CHAPTER 1

General Introduction

1.1 Tuberculosis history

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

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

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

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

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

1.2 Mycobacterium tuberculosis

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

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

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

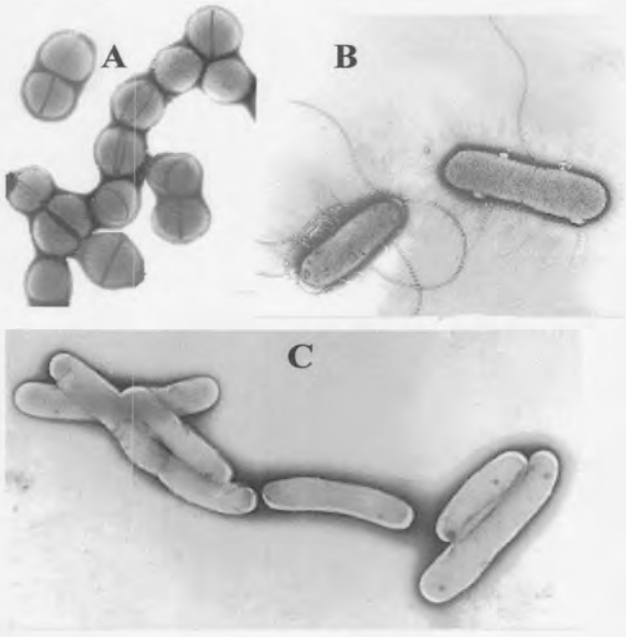


Figure 1.1: Electron micrographs of bacteria indicating the difference in appearances. Gram-positive bacteria (*S. aureus*) is depicted on A, Gram-negative bacteria (*E. coli*) is depicted on B and mycobacteria (*M. tuberculosis*) is depicted on C. (Adapted from http://www.uct.ac.za/depts/mmi/lstyn/cellwall)

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

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

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

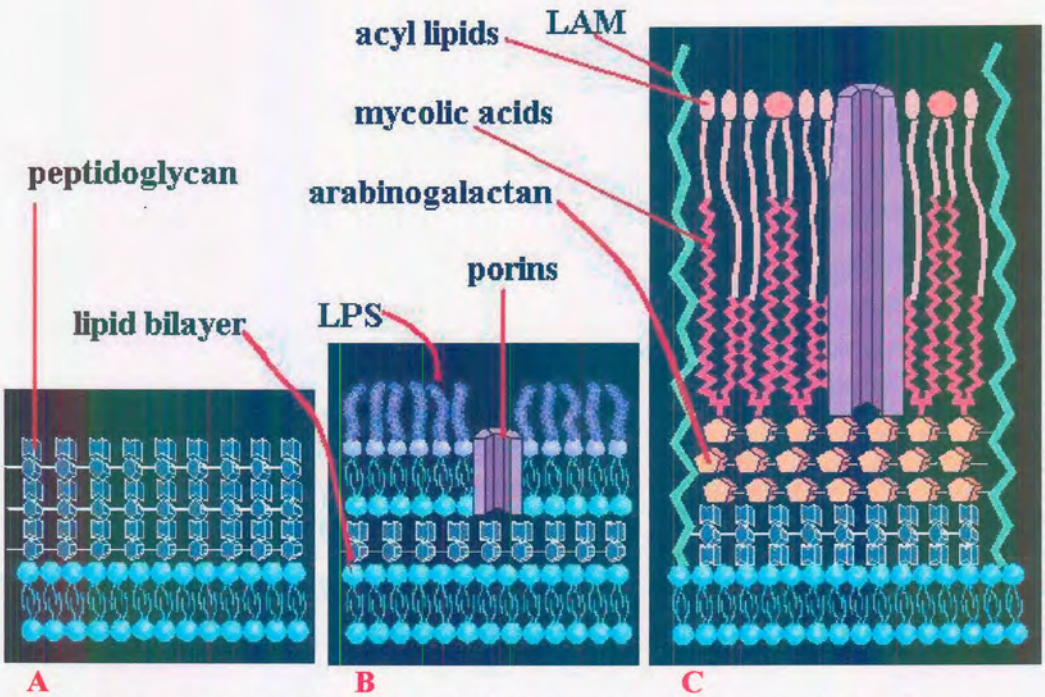


Figure 1.2: Bacterial cell walls of Gram-positive organisms (A), Gram-negative organisms with a double lipid layer (B) and mycobacteria rich in mycolic acids (C). (Adapted from http://www.uct.ac.za/depts/mmi/lstyn/cellwall)

1.3 Tuberculosis: an emergency that does not cease

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

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

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

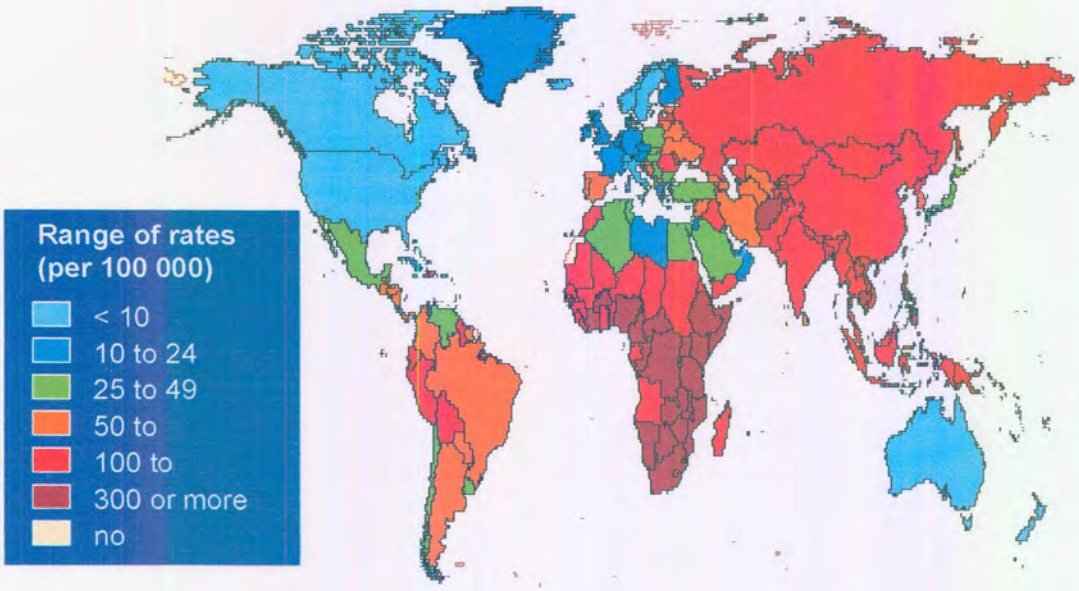


Figure 1.3: Countries with the highest tuberculosis cases in the world. (http://www.stoptb.org/tuberculosis/).

