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INVESTIGATION OF THE ROLE OF THE TRK POTASSIUM TRANSPORTER OF *MYCOBACTERIUM TUBERCULOSIS* IN INTRACELLULAR SURVIVAL

BY

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In

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Declaration

I declare that the work contained in this dissertation is my original work and has not been presented for a degree in any other institution. It is being submitted in fulfillment for MSc degree at the University of Pretoria.

Signed: ..... Date: .....

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SUMMARY

Mycobacterium tuberculosis (*M. tuberculosis*), the causative agent of tuberculosis (TB), is a microbial pathogen which has infected about one third of the world's population, with about eight million new cases of TB reported annually of which almost two million are fatal. Those coinfected with human immunodeficiency virus (HIV) are most vulnerable for development of severe disease. The disease is acquired through the respiratory route, whereby *M. tuberculosis* bacilli overcome the mechanical defences of the upper airways to reach the lungs where they infect alveolar macrophages. Although considerable progress has been made in identifying immune mechanisms which confer protection against *M. tuberculosis*, this has not resulted in the development of an effective vaccine, underscoring the fact that novel insights into the immunopathogenesis of *M. tuberculosis* infection are necessary. In this respect it is noteworthy that almost nothing is known about the involvement of the major mycobacterial potassium (K^+) transporters in microbial virulence.

M. tuberculosis possesses two major K^+-uptake systems, namely the Trk and Kdp systems. The Trk seems to be functional when the extracellular K^+ concentration is high, while the Kdp is an inducible back-up system. The Trk system consists of two proteins, CeoB and CeoC, which are encoded by the *ceoB* and *ceoC* genes, which have some degree of homology to the TrkA protein of *Escherichia coli*. These proteins share the NAD^+-binding motif, compatible with proton motive force as the driver of cation uptake, suggesting that the *M. tuberculosis* Trk K^+ transporter may operate as a K^+ and protons (H^+) symporter, raising the possibility that it may antagonize vacuolar acidification, a critical event in the eradication of this intracellular pathogen.

The possible involvement of the Trk system in the virulence of *M. tuberculosis* has been addressed in the current study by investigating the intracellular survival of a *trk*-gene knockout mutant of the microbial pathogen with that of the matched wild-type (WT) strain using human

monocyte-derived macrophages. In addition, the cytokine profiles of macrophages infected by both strains have also been investigated.

Macrophages were prepared from isolated human blood monocytes following sequential differential adherence of $CD14^+$ monocytes. These were matured into large monocytes-derived macrophages co-expressing $CD14^+$ / $CD16^+$ following a 7 day incubation period. These cells were then infected with either the WT (H37Rv) or *trk*-gene knockout strains of *M. tuberculosis* at a 1:10 cell: bacteria ratio and intracellular survival as well as cytokine (IL-1β, IL-6, IL8, IL-10, TNF-α) secretion profiles monitored over a 3 day period using a viable colony-counting procedure and multiplex bead array technology, respectively.

No significant differences with respect to either intracellular survival or cytokine secretion profiles were detected following infection of human monocyte-derived macrophages with the WT or *trk*-gene knockout strains of *M. tuberculosis*. Although the observations are compatible with lack of involvement of the Trk system in the intracellular survival and virulence of *M. tuberculosis,* this study may lay groundwork for future studies which simultaneously encompass both of the major K^+ transporters of this microbial pathogen.

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α	Alpha
ADCC	Antibody-Dependent Cell-mediated Cytotoxicity
APC	Antigen Presenting Cells
β	Beta
BCG	Bacillus Calmette-Guerin
C	Complement
Ca^{2+}	Calcium
CAM	Cell Adhesion Molecules
CD	Cluster of Differentiation
CDC	Complement-Dependent Cytotoxicity
Cl^{2+}	Chlorine
CMI	Cell-Mediated Immunity
CR	Complement Receptors
CTLA	Cytotoxic Lymphocyte-Associated Antigen
Cu^{2+}	Copper
DC	Dendritic cells
DOTS	Directly Observed Treatment Short-course
EAA	Early Endosomal Antigen
ELISA	Enzyme-Linked Immunosorbent Assay
γ	Gamma
G-CSF	Granulocyte-Colony-Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
H^{+}	Protons

HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
ICAM	Intercellular Adhesion Molecule
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
K^+	Potassium
LPS	Lipopolysaccharide
LAM	Lipoarabinomannan
LM	lipomannan
M. tuberculosis	*Mycobacterium tuberculosis*
ManLAM	Mannosylated Lipoarabinomannan
MCP	Monocyte Chemoattractant Protein
MDR-TB	Multi-Drug Resistant Tuberculosis
Mg^{2+}	Manganese
MHC	Major Histocompatibility Complex
MHC-IIC	Major Histocompatibility Complex-II compartments
MIP	Monocyte Inflammatory Protein
MNL	Mononuclear Leukocytes
MR	Mannose Receptors
mRNA	Messenger Ribonucleic Acid
Na^+	Sodium
NK	Natural Killer
NO	Nitric Oxide

Nramp	Natural resistance associated macrophage protein
OD	Optical Density
PI3P	Phosphatidylinositol 3-Phosphate
PI3PK	Phosphoinositide 3-Kinase
PIMs	Phosphatidylinositol Mannosides
PknG	Protein serine/threonine Kinase G
RANTES	Regulated on Activation Normal T Expressed and Secreted
ROI	Reactive Oxygen Intermediates
SapM	Secreted acid phosphatase M
SEM	Standard Error of Mean
SOD	Superoxide Dismutase
Spp	Species
TB	Tuberculosis
TGF	Transforming Growth Factor
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
TST	Tuberculin Skin Test
WHO	World Health Organization
Zn^{2+}	Zinc

INTRODUCTION

This dissertation consists of a literature review followed by a description of the laboratory methodology, presentation and analysis of results and discussion and conclusion. The literature review consists of two major sections. Firstly, a review of the immunopathogenesis of *M. tuberculosis* infection, and secondly, albeit somewhat shorter, a description of the major potassium (K^+) transporters of this microbial pathogen. Some of the references cited are fairly old. However, these are landmark studies which merit inclusion.

CHAPTER 1

1 LITERATURE REVIEW

1.1 THE BURDEN OF TB GLOBALLY

Mycobacterium tuberculosis (M. tuberculosis) is the major infectious cause of morbidity and mortality globally yet it is preventable and treatable (Valadas and Antenes, 2005). In 2009, the estimated burden of disease caused by tuberculosis (TB) was 9.4 million incident cases, 14 million prevalence cases and 1.3 million deaths amongst human immunodeficiency virus (HIV) - negative people. The majority of these cases were reported from South-East Asia, sub-Saharan Africa and the Pacific region. About 13% of incidence cases were HIV-positive and the Africa region accounted for about 80% of these incident cases. From the TB patient reports in 2009, an estimated 250 000 had multi-drug resistant TB (MDR-TB). The frequency, mortality and prevalence rates are falling globally, but in South East Asia the incidence rate is stable. Mortality is reported to drop at least by 35% from 1990 to 2009, and possibly decrease by 50% by 2015 in at least six of the world health organization's (WHO) regions. China, India, the Russian Federation and South Africa are the four countries with the largest number of estimated cases of MDR-TB (WHO, Global Tuberculosis Control, 2011).

