*, 46**: 495-499

KIM B.C and KIM J.H. (1998). Exogenous C2-ceramide activates c-fos serum response element via Rac-dependent signalling pathway.
***Biochem J*, 330**:1009-14

KING T. P, and CRAIG L.C. (1962). Countercurrent distribution. In : Methods of Biochemical Analysis. Edited by D. Click, Vol. X, 201-228
Interscience Publishers, New York, London.

KNUTSON K.L, HMAMA Z, HERRERA-VELIT P, ROCHFORD R. and REINER N.E. (1998). Lipoarabinomannan of *Mycobacterium tuberculosis* promotes protein tyrosine dephosphorylation and inhibition

of mitogen-activated protein kinase in human mononuclear phagocytes. Role of the Src homology 2 containing tyrosine phosphatase 1.
J Biol Chem, **273**:645-652

KOBAYASHI M, FRITZ L, RYAN M, HEWIC R.M, CLARK S.C, CHAN S, LOUDON R, SHERMAN F, PERUSSIA B. and TRINCHERIE T. (1989). Identification and purification of natural killer stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes.
J Exp Med, **170:** 827

KOBAYASHI K, NAKATA N, KAI M, KASAMA T, HANYUDA Y and HATANO Y. (1997). Decreased expression of cytokines that induce type 1 helper T cell/interferon-gamma responses in genetically susceptible mice infected with *Mycobacterium avium.*
Clin Immunol Immunopathol, **85**:112-116

KOHNO K, KATAOKA J, OHTSUKI T, SUEMOTO Y, OKAMOTO I, USUI M, IKEDA M and KURIMOTO M. (1997). IFN-gamma-inducing factor (IGIF) is a costimulatory factor on the activation of Th1 but not Th2 cells and exerts its effect independently of IL-12.
J Immunol, **158**:1541-50

LADEL C.H, SZALAY G, RIEDEL D and KAUFMANN S.H.E. (1997). Interleukin-12 secretion by *Mycobacterium tuberculosis*-infected macrophages.
Infect Immun, **65**:1936-1938

LEENEN P.J, CANONO B.P, DREVETS D.A, VOERMAN J.S and CAMPBELL P.A. (1994). TNF-alpha and IFN-gamma stimulate a macrophage precursor cell line to kill Listeria monocytogenes in a nitric oxide-independent manner.
J Immunol, Dec 1;**153:** 5141-5147

MA Y, SEILER K.P, TAI K.F, YANF L.M, WOODS M. and WEI S. (1994). Outer surface lipoproteins of *Borrelia burgdorferi* stimulate nitric-oxide production by the cytokine-inducible pathway
Infect Immun, **62:** 3663-3671

MALAVIYA R, IKEDA T, ROSS E and ABRAHAM S.N. (1996). Mast cell modulation of neutrofil influx and bacterial clearance at sites of infection through TNF-α.
Nature, **381:** 77

MATSUNGA I, OKA S, FUJIWARA N. and YANO I. (1996). Relationship between induction of macrophage chemotactic factors and formation of granulomas caused by mycoloyl glycolipids from *Rhodococous ruber.*
J Biochem, **120:** 663-670

MAURI C, WILLIAMS R.O, WALMSLEY M. and FELDMAN N. (1996). The relationship between Th1/Th2 cytokine patterns and the arthritogenic response in collagen induced arthritis.
Eur J Immunol, **26:**1511-1518

MAYER B, PITTERS E, PFEIFFER S, KUKOVETZ W.R and SCHMIDT K. (1997). A synthetic peptide corresponding to the putative dihidrofolate reductase domain of nitric oxide syntase inhibits uncoupled NADPH oxidation.
NO Biol Chem, **1:** 50-61

MCDONOUGH K.A, KRESS Y and BLOOM B.R (1993). Pathogenesis of tuberculosis: interaction of *Mycobacterium tuberculosis* with macrophages.
Infect Immun, **61**: 2763-2773

MEDINA E and NORTH R.J. (1996). Evidence inconsistent with a role for the Bcg gene (Nramp1) in resistance of mice to infection with virulent Mycobacterium.
J Exp Med, **183:** 1045 -1051