1.4 Tuberculosis and HIV

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

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

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

1.1 Infection and transmission of *M. tuberculosis*

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

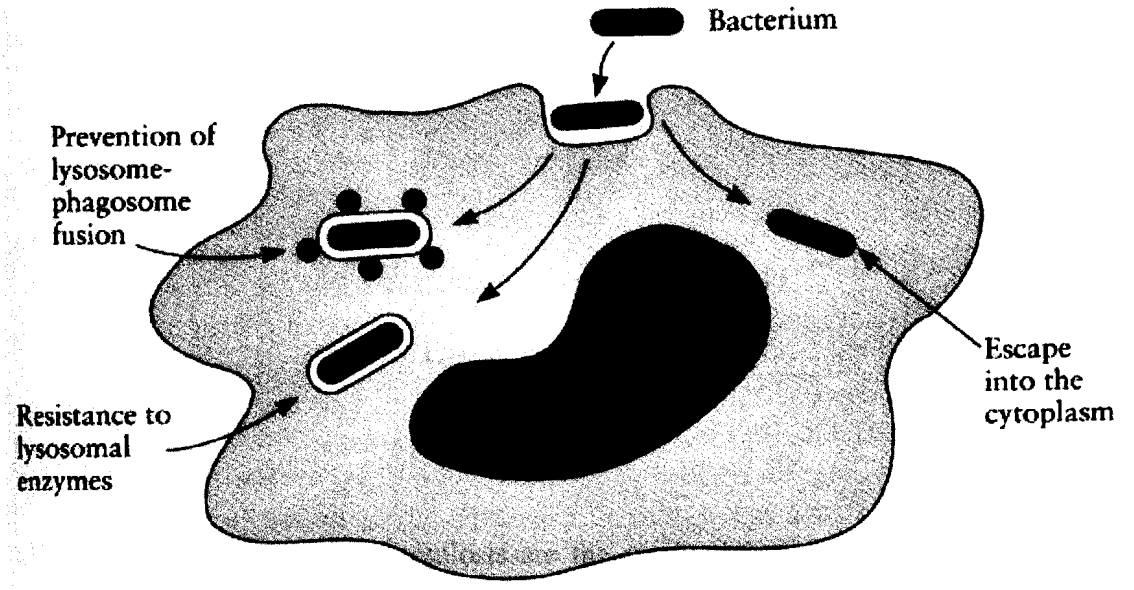


Figure 1.4: Mechanism of *M. tuberculosis* survival and escape into the cytoplasm.

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

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

1.6 Tuberculosis

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

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

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

1.2.1 Stages of tuberculosis

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

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

1.3 Tuberculosis immunity

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

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

1.4 Cell-mediated immune response

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

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

1.4.1 CD4+ T-cells in tuberculosis

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

1.4.2 CD8+ T-cells in tuberculosis

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

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

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

1.4.3 Double-negative αβ T-cells

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

1.4.4 γδ T-cells in tuberculosis

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

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

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

1.4.5 Other cells in tuberculosis

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

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

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

(Adapted from Dannenberg, 1999)

1.9 Cholesterol and mycobacterial survival

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

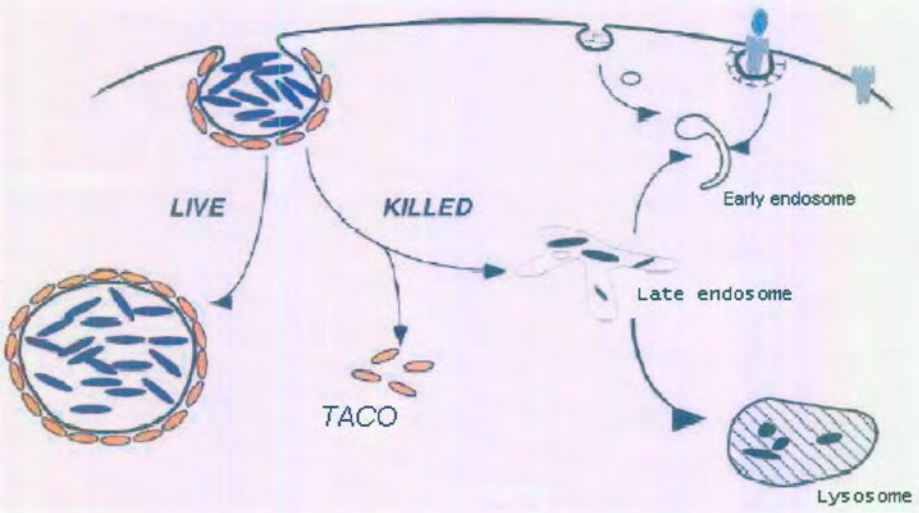


Figure 1.6: Mycobacterial evasion of the host macrophage. (Adapted from Gatfield, Ferrari and Pieters, 2000).

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

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

1.6 The macrophage (MΦ)

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

1.6.1 Macrophage (MΦ) activation

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

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

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

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

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

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

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

1.7 Inflammation

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

1.8 Cytokines

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

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

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

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

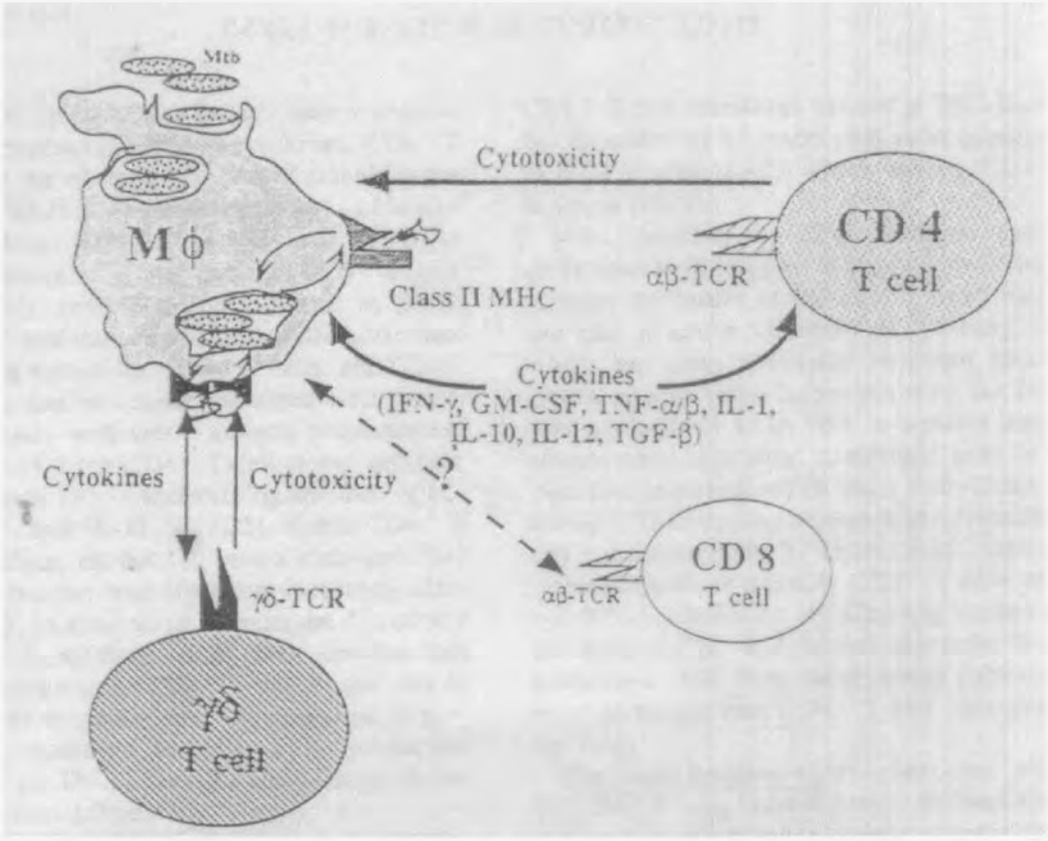


Figure 1.7: Model of the bi-directional cellular and cytokine interactions between T-cell subsets and macrophages (MΦ) infected with *M. tuberculosis* (Mtb) (Boom, 1996).