M. tuberculosis is a member of the *M. tuberculosis* complex which also includes six species that are closely related such as *M. africanum, M. bovis, M. canetti, M. caprae, M. macroti and M. pinnipedii* (Ahmad, 2010). There is still an increase in new TB cases each year despite the coverage of Bacillus Calmette-Guerin (BCG) vaccination, which does not protect against the reactivation of latent TB especially in HIV/TB co-infected individuals. HIV does not only cause reactivation of latent TB, but it also increases TB progression soon after infection and re-infection (Corbett et al., 2003; Sutherland et al., 2009). It has been estimated that one in three people around the world is infected by *M. tuberculosis* and they are also at risk of developing active disease (Valadas and Antenes, 2005).

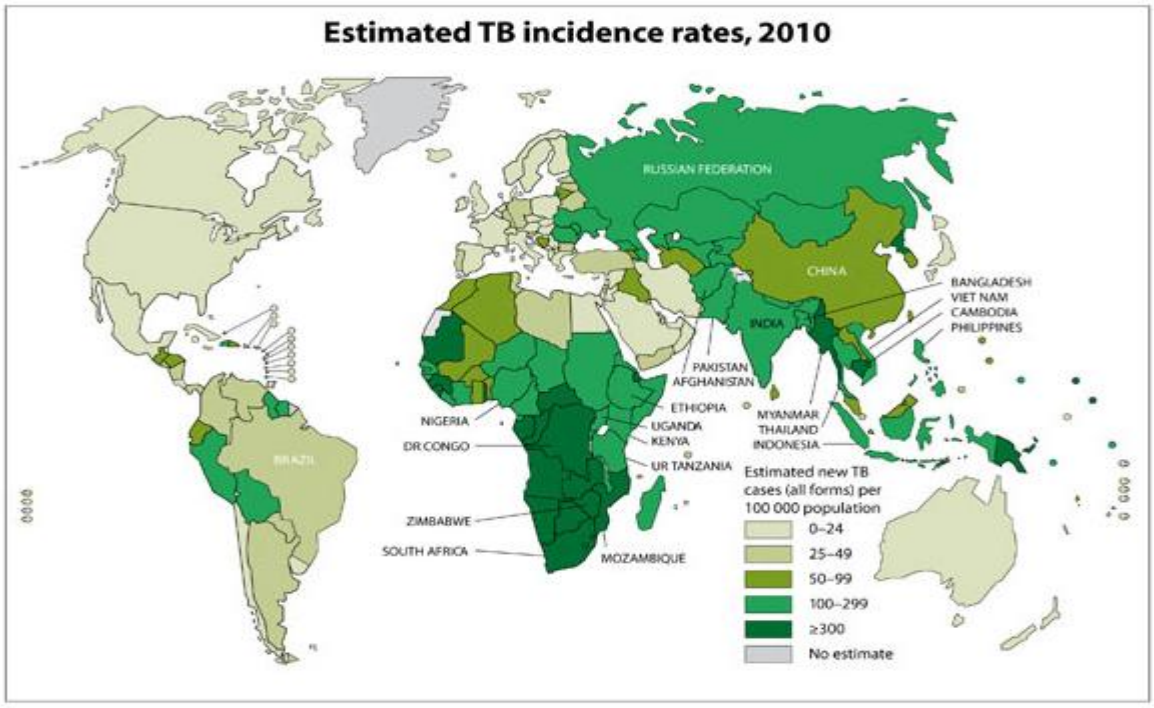

Figure 1.1: Estimated TB incidence rates in 2010. (W.H.O. Global TB Control. 2011).

In those who are infected with *M. tuberculosis* alone the lifetime risk of developing TB is estimated to be up to 20%, while in those co-infected with HIV/TB the annual risk may be up to 10%. Over the past decade, the burden of HIV/TB co-infection has increased, with 90% of TB cases reported in developing countries such as those of sub-Saharan Africa (Corbett et al, 2003; Valadas and Antenes, 2005). The incidence of TB in sub-Saharan Africa is increasing at about 4%, while in eastern and southern Africa it is estimated at 6% annually. In many countries, HIV has been associated with TB outbreaks, threatening the health and survival of HIV- negative individuals with many of the reported outbreak cases being with multidrug-resistant strains. In the long-term, the development of effective vaccines and antibiotics are required in order to improve TB control.

1.1.1 Prevalence of TB in South Africa

South Africa has the second highest TB incidence per capita globally (WHO: Global Tuberculosis Control Report, 2009). It has been estimated that the TB incidence in South Africa may be 692 per 100 000 people and the WHO has classified this as a serious epidemic (WHO, Global Tuberculosis Control, 2009). Although the Directly Observed Treatment Short-course (DOTS) program has been established and activated since the year 1995, TB is still a major health problem in South Africa especially the Eastern Cape Province (Walzl et al., 2005). The cure rate is low, being 65% compared to the 85% rate which was recommended by the WHO (WHO, Global Tuberculosis Control, 2009).

1.1.2 Mycobacterium tuberculosis

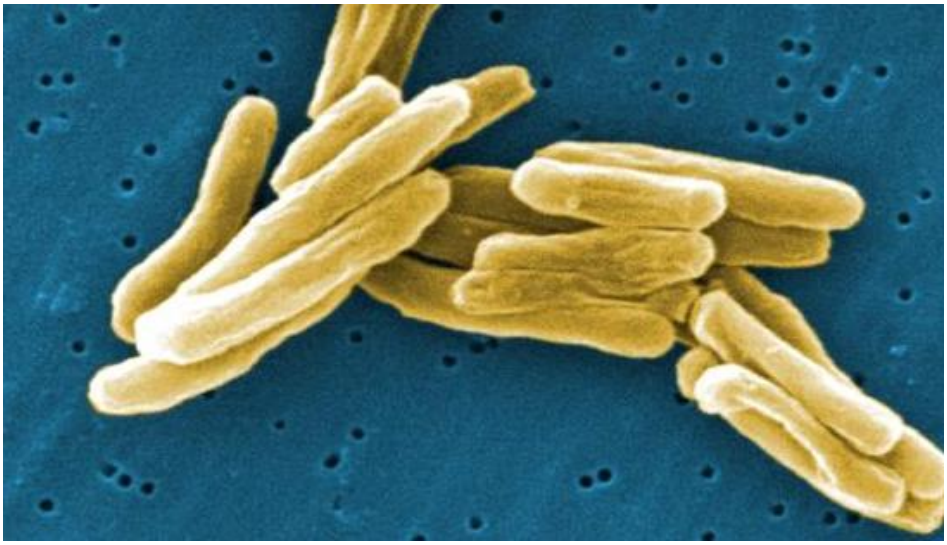

Figure 1.2: Electron micrograph of *M. tuberculosis* bacilli (www.katiephd.com/wp-content/uploads/2011/03*Mycobacterium tuberculosis*. jpg).

M. tuberculosis is a non-motile, Gram-positive bacillus (Figure 1.2) that is approximately 2-5µm long and 0.2-0.3µm in diameter (Segovia-Juarez et al., 2004). These bacilli are obligate human parasites that can infect almost all mammals, but are only transmitted by humans. It is one of the oldest micro-organisms known to mankind. *M. tuberculosis* has evolved to be one of the most successful pathogens, able to infect about one third of the human population worldwide. This bacillus has a stable genome and is a slow grower (Victor et al., 1997).