MEDINA E and NORTH R.J. (1998). Resistance ranking of some common inbred mouse strains to *Mycobacterium tuberculosis* and relationship to major histocompatibility complex haplotype and Nramp1 genotype.
***Immunology,* 93**: 270-274

MENDEZ-SAMPERIO P. GONZALEZ-GARCIA L. PINEDA-FRAGOSO P.R. and RAMOS-SANCHEZ E. (1995). Specificity of T cells in human resistance to *Mycobacterium tuberculosis* infection.
***Cell Immun,* 162:**194

MENDEZ-SAMPERIO P, TREJO-ECHEVERRIA A and AYALA-VERDIN H (1998). Regulation of Interleukin-12 Production in Human Cells Stimulated with *Mycobacterium bovis* BCG.
***Cell Immunol,* 189**:25-30

MICALLEF M.J, OHTSUKI T, KOHNO K, TANABE F, USHIO S, NAMBA M, TANIMOTO T, TORIGOE K, FUJII M, IKEDA M, FUKUDA S and KURIMOTO M. (1996). Interferon-gamma-inducing factor enhances T helper 1 cytokine production by stimulated human T cells: synergism with interleukin-12 for interferon-gamma production.
***Eur J Immunol,* 26:**1647-1651

MINNIKIN D.E. and GOODFELLOW M. (1980). Microbiological Classification and Identification of Acid-fast Bacteria. (eds Goodfellow, M. & Board, R.G.) 189-256 (Academic, London, 1980).

MOSMANN T.R and COFFMAN R.L. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties.
***Annu Rev Immunol,* 7**:145-173

MUNK M.E and EMOTO M (1995). Functions of T-cell subsets and cytokines in mycobacterial infections.
***Eur Respir J Suppl,* 20**: 668S-675S

MURCH S.H (1995). Cellular mediators of lung damage.
***Brit J Intensive Care*, 15:** 27

NAKANO Y, TOBE T, CHOI-MIURA N, MAZDA T. and TOMITA M. (1996). Isolation and characterisation of GBP28, a novel gelatine binding protein purified from human plasma.
***J Biochem,* 120:** 803-812

OGASAWARA K, TAKEDA K, HASHIMOTO W, SATOH M, OKUYAMA R, YANAI N, OBINATA M, KUMAGAI K, TAKADA H, HIRAIDE H and SEKI S. (1998). Involvement of NK1+ T cells and their IFN-gamma production in the generalised Shwartzman reaction.
***J Immunol,* 160:** 3522-3527

ORANGE J.S and BIRON C.A. (1996). Characterisation of early IL-12, IFN-alpha, -beta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection.
***J Immunol,* 156**: 4746-4756

ORBACH-ARBOUYS S, TENU J.P. and PETIT J.F. (1983). Enhancement of in vitro and in vivo antitumour activity by cord factor administred suspended in saline.
***Int J allergy Appl Immun*,71:**67-73

ORME I.M. (1987). The dynamics of infection following BCG and *Mycobacterium tuberculosis* challenge in T-cell-deficient mice.
***Tubercle,* 68**: 277-283

ORME I.M, ROBERTS A.D, GRIFFIN J.P and ABRAMS J.S. (1993). Cytokine secretion by CD4 T lymphocytes acquired in response to *Mycobacterium tuberculosis* infection.
***J Immunol*, 151**: 518-525

ORME I.M, ANDERSEN P and BOOM W.H. (1993). T cell response to *Mycobacterium tuberculosis*.

J Infect Dis, **167:** 1481-1497

ORME I.M. (1993). The role of CD8+ T cells in immunity to tuberculosis infection.
***Trends Microbiol,* 1**:77-78

ORME I.M. (1996). Immune responses in animal models.
***Curr Top Microbiol Immunol,* 215**:181-196

ORTHALO-MAGNE A, LEMASSU A, LANEELLE M, BARDOU F, SILVE G, GOUNON P, MARCHAL G. and DAFFE M. (1996). Identification of the surface-exposed lipids on the cell envelopes of *Mycobacterium tuberculosis* and other mycobacterial species.
***J Bact,* 178:** 456-461