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

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

1.8.1 Interleukin-1

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

1.8.2 Interleukin-4

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

1.8.3 Interleukin-10

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

1.8.4 Interleukin-12

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

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

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

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

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

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

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

1.8.5 Interferon-gamma (IFN-γ)

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

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

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

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

1.12.6 Tumour necrosis factor-alpha (TNF-α)

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

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

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

1.13 Tuberculosis control

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

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

1.9.1 Tuberculosis notification and treatment

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

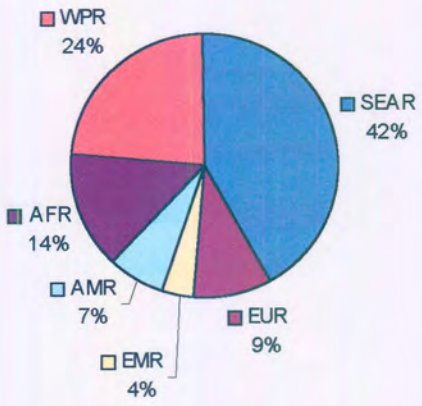


Figure 1.8: World distribution of notified tuberculosis cases by World Health Organisation (WHO) region. AFR-sub-Saharan Africa; AMR, Americas; EMR, Eastern Mediterranean; EUR, Europe; SEAR, South-East Asia; WPR, Western pacific (http://www.who.int/).

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

1.9.2 Tuberculosis diagnosis

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

1.9.2.1 Tuberculin test

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

1.9.2.2 Acid-fast staining

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

1.9.2.3 Chromatographic identification

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

1.9.2.4 DNA based techniques

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

1.9.2.5 The demand for a serodiagnostic tuberculosis test

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

1.10 Mycobacterial mycolic acids in tuberculosis resistance

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

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

1.11 Perspectives of this study

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

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

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

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

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

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

CHAPTER 2

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

2.1 Introduction

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

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

2.1.1 Cytokine profiling

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

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

2.1.2 Quantitative cytokine analysis

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

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

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

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

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

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

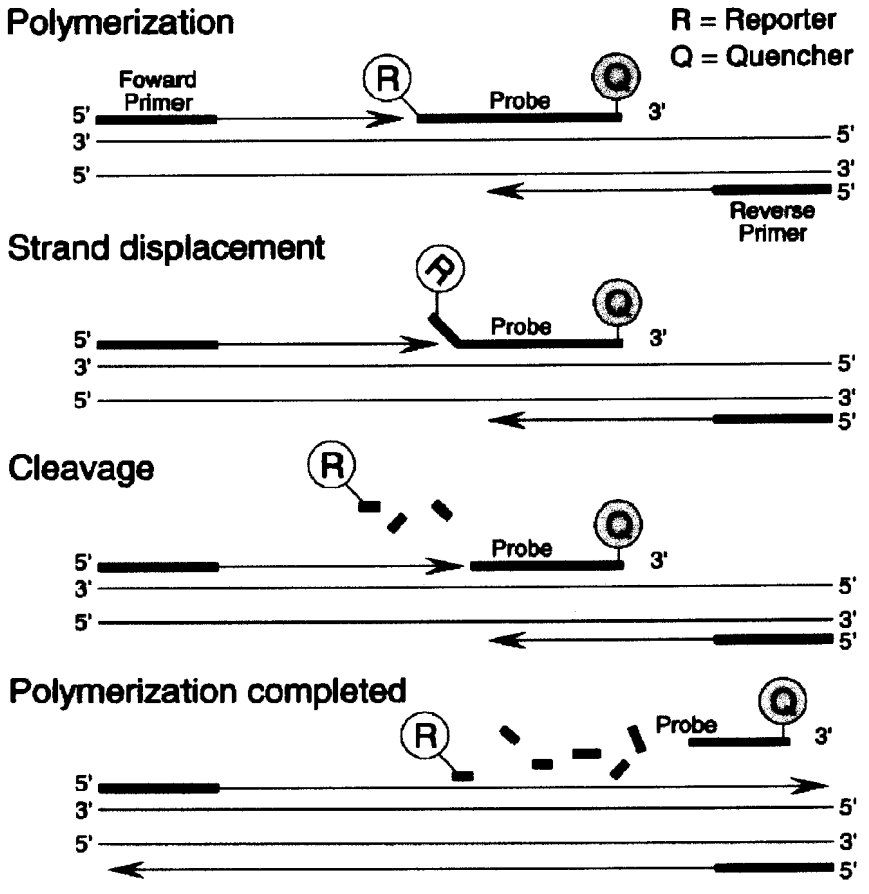


Figure 2.1: The 5' nuclease activity of the DNA polymerase enzyme exploited to detect amplified DNA. (Applied Biosystems, 2000)

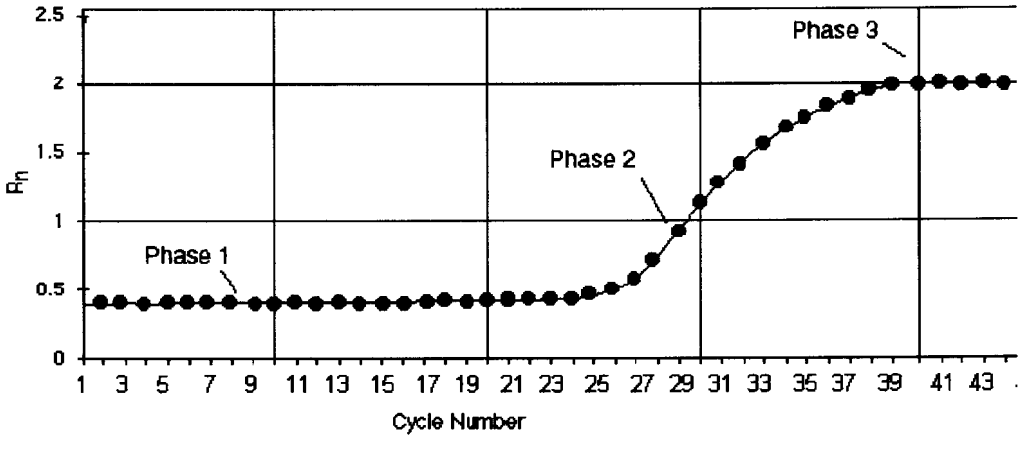


Figure 2.2: Schematic representation of normalised reporter (Rn) versus cycle number. (Adapted from Applied Biosystems, 2000).