1.2 PATHOGENESIS OF TUBERCULOSIS

1.2.1 Transmission of TB

TB is a communicable disease that is acquired by inhaling aerosolized droplet nuclei into the lungs. The bacilli may spread from the initial site of infection in the lungs via the lymphatics or blood to other parts of the body. *M. tuberculosis* is a human pathogen since it can stay alive in humans for a long period of time (Hunter et al., 2006). The ability of *M. tuberculosis* to cause disease is, however, dependent on the series of circumstances such as bacillary load, infectivity of the source case, and the immune status of the potential host (Ahmad, 2010). Because of their small size, the droplet nuclei can remain suspended in the air for long periods and can avoid the mechanical defenses of the respiratory tract, penetrating the alveoli where they are engulfed by the alveolar macrophages or the dendritic cells (DC) (Danelishvili et al., 2003; Mehta et al., 2006; Kinhikar et al., 2010).

In the early stages of infection, *M. tuberculosis* is taken up by phagocytic immune cells, where it replicates intracellularly. These bacteria-laden cells can cross the alveolar barrier, causing systemic dissemination (Teitelbaum et al., 1999, Bermudez et al., 2002). In the majority of individuals an effective cell-mediated immune response will develop within a period of 2-8 weeks after infection. T-lymphocytes, macrophages and other cells of the immune system form a granuloma that walls off the growing necrotic tissue, stopping further replication and spread of *M. tuberculosis* (Frieden et al., 2003; Hingley-Wilson et al., 2003).

Macrophages are not the only immune cells involved in *M. tuberculosis* killing. Non-phagocytic cells found in the alveolar space, such as alveolar endothelial, type 1 and type 2 epithelial cells may also contribute (Bermudez and Goodman, 1996).

1.3 INFLAMMATION

Inflammation is defined as a restricted, but protective response to microbial invasion or injury. Inflammation must be fine-tuned and precisely controlled because absence or excesses of the inflammatory response may lead to infection and cancer, causing morbidity and even a shorter life-span (Tracy, 2002).

1.4 EVENTS INVOLVED IN THE PATHOGENESIS OF INFECTION WITH *M. TUBERCULOSIS*

Based on the studies done by Lurie in rabbits there are at least four stages of pulmonary TB (Lurie, 1964). The first stage is the inhalation of the tubercle bacilli and the recognition by the alveolar macrophages. The alveolar macrophages then ingest and at times kill the pathogen. The destruction of the mycobacteria depends on the intrinsic microbicidal capacity of these host phagocytes, as well as the virulence factors of the ingested mycobacteria. When the mycobacteria escape microbicidal mechanisms, they multiply intracellularly which leads to the disruption of the macrophages.

M. tuberculosis primarily infects the alveolar macrophages. Mycobacteria which have escaped the initial intracellular destruction by blood monocytes or macrophages and the other inflammatory cells are attracted to the lungs. Monocytes differentiate into macrophages which again eagerly ingest, but do not destroy the mycobacteria (van Crevel et al., 2002). At this point the mycobacteria grow at a logarithmic rate and the blood-derived macrophages accumulate with little tissue damage.

After two weeks of initial infection T-cell immunity develops with antigen specific T-lymphocytes proliferating within the early lesion. Macrophages are then activated to destroy the intracellular mycobacteria. Just after this stage, the early logarithmic bacillary growth ends. At this point, the central solid necrosis in these primary lesions inhibits extracellular growth of mycobacteria. As a result, the infection either becomes stationary or dormant. TB may progress and haematogenous distribution may take place after primary infection, or alternatively, an only year later if a person becomes immunocompromised.

The formation of cavities may lead to rupture into nearby bronchi, allowing the spread of mycobacteria through the airways to other parts of the lung and the external environment. The final outcome of the infection with *M. tuberculosis* depends on the balance between the killing of the *M. tuberculosis* organisms and their ability to evade host defenses. Uptake of the bacilli by macrophages may occur through a variety of different receptor molecules such as complement receptors (CR), mannose receptors (MR), the dendritic cell-specific intercellular adhesion molecule (ICAM)-3-grabbing nonintegrin (DC-SIGN) and Fc receptors.

The mycobacteria remain enclosed in a membrane-bound vacuole called the phagosome in which they may survive and proliferate, being released into the airways after an enormous increase in number because *M. tuberculosis* modifies the maturation of this phagosome in order to develop its own intracellular survival (Clemens and Horwitz, 1995; Raja, 2004).

1.5 INNATE IMMUNITY

Phagocytosis and consequent production of interleukin (IL) -12 are processes initiated in the absence of prior exposure to an antigen and therefore form a component of innate immunity. The other components include natural resistance associated macrophage protein (Nramp), neutrophils, and natural killer (NK) cells (Raja, 2004). Nramp-1 is an integral membrane protein which belongs to the metal ion transporter protein family. After *M. tuberculosis* is engulfed, Nramp-1 becomes part of the phagosome (van Crevel et al., 2002).

It is vital in the transportation of nitrite from the intracellular compartment such as the cytosol to the phagolysosome where it can be converted into the antimicrobial agent, nitric oxide (NO). A defect in the production of Nramp1 increases susceptibility to mycobacterial infection (Raja, 2004).

1.5.1 Macrophages

Macrophages originate from the bone marrow precursors of circulating monocytes. The macrophage maturation process is accompanied by progressive morphological (Figure 1.3) and functional changes which continue even after they enter the tissues (Nichols and Bainton, 1975; Van Furth et al., 1980). Monocytes are produced in the bone marrow under both positive and negative feedback control, with peripheral macrophages and lymphocytes secreting factors that are stimulatory and also inhibitory to proliferation of the stem cells in the bone marrow (Quesenberry et al., 1979). Under a light microscope macrophages are round, oval or spindle shaped in outline with a cytoplasm which varies from eosinophilic and finely granular, to clear and vesicular. The nucleus of the macrophage is smooth, but sometimes it is indented with marginated heterochromatin and usually a single nucleolus (Nichols and Bainton, 1975).

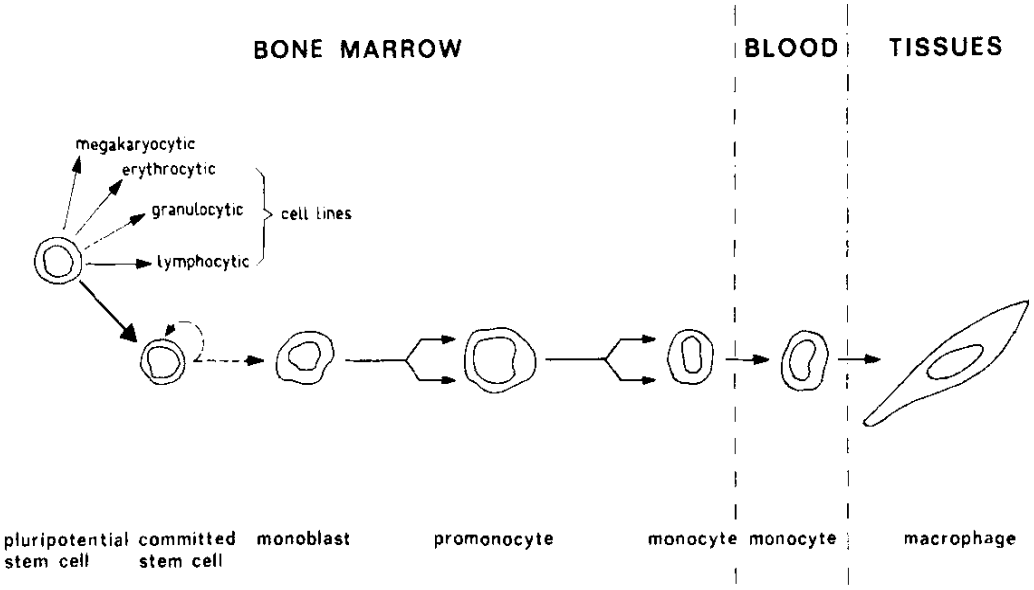

Figure 1.3: Schematic representation of the origin of tissue macrophages from the bone marrow

Recruitment and localization of monocytes into the inflammatory lesion is assisted by two types of mediator. These mediators include firstly, microbial products, complement cleavage products, fibrin degradation products, and chemokines, while secondly, immobilization of macrophages within a lesion is assisted by cytokines, including migration inhibitory and pro-adhesion factors (Ward, 1974). Macrophages are the major components of inflammatory and immunologic reaction in the lungs. They are also involved in tissue homeostasis through clearance of apoptotic cells and the production of growth factors (Twigg, 2004; Chitu and Stanley, 2006).