OSWALD I.P, DOZOIS C.M, PETIT J. and LEMAIRE G. (1997). Interleukin-12 synthesis is a required step in trehalose dimycolate induced activation of mouse peritoneal macrophages.
***Infect Immun,* 65:**1364-1369

PARANT M, PARANT F, CHEDID L, DRAPIER J.C, PETIT J.F. WIETZERBIN J. and LEDERER E. (1977). Enhancement of non-specific immunity to bacterial infection by cord factor (6'6-trehalose dimycolate).
***J Infect Dis,* 135:**771-777

PARK S.H, ROARK J.H and BENDELAC A. (1998). Tissue-specific recognition of mouse CD1 molecules.
***J Immunol,* 160:** 3128-34

PASPARAKIS M, ALEXOPOULOU L, EPSOKOPOU V and KOLLIAS G. (1996). Immune and inflammatory responses in TNF-α-deficient mice: a critical role for TNF-α in the formation of primary b cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response.
***J Exp Med,* 184:** 1397

PECE S, GIULIANI G, FUMAROLA D, MASTROIANNI C.M, LICHTNER M, VULLO V, ANTONACI S and JIRILLO E (1997). In vitro production of tumour necrosis factor-alpha, interleukin-6 and interleukin-8 from normal human peripheral blood mononuclear cells stimulated by Rhodococcus equi.
***Vet Microbiol,* 56**: 277-285

PORCELLI S, BRENNER M.B, GREENSTEIN J.L, BALK S.P, TERHORST C. and BLEICHER P.A. (1989). Recognition of CD1 antigens by human CD4(-)CD8(-) cytolytic T lymphocytes.
***Nature,* 341:** 447-450

PORCELLI S.A, MORITA C.T. and BRENNER M.B. (1992). CD1b restricts the response of human CD4(-)8(-) T lymphocytes to a microbial antigen.
***Nature,* 360,** 539-597

PORCELLI S.A, and MODLIN R.L. (1995). CD1 and the expanding universe of T cell antigens.
***J Immunol,* 152:** 3709-3710

PORTER J.D.H. and McADAM K.P.W.J. (1994). Tuberculosis back to the future.
***John Wiley and sons.* New York.** Chapter 3, pp 53

POWRIE F and COFFMEN R.L. (1993). Cytokine regulation of T cell function: potential for therapeutic intervention.
***Immunol Today*, 14:** 270-274

PROBST P, SKEIKY Y.A, STEEVES M, GERVASSI A, GRABSTEIN K.H and REED S.G. (1997) A Leishmania protein that modulates interleukin (IL)-12, IL-10 and tumour necrosis factor-alpha production and expression of B7-1 in human monocyte-derived antigen-presenting cells.
***Eur J Immunol*, 27**:2634-2642

REINER S.L, ZHENG S, CORRY D.B and LOCKSLEY R.M. (1994). Constructing polycompetitor cDNAs for quantitative PCR.
J'Immunol Methods,**175:** 275-280

RHOADES E.R, COOPER A.M and ORME I.M. (1995). Chemokine response in mice infected with *Mycobacterium tuberculosis.*
***Infect Immun,* 63**, 3871

RIBI E, GRANGER D.L, MILNER K.C, YAMAMOTO K, STRAIN S.M, PARKER R, SMITH R.W, BREHMER W and AZUMA I. (1982). Induction of resistance to tuberculosis in mice with defined components of mycobacteria and with some unrelated materials.
***Immunology,* 46**: 297-305

ROITT I, BROSTOFF J and MALE D. (1993). Immunology.
Third edition.
Mosby. **Boston and London.**

ROJAS M, BARRERA L.F, PUZO G, GARCIA L.F. (1997). Differential induction of apoptosis by virulent *Mycobacterium tuberculosis* in resistant and susceptible murine macrophages: role of nitric oxide and mycobacterial products.
***J Immunol,* 159**: 1352-1361

ROOK G.A.W. and HERNANDEZ-PANDO R. (1996). The pathogenesis of tuberculosis.
***Annu Rev Microbiol,* 50:** 259-284

SAIKI R.K, GELFAND D.H , STOFFEL S, SCHARF S.J, HIGUCHI R, HORN G.T, MULLIS K.B and ERLICH H.A. (1988). Primer-directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase.
***Science,* 239**: 487 -491

SAMBROOK J, FRITSCH E.F and MANIATIS T. (1989). Molecular Cloning. A Laboratory Manual. Second Edition.
Cold Spring Laboratory Publications. New York.