2.2 Study Approach

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

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

2.3 Aims:

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

2.4 Materials

2.4.1 Bacteria

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

2.4.2 Animals

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

2.4.3 Reagents

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

Ethidium bromide (Boehringer Mannheim, Germany)

Formamide and formaldehyde (BDH, Poole UK)

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

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

Sodium acetate (Merck, Darmstadt Germany)

TRI-reagent (Molecular Research Centre Inc, USA)

Formazol (Molecular Research Centre Inc, USA)

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

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

Oligo dT primers (Life Technologies Inc., Scotland)

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

Recombinant RNasin (Promega Corporation, Woods USA)

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

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

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

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

2.5 Methods

2.5.1 Preparation of mycobacteria and Preparation of mycobacterial suspensions

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

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

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

2.5.2 Mycolic acids preparation

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

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

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

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

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

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

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

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

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

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

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

2.5.3 Treatment, infection and preparation of the organs

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

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

2.5.4 RNA extraction and quantification

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

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

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

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

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

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

2.5.6 Real-time quantitative PCR

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

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

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

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

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

2.6 Results

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

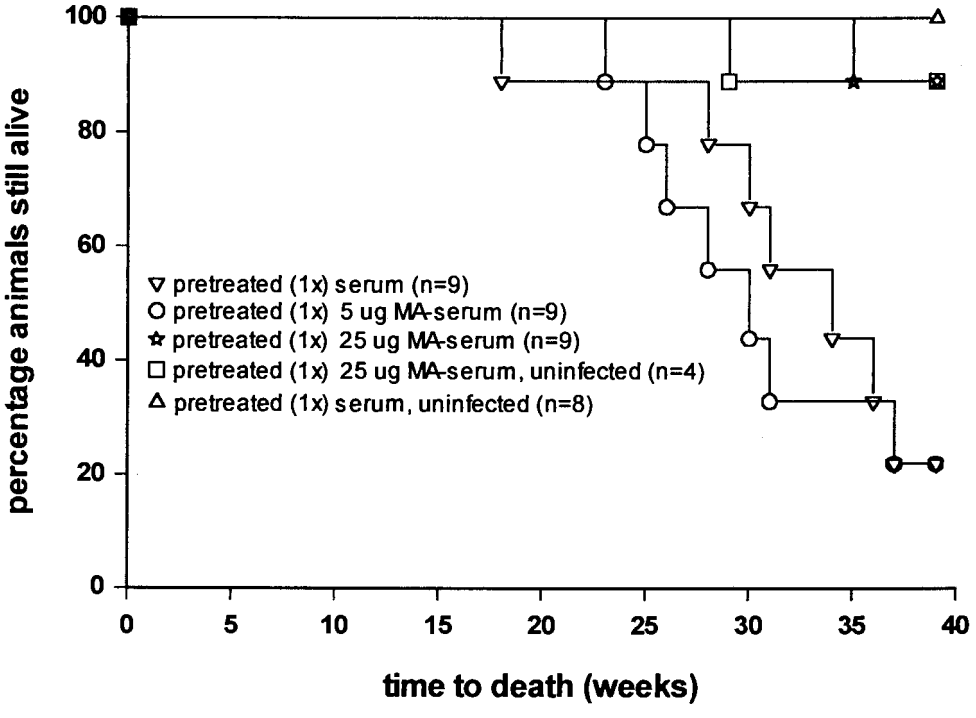
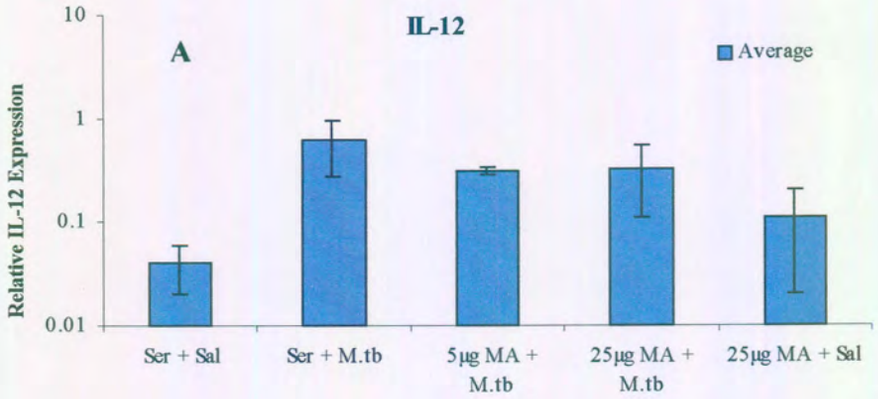


Figure 2.3: Survival of BALB/c mice infected intranasally with 2.5×10^5 *M. tuberculosis* H37Rv after pretreatment with purified mycobacterial mycolic acids (MA) conjugated onto normal mouse serum.

2.6.1 Interleukin-12 (IL-12) mRNA expression

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

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



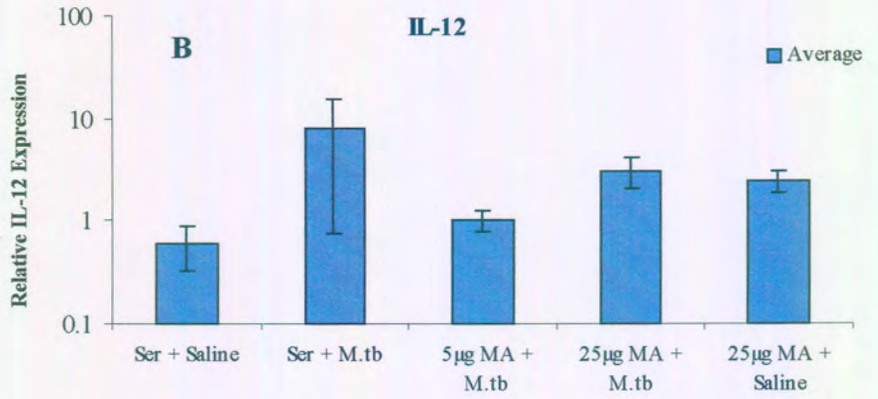


Figure 2.4: Relative IL-12 levels in the lungs of Balb/c mice removed five weeks after *M. tuberculosis* infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using the end-point PCR method (A) and the TaqMan™ Real-time Quantitative PCR method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