Macrophages have the ability to ingest a wide variety of substances into membrane-bound vacuoles (endocytosis). This process involves two mechanisms: pinocytosis and phagocytosis. Fluids, soluble proteins, immune complexes, hormones, lectins and other macromolecules are taken up by pinocytosis, while large particles are engulfed by phagocytosis (Gordon and Cohn, 1973; Michl, 1980). These processes are initiated by interactions between the particle and surface receptors which then triggers intracytoplasmic contractile proteins such as myosin and actin to create the movement of membrane and pseudopodial ingestion (Stossel, 1975; Michl, 1980; Silverstein and Loike, 1980). Ingestion is then followed by the fusion of the lysosome and phagosome (phagolysosome) allowing the intracellular degradation and killing of the microbes (Spector, 1974).

The killing of micro-organisms by macrophages depends on the production of superoxide, hydrogen peroxide, hydroxyl radical, nitric oxide and other microbicidal substances. This process is also assisted by the presence of antibodies such as IgG for bacteria and IgE for parasites and protozoa (Babior, 1978; Nelson, 1981). The killing of intracellular micro-organisms is largely enhanced by the process of macrophage activation, resulting in morphological functional alterations such as secretion of antimicrobial agents and enhanced phagocytic capacity (Nichols and Bainton, 1975; Allison, 1978).

Some micro-organisms such as *Mycobacterial* spp., *Nocardia* spp. and *Legionella* spp. are resistant to killing by macrophages and are capable of replicating intracellularly and therefore the eradication of these micro-organisms requires the development of cell-mediated immunity (Zhang et al., 2000). Lung macrophages are morphologically and functionally heterogeneous,

and include interstitial, intravascular, alveolar and airway macrophages, each having a distinct morphology and functional features. The lung macrophages have surface receptors for many ligands which enable them to react to environmental factors and promote the clearance of microparticles and microorganisms in the distal airways and the alveolar space (Sibille, 1990).

Macrophages move around the tissues internalizing tissue debris and apoptotic cells. This is accomplished in a discrete, non-inflammatory manner to limit tissue damage (Rohde et al., 2007). The stimulation of Toll-like receptors (TLR)-mediated signals is the initial step in the transition of macrophages to their role as immune effector cells, even though non-activated macrophages express low levels of major histocompatibility complex (MHC) class II molecules, an activated macrophage upregulates its antigen presenting machinery considerably (Twigg, 2004; Chitu and Stanley, 2006).

Macrophages have a broad range of pathogen-recognition receptors that allows them to function as phagocytic cells and to induce the production of inflammatory cytokines (Teitelbaum et al., 1999; Bermudez et al, 2002). Macrophages release cytokines such as growth-promoting and inhibiting factors. They may also mediate the damage and repair of matrix processes (Sibille, 1990).

1.5.1.1 The role of Toll-like receptors in recognition of *M. tuberculosis*

In addition to phagocytosis, identification of *M. tuberculosis* or other mycobacteria is an important step in an effective host response. The immune identification of the major mycobacterial cell wall component, lipoarabinomannan (LAM), is similar to that of the Gram-negative bacterial lipopolysaccharide (LPS) (Zhang et al., 1993). There are a number of receptors and circulating factors that are involved. Toll-like receptors are expressed by many cell types and recognize a number of pathogen-associated molecular patterns such as LPS, lipoprotein and flagellin (Akira and Takeda, 2004; Halayko and Ghavami, 2009; Wheeler et al., 2009).

Some studies have reported that the activation of TLR is initiated by ligand-bridged dimerization of extracellular receptor domains. Following binding to their ligands, they form homodimer or heterodimer triggering recruitment of adaptor molecules to activate downstream signaling pathways (O'Neill and Bowie, 2007; Dowling et al., 2008).

1.5.1.2 The role of mannose receptors in recognition of *M. tuberculosis*

Mannose receptors (MR) are defined as homeostatic or clearance or immunomodulatory receptors for endogenous serum glycoproteins with high *N*-linked mannose content which are increased during inflammation (Martinez-Pomares et al., 2001). The *M. tuberculosis* cell envelope is rich in mannose-containing biomolecules such as ManLAM, lipomannan (LM), higher order PIMs, arabinomannan, mannan and mannosylated glycoproteins. ManLAM, LM and higher-order PIMs are found in the *M. tuberculosis* cell-wall acting as ligands for host cell receptors and contributing to the immunopathogenesis of *M. tuberculosis* infection (Torrelles and Schlesinger, 2010).

Some authors have proposed that *M. tuberculosis* may take advantage of its mannosylated cell surface components in order to survive within the host by binding the MR Kang et al., 2005 and Torrelles et al., 2006 have both demonstrated that the ManLAM/MR and higher-order PIMs/MR phagocytic pathways lead to phagosome maturation arrest. Identification of mannose residues by MR has also been demonstrated to reduce the microbicidal activities of macrophages by inhibiting nitric oxide, oxygen radical and pro-inflammatory cytokine production and also by blocking *M. tuberculosis*-induced apoptosis via modification of Ca^{2+} -dependent signaling pathways (Torrelles et al., 2008).

1.5.1.3 The role of complement receptors in recognition of *M. tuberculosis*

Complement receptors (CR) are expressed on all mononuclear phagocytes and mediate the phagocytosis of a diverse group of intracellular pathogens. Most studies have established the critical function of C3b- mediated opsonization and the contribution of CR1, CR3 and CR4 in *M. tuberculosis* phagocytosis (Fenton et al., 2005). Deposition of C3b on *M. tuberculosis* happens quickly and is initiated by activation of both the classical and alternative pathways through covalent linkages to *M. tuberculosis* surface targets in the form of C3b and C3bi (Ferguson et al., 2004). However, it is still not known whether C3-mediated opsonization differs quantitatively and qualitatively at different stages and tissue sites of *M. tuberculosis* infection. Even though CR3 mediates both opsonic and non-opsonic uptake *of M. tuberculosis* by macrophages, its function during human infection is still not known.

1.5.1.4 The role of CD14 and scavenger receptors in recognition of *M. tuberculosis*

Cluster of differentiation (CD) 14 and scavenger receptors are also involved in the recognition of *M. tuberculosis* and related inflammation. These receptors participate in the uptake of non-opsonized bacilli by tissue specific macrophages (Khanna et al., 1996; Zimmerli et al., 1996). The role of these receptors in inflammation differs depending on the species-specific cell type used. The plasma LPS-binding protein increases the response of macrophages to LPS and LAM by translocating these microbial products to the cell-surface receptor, CD14 (Fenton and Golenbock, 1998). Soluble CD14 also promotes responsiveness to both LPS and LAM in CD14-negative cells (Yu et al., 1998).