SATO K, AKAKI T and TOMIOKA H (1998). Differential potentiation of anti-mycobacterial activity and reactive nitrogen intermediate-producing ability of murine peritoneal macrophages activated by interferon-gamma (IFN-gamma) and tumour necrosis factor-alpha (TNF-alpha).
***Clin Exp Immunol,* 112:** 63-68

SAUNDERS B.M. and CHEERS C. (1994). Inflammatory response following intanasal infection with *Mycobacterium avium* complex: role of T-cell subsets and gamma inteferon.
***Infect Immun,* 63,** 2282

SCHEBESCH C, KODELJA V, MULLER C, HAKIJ N, BISSON S, ORFANOS C.E and GOERDT S (1997). Alternatively activated macrophages actively inhibit proliferation of peripheral blood lymphocytes and CD4+ T cells in vitro.
***Immunology,* 92**: 478-486

SCHOENHAUT D.S, CHUA A.O, WOLITZKY A.G, QUINN P.M, DWYER C.M, McCOMAS W, FAMILLETTI P.C, GATELY M.K. and GUBLER U. (1992). Cloning and Expression of Murine IL-12.
***J Immunol,* 146:** 3433-3440.

SCOTT P. (1993). IL-12: Initiation cytokine for cell-mediated immunity.
***Science,* 260:** 496

SIELING P.A, CHATTERJEE D, PORCELLI S.A, PRIGOZY T.I, MAZZACCARO R.J, SORIANOT, BLOOM B.R, BRENNER M.B, KRONENEBERG M, BRENNAN P.J. and MODLIN R.L. (1995). CD1-Restricted T cell recognition of microbial lipoglycan antigens.
***Science,* 269:** 227-230

SIKO, G. (1999) M Sc. dissertation.
Department of Biochemistry, University of Pretoria.

SILVA R.A, PAIS T.F and APPELBERG R. (1998) Evaluation of IL-12 in immunotherapy and vaccine design in experimental *Mycobacterium avium* infections.
***J Immunol*, 161**: 5578-85

SIMMONNEY N, MOLINA J.M, MOLIMARD M, OKSENHENDLER E, PERRONNE C. and LAGRANGE P.H. (1995). Analysis of the immunological humoral response to *Mycobacterium tuberculosis* glycolipid antigens (DAT, PGLTb1) for diagnosis in HIV-seropositive and seronegative patients.
***Eur J Clin Microbiol Infect Dis*, 14:** 883-891

SKEEN M.J, MILLER M.A, SHINNICK T.M and ZIEGLER H.K (1996) Regulation of murine macrophage IL-12 production. Activation of macrophages in vivo, restimulation in vitro, and modulation by other cytokines.
***J Immunol*, 156**:1196-1206

SKEIKY Y.A, GUDERIAN J.A, BENSON D.R, BACELAR O, CARVALHO E.M, KUBIN M, BADARO R, TRINCHIERI G and REED S.G. (1995). A recombinant Leishmania antigen that stimulates human peripheral blood mononuclear cells to express a Th1-type cytokine profile and to produce interleukin 12.
***Exp Med*, 181**:1527-1537

SKINNER M.A, YUAN S, PRESTIDGE R, CHUK D, WATSON J.D and TAN P.L. (1997). Immunization with heat-killed *Mycobacterium vaccae* stimulates CD8+ cytotoxic T cells specific for macrophages infected with *Mycobacterium tuberculosis*.
***Infect Immun*, 65**:4525-30

SNIJDERS A, ILKENS C.M.U, VAN DER POUW KRAAN T.C.T.M, ENGEL M, AARDEN L.A. and KAPSENBURG M.L. (1996). Regulation of Bioactive IL-12 Production in Lipopolysaccharide-Stimulated Human Monocytes is Determined by the Expression of the p35 Subunit.
***J Immunol*, 156:** 1207-1212

SOUSA A.O, LEE F.K, FREIJI R, LAGRANGE P.H, NAHMIAS A (1998). Human immunodeficiency virus infection alters antigen-induced cytokine responses in patients with active mycobacterial diseases.
***J Infect Dis*, 177**:1554-62