2.6.2 Interferon gamma (IFN-γ)

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

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

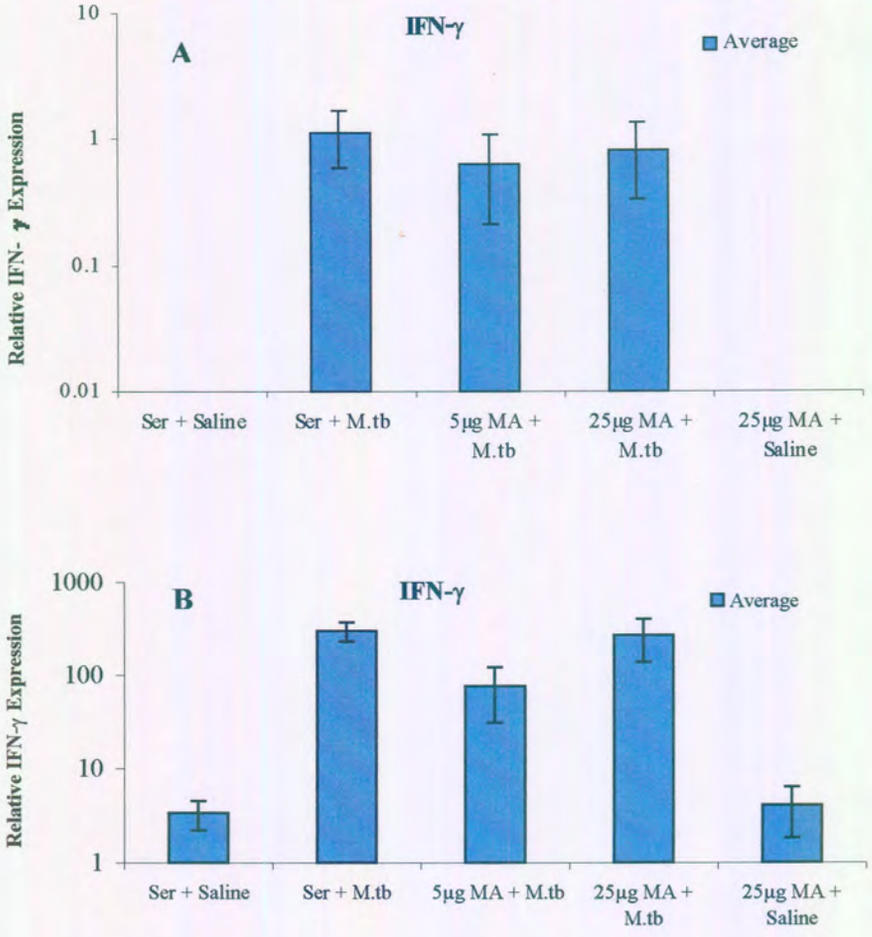


Figure 2.5: Relative IFN-γ levels in the lungs of Balb/c mice removed after five weeks of infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using the end-point PCR method (A) and the TaqMan™ Real-time Quantitative PCR method (B). The bars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

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

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

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

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

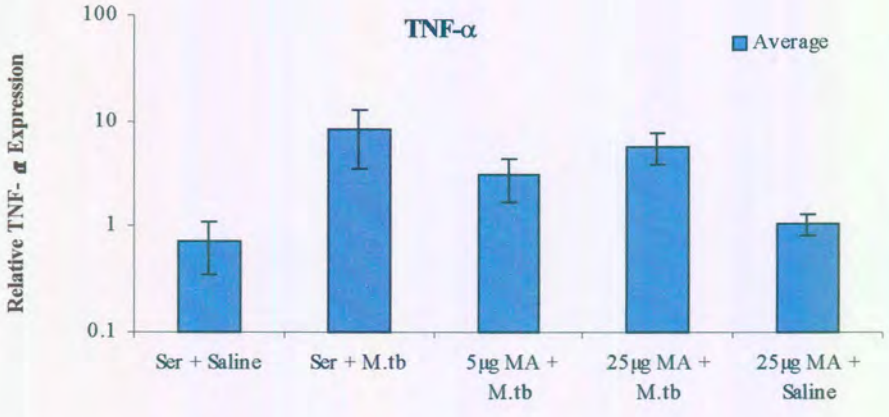


Figure 2.6: Relative TNF-α levels in the lungs of Balb/c mice removed after five weeks of infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using Taqman™ Real-time Quantitative PCR method. The bars on the graph represent an average of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

2.6.4 Interleukin-4 (IL-4) expression

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

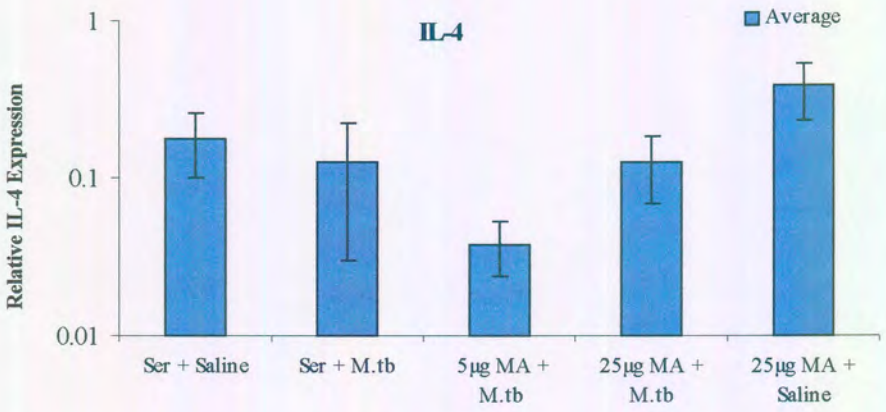


Figure 2.7: Relative IL-4 levels in the lungs of Balb/c mice removed after five weeks of infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using Taqman™ Real-time Quantitative PCR method. The bars on the graph represent an average of three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

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

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

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

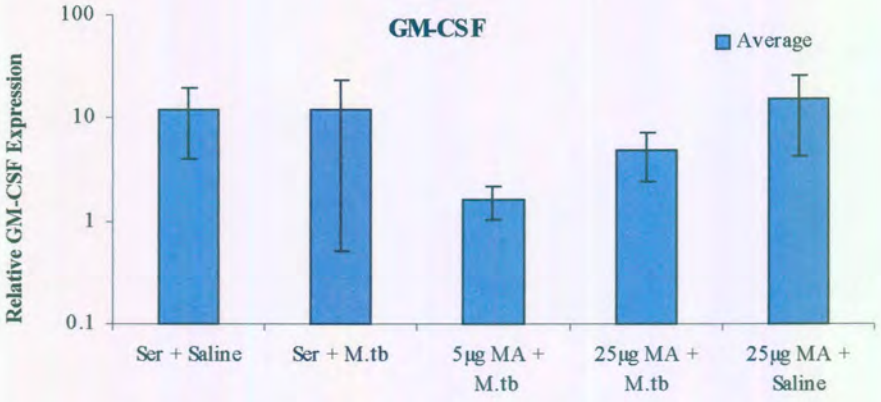


Figure 2.8: Relative GM-CSF levels in the lungs of Balb/c mice removed after five weeks of infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using Taqman™ Real-time Quantitative PCR method. The bars on the graph represent an average of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