1.5.2 Phagocytosis of *M. tuberculosis* by macrophages

Alveolar macrophages are the primary targets and residence of *M. tuberculosis.* After *M. tuberculosis* has encountered the alveolar macrophages, dendritic cells and monocyte-derived macrophages also take part in the phagocytic process (Weikert et al., 1997). The intracellular replication of *M. tuberculosis* occurs prior to the development of an adaptive immune response, which in turn allows *M. tuberculosis* to create a niche where it can avoid the adaptive immune system, allowing it to persist in a dormant state (Teitelbaum et al., 1999; Bermudez et al., 2002). Endocytosis of *M. tuberculosis* involves different receptors on the phagocytic cell to those which promote phagocytosis, with the former binding non-opsonised *M. tuberculosis* and the latter to opsonins on the surface of *M. tuberculosis* (Figure 1.4). As an example of the latter mechanism, *M. tuberculosis* can infect host macrophages after opsonisation with complement factor C3, which is later followed by binding and uptake through CR1, CR3, and CR4 (Hirsch et al., 1994; Aderem et al., 1999).

The CR1, CR2, CR3 and CR4, MR, Fc receptors and other cell receptors all play a significant role in binding of the organism to the phagocytes (Glickman and Jacobs, 2001; Raja, 2004). *M. tuberculosis* is extremely promiscuous in its use of multiple cell surface receptors to gain entry into macrophages (Glickman and Jacobs, 2001). The interaction between MR on phagocytic cells and mycobacteria seems to be mediated via the mycobacterial surface glycoprotein, LAM (Raja, 2004). The comparative importance of the various receptors for complement factor C3 is apparent from experiments *in vitro*, in which in the absence of CR3, phagocytosis of *M. tuberculosis* by human macrophages and monocytes is reduced by approximately 70 to 80% (Schlesinger et al., 1990; Schlesinger, 1993). *M. tuberculosis* also makes use of the classical pathway of complement activation by directly binding to C2a, even in the absence of C4b. In this way the C3b necessary for binding to CR1 is formed (Schorey et al., 1997).

On the other hand, the best-characterised receptor for non-opsonin-mediated phagocytosis of *M. tuberculosis* is the macrophage MR, which binds the terminal mannose residues on mycobacteria (Schlesinger, 1993**,** Schlesinger et al., 1996). If the uptake by the CR and MR is blocked, macrophages can also internalise the *M. tuberculosis* via the type A scavenger receptor (Zimmerli, 1996). Binding of *M. tuberculosis* to airway epithelial cells or alveolar macrophages may represent a risk factor for developing the clinical tuberculosis (van Crevel et al., 2002).

The TLR also participate in *M. tuberculosis* immune recognition as mentioned earlier. In the case of monocytes, TLR2 binds to LAM, while a heterodimer of TLR2 and TLR 6 binds to *M. tuberculosis* lipoprotein, and TLR4 binds to a vague heat-labile, cell-associated factor. Subsequent to binding to TLRs, intracellular signalling pathways lead to cell activation and the production of cytokines. Phagocytosis alone probably does not lead to immune activation without the contribution of TLRs (van Crevel et al., 2002). Prostaglandin E2 (PGE2) and interleukin (IL-)-4, a Th2-type-2 cytokine, upregulate CR and MR receptor expression and function, while interferon (IFN) -γ decreases receptor expression, resulting in diminished capacity of the mycobacteria to attach to macrophages (Raja, 2004). These events are summarized in figure 1.4.

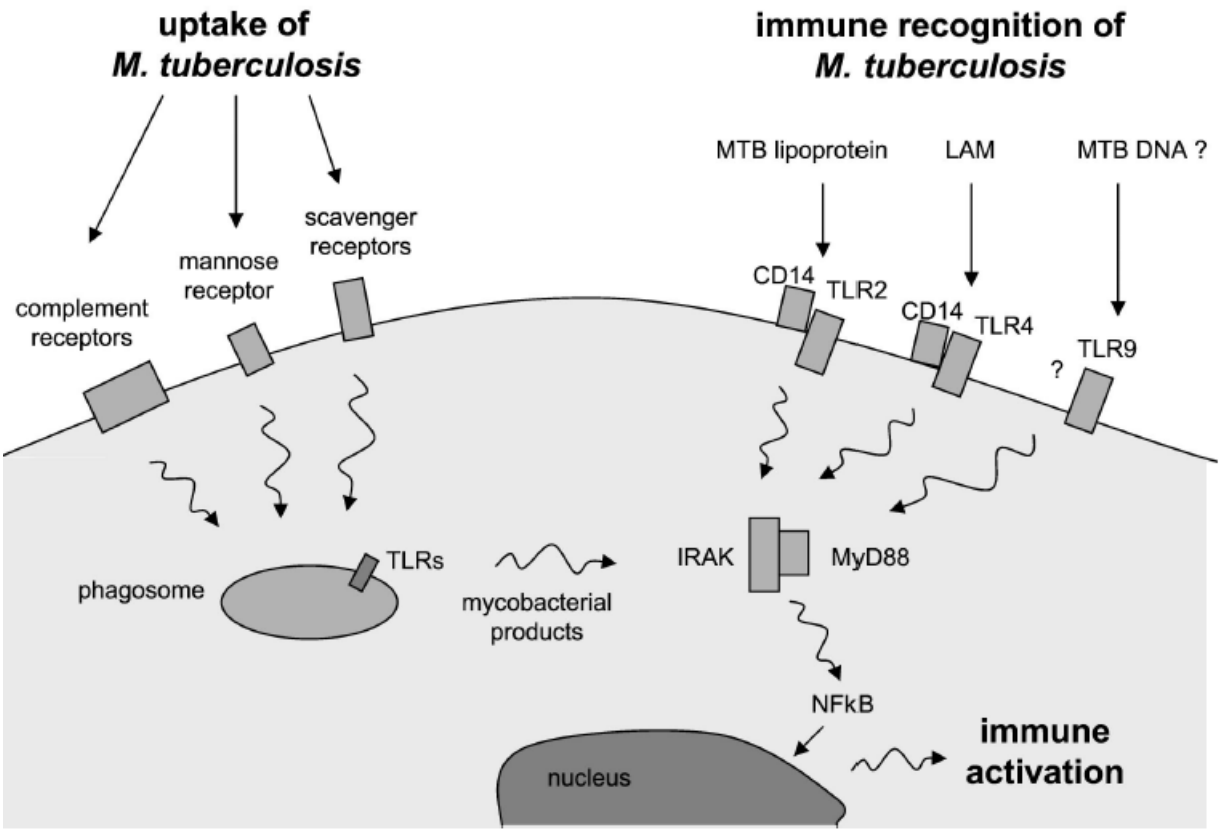

Figure 1.4: Phagocytosis and immune recognition of *M. tuberculosis*. A number of receptors has been indentified which mediate *M. tuberculosis* phagocytosis by macrophages; simultaneous activation of both mechanisms is necessary for macrophage activation and induction of adaptive immunity. (van Crevel et al., 2002. *Clin Microbiol Rev*).

1.5.3 Maturation of the phagosome

When a phagosome containing *M. tuberculosis* fuses with lysosomes it acidifies, exposing the mycobacteria to lysosomal hydrolases, as well as to reactive oxygen and nitrogen intermediates (Rhode et al., 2007). However, the bacilli may escape this process. A variety of genes have been associated with inhibition of phagosome maturation (Deretic et al., 2006). *M. tuberculosis* inhibits the maturation of the phagosome. For normal maturation of the phagosome, translocation of phagosome Rab5 to the phagosomal membrane is necessary, which is followed by production of phosphatidylinositol-3, 4, 5 phosphate (PI3P3) by the type III PI3P

kinase (PI3PK) vsp34. The mycobacterial cell-wall LAM is believed to inhibit production of PIP3 by PI3PK vsp34 (Vergne et al., 2003; Vergne et al., 2004; Kang et al., 2005). This, in turn stops the recruitment of early endosomal antigen 1 (EEA1) and other Rab5 effectors to the phagosomal membrane inhibiting recruitment of Rab7 and fusion with late endosomes and lysosomes (Vergne et al., 2005).