SPARGO B.J, CROWE L.M, IONEDA T, BEAMAN B.L and CROWE J.H (1991). Cord factor inhibits fusion between phospholipid vesicles.
***Proc Natl Acad Sci USA*, 88:**737-740

STAMLER J.S, SINGEL D.J. and LOSCALZO J. (1992). Biochemistry of nitric oxide and its redox-activated forms.
***Science*, 258:** 1898

STANFORD J.L. and STANFORD C.A. (1996a). Immunotherapy with *M. vaccae* and the treatment of tuberculosis.
***J Appl Bact*, 81:** 81s-86s

STANFORD J.L. and STANFORD C.A. (1996b). Immunotherapy for tuberculosis with *M. vaccae.*
***J Med Microbiol*, 944:** 24-34

STERN A.S, PODLASKI F.J, HULMES J.D, PAN Y.C.E, QUINN P.M, WOLITZKEY A.G. FAMILLETTI P.C, STREMBO D.L TRUITT T, CHIZZONITE R and GATELY M.K (1990). Purification to homogeneity and partial characterisation of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells.
***Proc Natl Acad Sci USA*, 87:** 6808

SURCEL H.M, TRYPE-BLOMBERG M. and PAULIE S. (1994). Th1/Th2 profiles in tuberculosis, based on the proliferation and cytokine response of blood lymphocytes to mycobacterial antigens.
***Immunol*, 81:** 171-176

SYPEC J.P, CHUNG C.L, MAYOR S.H.E, SUBRAMANYAM J.M, GOLDMAN S.J, SIEBURT D.S, WOLF, S.F. and SCHAUB, R.G. (1993). Resolution of cutaneuos Leishmaniasis: Interleukin 12 initiates a protective T helper type 1 immune response.
***J Exp Med,* 177:** 1797

TABATA A, KENDA K, WATANABE H, ABO T and YANO I. (1996). Kinetics of organ-associated natural killer cells and intermediate CD3 cells during pulmonary and hepatic granulomatous inflammation induced by Mycobacterial cord factor.
***Microbiol Immunol,* 40:**651-658

TANAKA Y, MORITA C.T, TANAKA Y, NIEVES E, BRENNER M.B. and BLOOM B.R. (1995). Natural and synthetic non-peptide antigens recognised by human gamma-delta T cells.
***Nature,* 375,** 155-158

TASCON R.E, STAVROPOULOS E, LUKACS K.V and COLSTON M.J. (1998). Protection against *Mycobacterium tuberculosis* infection by CD8+ T cells requires the production of gamma interferon.
***Infect Immun,* 66**: 830-834

THOMPSEN H, KAHAN M. and LONDEI M. (1995). Differential effects of Interleukin-10 on the expression of HLA class II and CD1 Molecules induced by Granulocyte-Macrophage Colony Stimulating Factor/Interleukin-4.
***Eur J Immunol,* 25:** 2465-2470

THOMPSON-SNIPES L, SKAMENE E and RADZIOCH D. (1998). Acquired resistance but not innate resistance to *Mycobacterium bovis* bacillus Calmette-Guerin is compromised by interleukin-12 ablation.
***Infect Immun,* 66:** 5268-74

TOOSSI Z, MINCEK M, SEEHOLTZER E, FULTON S.A, HAMILTON B.D and HIRSCH C.S. (1998) Modulation of IL-12 by transforming growth factor-beta (TGF-beta) in *Mycobacterium tuberculosis*-infected mononuclear phagocytes and in patients with active tuberculosis.
***J Clin Lab Immunol,* 49:** 59-75

TOOSSI Z. (1996). Cytokine circuits in tuberculosis.
***Infect Agent Dis,* 5:** 98

TOOSSI Z, HIRSCH S, HAMILTON B.D, KNUTH C.K, FRIEDLANDER M.A. and RICH E.A. (1996). Decreased production of TGF-β by human alveolar macrophages compared to blood monocytes.
***J Immunol,* 156:** 3461