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

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

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

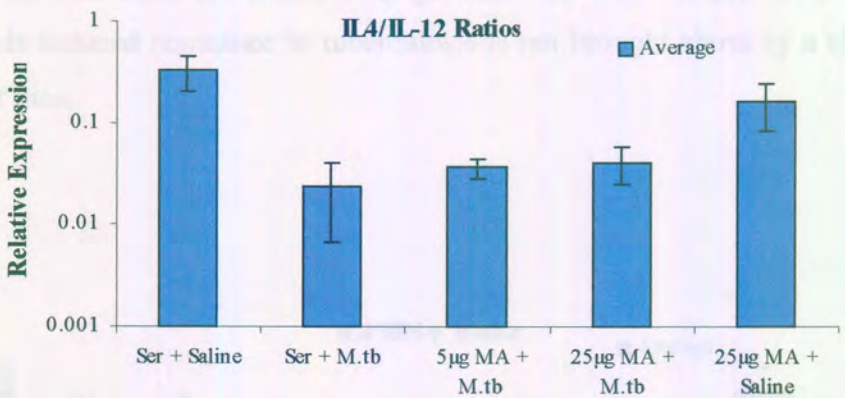


Figure 2.9.1: Relative expression of IL-4/IL-12 cytokine levels in the lungs of Balb/c mice removed after five weeks of infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using Taqman™ Real-time Quantitative PCR method. The bars on the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

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

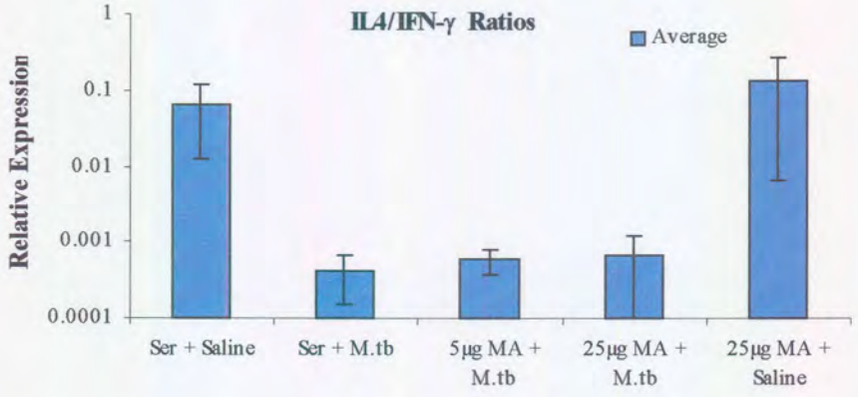

Figure 2.9.2: Relative expression of IL-4/IFN-γ cytokine levels in the lungs of Balb/c mice removed after five weeks of infection. The cytokines were assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using the Taqman™ Real-time Quantitative PCR method. The bars on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

2.7 Discussion

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

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

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

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

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

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

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

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

CHAPTER 3

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

3.1 Introduction

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

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

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

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

3.1.1 Animal models

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

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

3.1.2 Murine AIDS

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

Table3.1: Comparisons of MAIDS and AIDS

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

3.1.3 Immune response during MAIDS

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

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

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

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

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

3.1.4 Cytokines in MAIDS

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

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

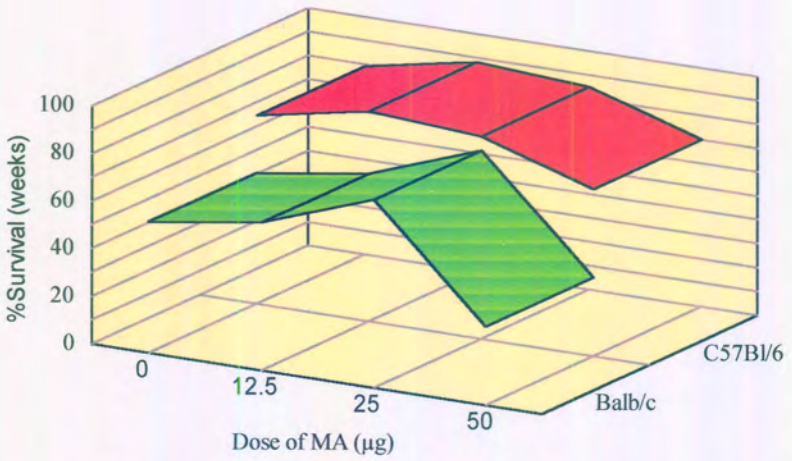

Figure 3.1: Comparison of Balb/c and C57Bl/6 mice that were pre-treated with different dosages of mycolic acids prior to infection with *M.tuberculosis*. Mice were kept in a glove isolator and in groups of 8 while being daily monitored and maintained for 40 weeks of infection. (Adapted from Siko, 1999)

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

3.2 Aims

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

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

3.3 Materials

3.3.1 Bacteria

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

3.3.2 Virus

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

3.3.3 Animals

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

3.3.4 Reagents

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

Ethidium bromide (Roche Molecular Systems, New Jersey USA)

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

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

Sodium acetate (Merck, Darmstadt Germany)

RNAZol ™-reagent (Biotech, Italy)

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

Oligo dT primers (Life Technologies Inc., Scotland)

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

Recombinant RNasin (Promega Corporation, Woods USA)

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

Housekeeping gene (GAPDH) primers and probes

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

3.4 Methods

3.4.1 Virus preparation

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

3.4.2 Bacterial culture

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

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

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

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

3.4.3 Mycolic acids preparation

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

3.4.4 Infection and treatment

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

3.4.5 Organ preparation and survival study

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

3.4.6 RNA preparation

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

3.4.7 Real-time PCR

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

3.5 Results

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

3.5.1 Survival study

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

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

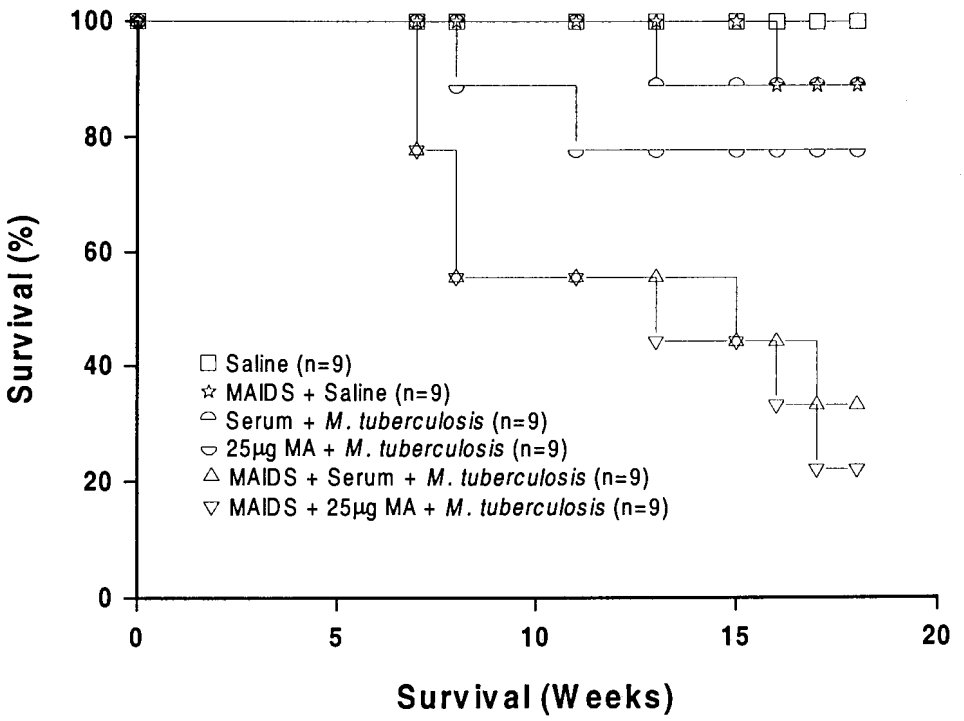

Figure 3.2: Relative survival of C57Bl/6 mice infected with LP-BM5 murine leukemia virus and *M. tuberculosis*. Mice were divided into groups of eight. M=LP-BM5 MuLV infection, Serum= serum treatment, MA= treatment with mycolic acids-serum conjugates and *M. tuberculosis*= infection with *M.tuberculosis*.