Recruitment of Rab7 permits fusion with lysosomes, acidification of the phagosome, and the release of lysosomal hydrolases into the lumen of the matured phagosome. *M. tuberculosis* inhibits this process by releasing LAM and the PI3P phosphatase secreted acid phosphatase M (sapM) which stops the generation and recruitment of PI3P to the phagosomal membrane. Although the phagosome stays immature, it can still fuse with recycling endosomes that may transport nutrients, therefore providing a protective environment for bacterial replication and survival (Harris and Keane, 2010).

On the other hand, macrophages that are activated by IFN-γ induce acidification and maturation of mycobacteria-containing phagosomes, which in turn leads to increased intracellular killing by macrophages *in vitro* (Schaible et al., 1998; Vial et al., 1998). Harris et al., 2008 have demonstrated that IFN-γ induces an increase in phagosome maturation which is inhibited in human macrophages treated with tumour necrosis factor (TNF) antagonists.

1.5.4 Granuloma formation during infection with *M. tuberculosis*

The granuloma is a structured cluster containing *M. tuberculosis*-infected macrophages in the center, surrounded by different types of immune cells, in particular macrophages and T-lymphocytes. (Pieters, 2008). The formation of the granuloma depends on a number of events such as the triggering of T-cells by antigen-presenting cells, particularly alveolar macrophages and dendritic cells, as well as the release of cytokines and chemokines by macrophages, activated lymphocytes, dendritic cells and polymorphonuclear cells. Following the stable dynamic accumulation of immunocompetent cells and the formation of the organized structure of the

granuloma, the final stage is fibrosis. This is characterized by the persistence of a chronic inflammatory response that is accompanied by failure of immune regulatory mechanisms, which in turn leads to invasion of pulmonary tissues by the granuloma and derangement of alveolar structures (Figure 1.5). Granulomas induced by *M. tuberculosis* infection are a collection of well-organized immune cells that offer a safe microenvironment conducive to latency. From the host perspective, the formation of the granuloma restricts the spread of *M. tuberculosis* infection. Granuloma formation begins with influx of polymorphonuclear leukocytes to the site of infection, followed thereafter by activated macrophages and lymphocytes (Ulrichs and Kaufmann, 2006; Russell, 2007). Within the granuloma, macrophages separate into epithelioid cells and or fuse to form multinucleated giant cells. Furthermore, highly-vacuolated, as well as lipid-rich foamy macrophages are also found (Ridley and Ridley, 1987; Cardona et al., 2000).

Granulomas are stabilized and maintained by events mediated by both host and pathogen. This is beneficial for the host since it helps to contain and localize the infection. Granuloma formation maintains the infection at an equilibrium which does not harm the host, but neither does it kill the bacteria. The matured granuloma has a distinct morphology, consisting of a central necrotic core surrounded by a concentric layer of macrophages, Langerhans giant cells and lymphocytes. These events are summarized in figure 1.5.

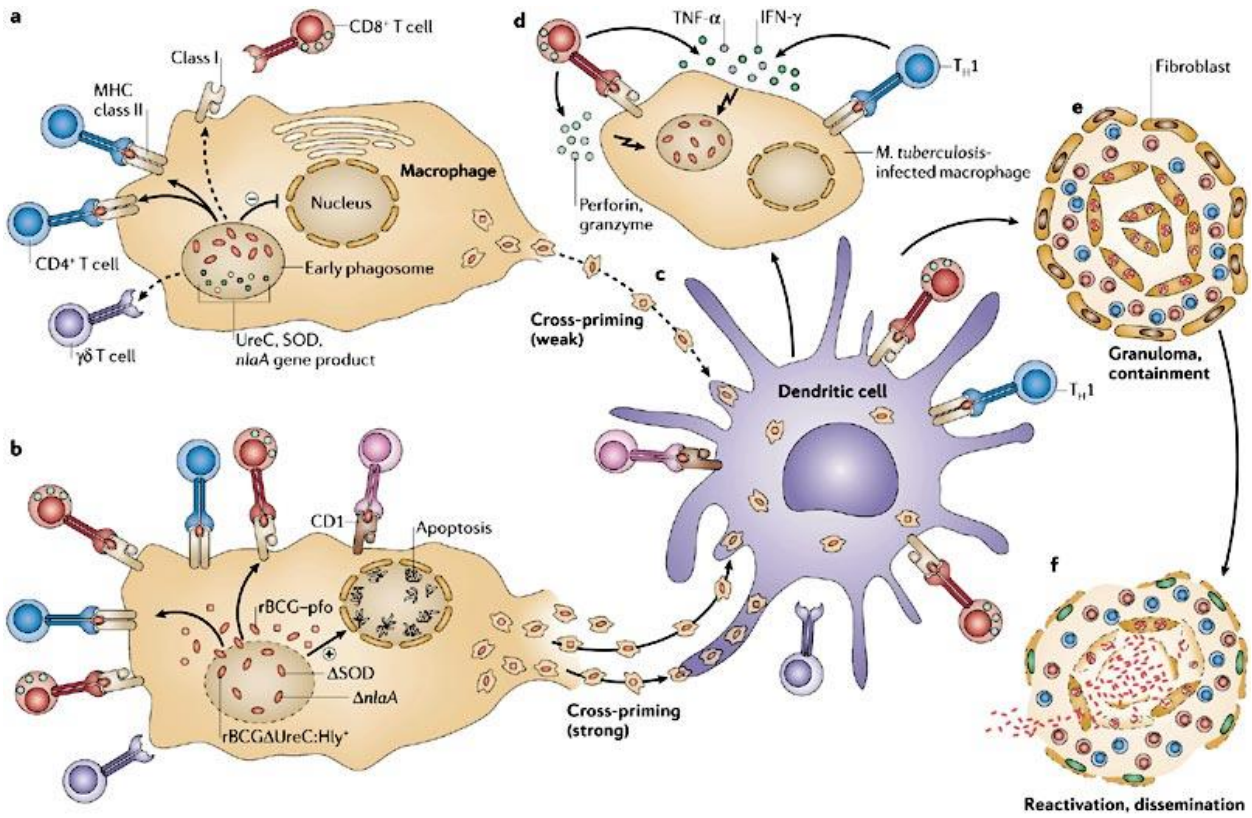

Figure 1.5: **A.** *M. tuberculosis* subverts macrophage phagosome maturation via the release of UreC, which inhibits the acidification of the early phagosome. *M. tuberculosis* also produces factors such as superoxide dismutase (SOD) and the *nlA* gene product, which might inhibit host defences by interfering with host cell apoptosis. **B**. enhancement of MHC class I antigen presentation and $CD8^+$ T-cell responses by modified BCG constructs. **C**. resulting in enhanced $CD8^+$ and $CD4^+$ T-cell responses as well as $\gamma\delta$T-cell and CD1-restricted $\alpha\beta$T-cell activation. **D.** $CD8^+$ T cells produce effector cytokines (IFN-γ and TNF-α) and lyse infected cells through the release of granules or by Fas-mediated lysis, whereas activated $CD4^+$ T_H1 cells produce IFN-γ, which activates macrophages to kill *M. tuberculosis*. **E.** containment by infected macrophages within granulomas leads to a state of mycobacterial dormancy which may persist for decades. **F.** however, *M. tuberculosis* can reactivate when the immune system is weakened, with release of organisms from the granuloma and progression to active clinical disease (Skeiky and Jerald, 2006. *J Immunol*)