TOOSSI Z, YOUNG T, AVERILL L.E, HAMILTON B.D, SHIRATSUCHI H. and ELLNER, J.J. (1995a). Induction of transforming growth factor β1 by PPD of *Mycobacterium tuberculosis.*
***Infect Immun,* 63:** 224

TOOSSI Z, GOGATE P, SHIRATSUCHI H, YOUNG T. and ELLNER J.J. (1995b). Enhanced production of TGF-β by blood monocytes from patients with active tuberculosis and presence of TGF-β in tuberculosis granulomatous lung lesions.
***J Immunol,* 154:** 465

TRINCHIERI G and SCOTT P. (1994). The Role of Interleukin-12 in the Immune Response, Disease and Therapy.
***Immunol Today,* 15:** 460-463

TRINCHIERI G. (1993). Interleukin-12 and its Role in the Generation of Th1 Cells.
***Immunol Today,* 14:** 335-337

TRIPP C. S, GATELY M.K, HAKIMI J, LING P and UNANUE E.R (1994). Neutralization of IL-12 decreases resistance to *Listeria* in SCID and C.B-17 mice. Reversal by IFN-gamma.
***J Immunol,* 152**: 1883-1887

VANHAM G, TOOSSI Z, HIRSCH C.S, WALLIS R.S, SCHWANDER S.K, RICH E.A and ELLNER J.J. (1997). Examining a paradox in the pathogenesis of human pulmonary tuberculosis: immune activation and suppression/anergy.
Tuber Lung Dis 78: 145-58

VAN OSS C.J. and VAN REGELMORTEL M.H.V. (1994). Immunochemistry.
Marcel Dekker, Inc. **New York.**
Chapter 38, p 995

VODOVOTZ Y. (1997) Control of nitric oxide production by transforming growth factor-β1: mechanistic insights and potential relevance to human disease.
NO Biol Chem, **1:** 3-10

WYNN T.A, JANKOVIC D, HIENY S, ZIONCHECK K, JARDIEU P, CHEEVER A.W, and SHER A. (1995). IL-12 exacerbates rather than suppresses T helper 2-dependent pathology in the absence of endogenous IFN-gamma.
J Immunol, **154**: 3999-4009

XING Z, WANG J, CROITORU K and WAKEHAM J (1998) Protection by CD4 or CD8 T cells against pulmonary *Mycobacterium bovis* bacillus Calmette-Guerin infection.
Infect Immun, **66:** 5537-42

YOSHIMOTO T, BENDELAC A, WATSON C, HU-LI J. and PAUL W.E. (1995a). Role of $NK1.1^+$ T cells in a Th2 response and in immuhoglobulin E production.
Science, **270:** 1845-1847

YOSHIMOTO T, BENDELAC A, WATSON C, HU-LI J. and PAUL W.E. (1995b). Defective IgE production by SLJ mice is linked to the absence of $CD4^+$, $NK1.1^+$ T cells that promptly produce interleukin 4.
Proc Natl Acad Sci USA, **492:** 11931-11934

YOHSIMOTO T, NAGASE H, ISHIDA T , INOUE J and NARIUSHI H. (1997). Induction of interleukin-12 p40 transcript activation of nuclear factor-kappa B.
Eur J Immunol **27**:3461-3470

YOUNG V.M, TOBOREK M, YANG F, MCCLAIN C.J and HENNIG B. (1998). Effect of linoleic acid on endothelial cell inflammatory mediators.
Metabolism, **47**:566-72

YUAN Y, CRANE D.C, MUSSER J.M, SREEVATSAN S and BARRY C.E 3rd (1997). MMAS-1, the branch point between cis- and trans-cyclopropane-containing oxygenated mycolates in *Mycobacterium tuberculosis.*
Biol Chem, **272**: 10041-10049

ZHANG M, GATELY M.K, WANG E, GONG J, WOLF S.F, L.U S, MODLIN R.L. and BARNES P.F (1994). Interleukin 12 at the site of disease in tuberculosis.
J Clin Invest, **93:** 1733

ZENG Z.H, CASTANO A.R, SEGELKE B.W, STURA E.A, PETERSON P.A and WILSON I.A. (1997). Crystal structure of mouse CD1: an MHC-like fold with a large hydrophobic binding groove.
Science, **278:** 1626 - 1629