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

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

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

3.5.2.1 Interleukin-12 (IL-12) mRNA expression

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

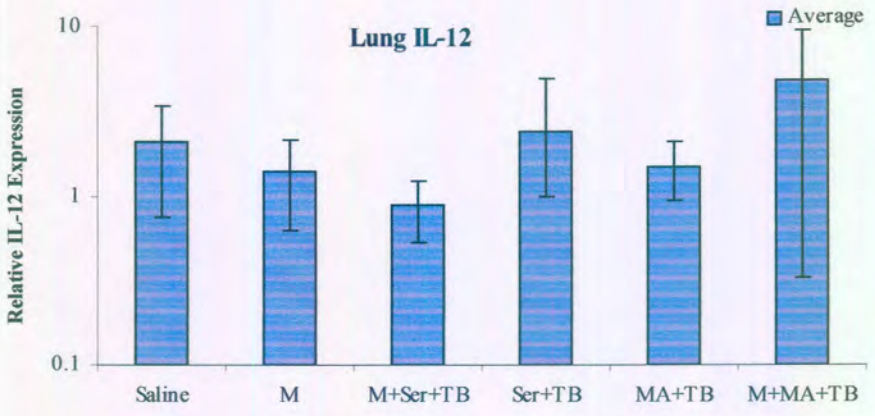

Figure 3.3: Relative IL-12 levels in the lungs of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed by the TaqMan™ Real time PCR method. The bars on the graph represent an average of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

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

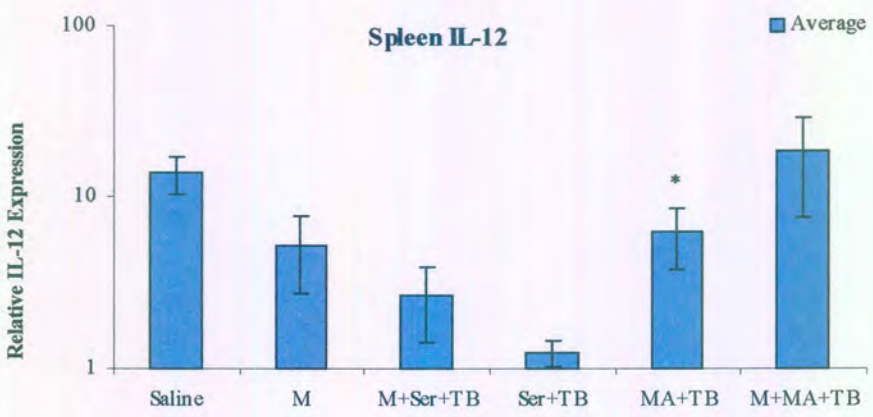

Figure 3.4: Relative IL-12 levels in the spleens of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using TaqMan™ Real time PCR method. The bars on the graph represent an average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

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

3.5.2.2 Interferon-gamma (IFN-γ) mRNA expression

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

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

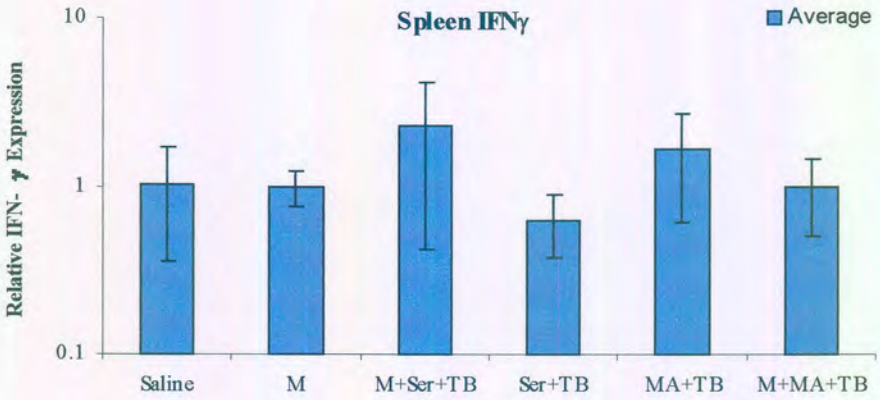

Figure 3.5: Relative IFN-γ mRNA levels in the spleens of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using TaqMan™ Real time PCR method. The bars on the graph represent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

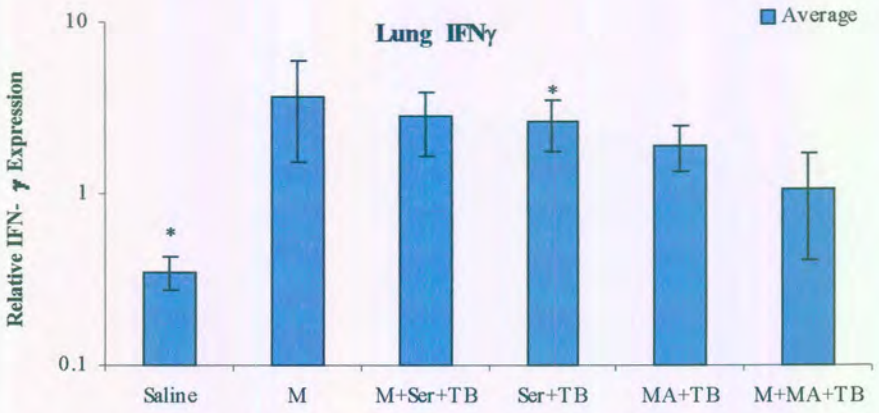

Figure 3.6: Relative IFN-γ mRNA levels in the lungs of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using TaqMan™ Real time PCR method. The bars on the graph represent an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

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

3.5.2.3 Interleukin-10 (IL-10) mRNA expression

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

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

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

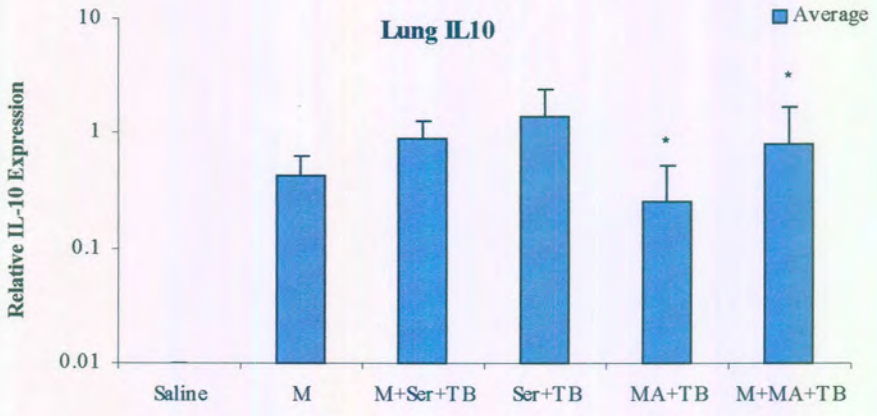

Figure 3.7: Relative IL-10 levels in the lungs of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using TaqMan™ Real time PCR method. The bars on the graph represent an average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