Surrounding the central necrotic centre of the granuloma are activated macrophages and a layer of $CD4^+$ and $CD8^+$ T cells that defines a dense cellular wall that restricts the dissemination of *M tuberculosis* (Saunders and Cooper, 2002). The activated macrophages present the mycobacterial antigens to the T-lymphocytes which in turn become activated through the triggering of T-cell receptors to produce different types of chemokines and cytokines (Flynn and Chan, 2001). In immunocompetent *M. tuberculosis*-infected individuals, *M. tuberculosis* granulomas are small, compact and are characterized by the presence of large amounts of IFN-γ -secreting CD4 T-cells, but in immunosuppressed persons *M. tube*rculosis granulomas are large and contain large amounts of activated macrophages with few surrounding lymphocytes (Ulrichs et al., 2005). The main reason for tissue injury and clinical manifestations of pulmonary tuberculosis are the presence of large caseating granulomas and fibrotic scarring due to the inflammation leading to granuloma formation (Daley, 2010).

The precise mechanisms and mediators which promote the formation of the granuloma are not yet fully understood. In response to *M. tuberculosis,* interactions with alveolar macrophages and dendritic cells, there is a discharge of inflammatory cytokines such as IL-12, IL-23, TNF-α and a number of different chemokines such as (C-C) motif ligand 2 (CCL2), CCL5 and (C-X-C) motif ligand 8 (CXCL8). IL-12 production and IL-23 production by the dendritic cells induces the Th1 response, which is essential for the assembly of the granuloma. This flow of inflammatory events is regulated by IFN-γ production and IL-2 by the activated Th1 cells that reach the site of infection (Seder et al., 1993; Oppmann et al., 2000; Cooper, 2009).

Studies done using CD4 T-cell deficient mice have shown that CD4 T-cells are needed for the recruitment of mononuclear cells to the site of infection and also for the protection that is needed for the long term survival of the host (Saunders and Cooper, 2002). However, a common characteristic between murine and human pulmonary TB is the presence of B lymphocyte clusters (Turner et al., 2001; Ulrichs et al., 2004; Tsai et al., 2006). Some studies have demonstrated that B-cell aggregates may serve different purposes in both species since murine macrophages encircle B-cells; however in humans B cell aggregates recruit T-cells that are evenly distributed in clusters (Tsai et al., 2006).

Even though this seems contradictory, it is known that the initial inflammatory response to *M. tuberculosis* is crucial for the formation of the granuloma; however it is also necessary for the long term survival of *M. tuberculosis* within its host. In this setting, pro-inflammatory cytokines reduce the bacterial burden, regulate the activity of other cytokines and chemokines, and generate and maintain the granulomas. When macrophages are infected with *M. tuberculosis* they are induced to produce TNF, IFN-γ, IL-12, RNI and ROI, which are known to be key regulators of the formation and the maintenance of the granuloma structure (Flynn and Chan, 2001; Cooper, 2009). There are many other cytokines which are involved in the formation of the granuloma; however their specific functions are not well understood (Feng et al., 2002).

Granuloma formation is considered to be a means by which the adaptive immune response achieves and maintains latency in *M. tuberculosis* infection (Segovia-Juarez et al., 2004). However, the bacteria have developed strategies which ensure their survival in this setting. *M. tuberculosis* can persist for the lifetime of the host, but as long as the host immune system is effective there is usually no adverse effect of *M. tuberculosis* on the host's health, which proves that the granuloma maintains the balance between the host and the pathogen (Saunders and Britton, 2007).

1.5.5 Effector mechanisms for killing of *M. tuberculosis*

The production of reactive oxygen intermediates (ROI) is a mechanism involved in the intracellular killing of mycobacteria by activated macrophages. Persistent intracellular growth of *M. tuberculosis* may depend on its ability to escape destruction by lysosomal enzymes, ROI and reactive nitrogen intermediates. As mentioned above, when bacteria have been engulfed by macrophages they enter into specialized phagosomes that undergo progressive acidification followed by fusion with lysosomes. However, in the case of *M. tuberculosis*, this pathogen has the ability to delay or inhibit phagolysosome function. In addition, *M. tuberculosis* prevents the maturation of the phagosome, as well as phagosome acidification, blocking the digestive activity of acidic hydrolases. The normal function of the macrophages is to engulf and destroy the organism (Ferrari et al., 1999).

The pathogens make use of a variety of mechanisms so that they may escape the delivery of the lysosomes. Some bacteria such as *Listeria* and *Shigella* spp. escape to the cytosol. In the case of *M. tuberculosis*, it is clear that the bacilli circumvent the hostile environment of the macrophages via modification of the maturation of the phagosomal compartment in order to ensure intracellular survival (Glickman and Jacobs, 2001). The phagosome containing *M. tuberculosis* behaves as though it has been arrested at an early endosomal stage of its maturation (Russell, 2001).

The mycobacteria survive inside the macrophage by inhibiting a variety of mechanisms such as phagolysosome fusion, vacuolar acidification, while resisting killing by the oxygenated metabolites (Basu, 2004). After phagocytosis, the microorganisms are subjected to degradation by intralysosomal acidic hydrolases upon phagolysosome fusion (Ferrari et al., 1999; Raja, 2004). The alteration of phagosomal maturation is said to be associated with the alteration in the protein content of the vacuole, including altered Rab GTPase composition (Raja, 2004). It has also been reported that in mycobacteria the phagosomes containing the living mycobacteria resist fusion with the organelles of the endosomal/ lysosomal system (Ferrari et al., 1999).

The key to the virulence of *M. tuberculosis* is its ability to prevent the incorporation of the ATP/proton pump into the phagosome membrane and to limit phagolysosome fusion (Rohde et al., 2007). Several molecules such as cell wall lipid lipoarabinomannan, trehalose dimycolate and phospholipids have been linked to the modulation of phagosome maturation and blocking phagosome/ lysosome fusion (Fratti et al., 2003; Vergne et al., 2005). Furthermore, bacterial secreted acid phosphatase (Sap) M and the serine/ threonine kinase protein kinase G (PknG) are also considered to be capable of regulating phagosome maturation. SapM is anticipated to function through dephosphorylation of PI3P and PknG via the phosphorylation of unknown host proteins.

In the case of *M. tuberculosis*, it is unclear as to how these enzymes access their respective cytolic substrates (Rohde et al., 2007). The *M. tuberculosis* strains that are defective in achieving full arrest in the maturation of their phagosomes are delivered to compartments with low pH of around pH 5.8 and are unable to enter into the replication phase (Pethe et al., 2004).

1.5.6 Apoptosis of macrophages during *M. tuberculosis* infection

At the first stage of infection by intracellular bacteria, control of proliferation of intracellular bacteria relies on natural resistance that is medicated by macrophages (Rojas, 1999). Other than innate effector mechanisms utilised by macrophages, some studies have suggested that apoptosis of infected macrophages amounts to an alternative strategy that may contribute in a number of ways to host defence (Molloy, 1994; Porcelli and Jacobs Jr, 2008).