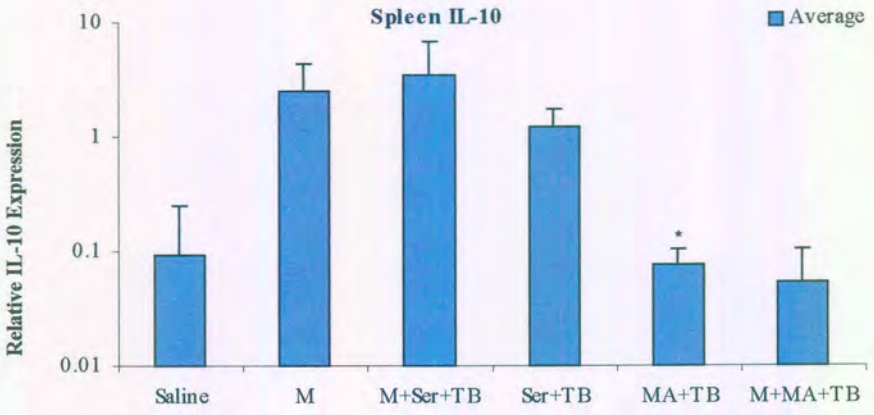

Figure 3.8: Relative IL-10 levels in the spleens of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The cytokine was assessed using RT to convert mRNA to cDNA. The amount of mRNA was then analysed through using TaqMan™ Real time PCR method. The bars on the graph represent an average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

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

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

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

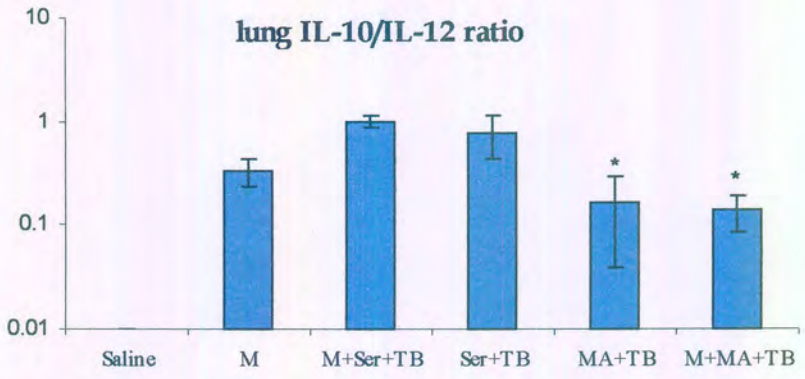

Figure 3.9: The ratios of IL-10 and IL-12 cytokine expression levels in the lungs of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The bars on the graph represent average ratios of the three mice per group obtained by random selection, while * denotes an average of two. M=MAIDS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

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

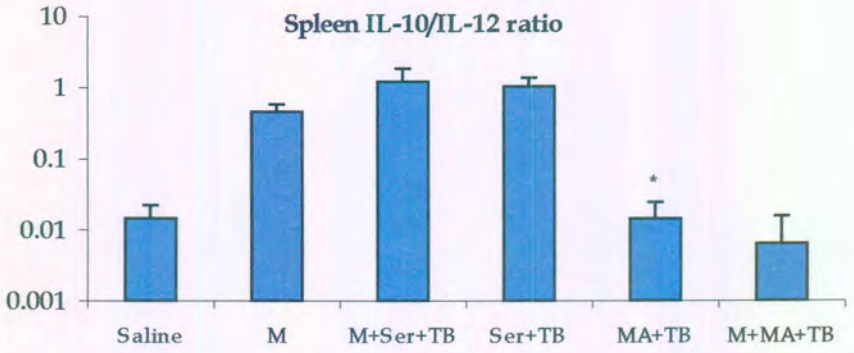

Figure 3.10: The ratios of IL-10 and IL-12 cytokine expression levels in the spleen of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The bars on the graph represent average ratios of the three mice per group obtained by random selection, while * denotes an average of two. M=MAIDS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

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

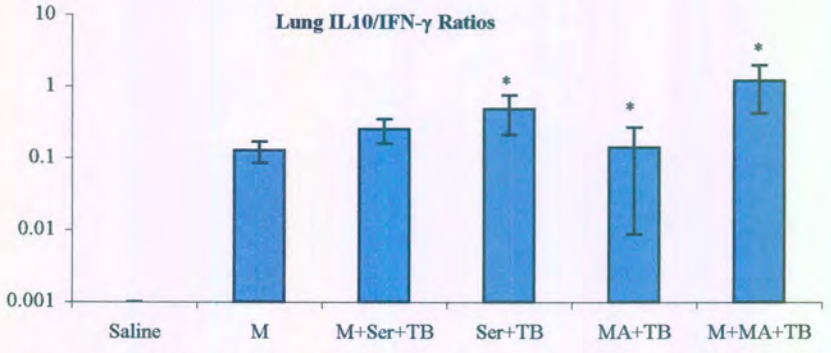

Figure 3.11: The ratios of IL-10 and IFN-γ cytokine expression levels in the lungs of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The bars on the graph represent average ratios of the three mice per group obtained by random selection, while * denotes an average of two. M=MAIDS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

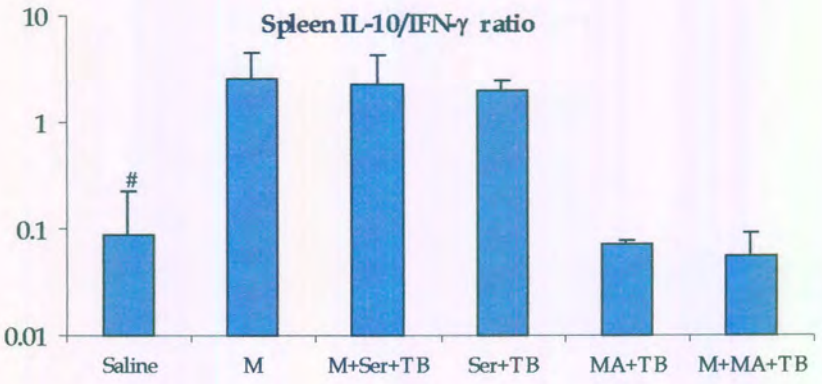

Figure 3.12: The ratios of IL-10 and IFN-γ cytokine expression levels in the spleens of C57Bl/6 mice removed after three weeks of *M. tuberculosis* infection. The bars on the graph represent average ratios of the three mice per group obtained by random selection. M=MAIDS induction, Sera= treated serum,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

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

3.6 Discussion

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

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

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

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

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

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

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

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

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

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

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