In macrophages that are infected with *M. tuberculosis,* apoptosis may be host protective by denying the bacilli their niche cell (Keane et al., 2000; Leemans et al., 2001), while preventing spread by sequestering and retaining the mycobacteria within the apoptotic bodies, which, in turn, are engulfed by recruited phagocytic cells (Molloy, 1994; Porcelli and Jacobs Jr, 2008). Several studies have reported that virulent strains of *M. tuberculosis* such as H37Rv, as well as *M. bovis* wild types, elicit less apoptosis of the infected phagocytic cell when compared to attenuated strains of *M. tuberculosis* such as H37Ra and *M. bovis* BCG, suggesting that inhibition of apoptosis of macrophages is a mycobacterial virulence strategy (Keane et al, 2000; Zhang et al, 2005).

Some studies have emphasized the significance of TNF-α and IL-10 in the inhibition of apoptosis of infected macrophages (Klingler et al, 1997; Rojas, 1999; Sly, 2003). Throughout the course of infection with mycobacteria, TNF-α and IL-10 have opposing effects on various functions of macrophages, including apoptosis elicited by the infection (Rojas, 1999). $CD8^+$ T-cells have also been demonstrated to play a significant role in mediating apoptosis of infected macrophages, resulting in the death of pathogens that use the macrophages as their habitat (Stegelmann, 2005; Bruns et al., 2009).

1.6 ADAPTIVE IMMUNITY

1.6.1 Initiation of adaptive immunity to *M. tuberculosis* infection

Adaptive and innate immunity are closely related. DC and macrophages are the main cells responsible for the innate immune response to mycobacteria. They also play a very significant role in the initiation of the adaptive immune response (van Crevel et al., 2002). The main cells in the adaptive immune response to intracellular bacteria include $CD8^+$ T-cells. When $CD8^+$ T-cells are stimulated, they provide a variety of effector functions, each aimed at clearing or restraining the pathogen (Sud et al., 2006).

The adaptive immune response to *M. tuberculosis* is involved in the development of the characteristic multicellular structures of the granuloma within the lung tissue of the infected individual. Moreover, it is a complex process that involves the spatial and temporal organization and interactions of a number of elements such as bacteria, chemokines, cytokines, adhesion molecules and immune effector cells as mentioned above (Segovia-Juarez et al., 2004). There are at least three processes that are involved in initiation of adaptive immunity: antigen presentation, costimulation, and production of cytokines (van Crevel et al., 2002).

As shown in Figure 1.6, infected macrophages release chemokines and cytokines that attract dendritic cells and blood monocytes. The DC engulfs bacteria, moving thereafter to the closest draining lymph node where they present antigen to the naïve T-cells. This induces differentiation and activation of T-cells, which then migrate to the lung to the site of infection, guided by adhesion molecules and chemokine signals produced by macrophages (Segovia-Juarez et al., 2004).

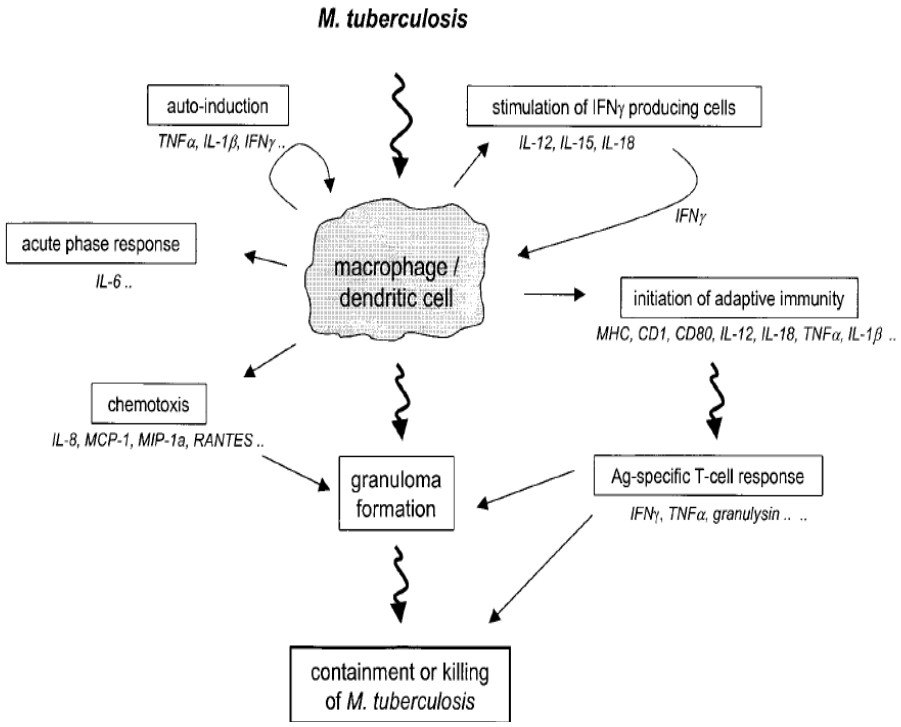

Figure 1.6: Inflammatory response occurring during phagocytosis of *M. tuberculosis* leading to macrophage activation: immune recognition by macrophages and dendritic cells is followed by an inflammatory response with a fundamental role in cytokine production. Anti-inflammatory cytokines are not represented in this diagram (van Crevel et al 2002. *Clin Microbiol Rev*)

1.6.2 The cell-mediated immune response during *M. tuberculosis* infection

Protective immunity to *M. tuberculosis* is primarily dependent on cell-mediated immunity (CMI). In murine models of experimental infection, following 1 week of infection with *M. tuberculosis*, the number of both CD4+ and CD8+ T cells in the regional lymph nodes increases (Feng et al., 1999), and at between 2-4 weeks of infection both CD4 + and CD8+ T-cells migrate towards the lungs and demonstrate an effector/memory phenotype. These findings indicate that activated T cells migrate to the site of infection following interactions with the antigen presenting cell (APC).

1.6.2.1 CD4$^+$ T-cells

M. tuberculosis is primarily a parasite of macrophages, with MHC class II presentation of mycobacterial antigens to CD4+ T cells being a clear result of infection. Apart from having memory and effector functions, CD4$^+$ T-cells are a good source of IFN-γ. IFN-γ is required for effective immunity to *M. tuberculosis* (Salgame, 2005). Even though CD4$^+$ T cells together with CD8 $^+$T cells and other cells, are major producers of IFN-γ, studies carried out in murine models have demonstrated that it is the early production of IFN-γ by the CD4$^+$ T-cells following activation of macrophages that determines the outcome of infection (Caruso et al., 1999; Cooper, 2009).

Studies have shown that depletion of CD4$^+$ T-cells was linked to reactivation of infection in chronically infected mice and resulted in pathological features and death, even though IFN-γ levels had remained high as a result of a strong response from the CD8$^+$ T cells and normal levels of inducible nitric oxide synthase (Scanga et al., 2000). The CD4$^+$ T-cells are also important in controlling infection in the granuloma (Chan and Flynn, 2004; Cooper, 2009).

Studies have reported that CD4$^+$ T cells can also control the intracellular growth of *M. tuberculosis* by a nitric oxide-dependent mechanism that is independent of IFN-γ production (Scanga et al., 2000; Cowley and Elkins, 2003). Thus, CD4$^+$ T-cells, in addition to early production of IFN-γ, seem to have a number of other secondary functions that are critical in the control of the *M. tuberculosis* infection (Ahmad, 2010).

1.6.2.2 CD8 T$^+$ cells

CD8$^+$ T-cells are recruited by TNF-dependent and independent pathways (Sud et al., 2006). These cells are activated by the endogenous human leukocyte antigen (HLA) class I-dependent mechanism of antigen processing and presentation (Weerdenburg et al., 2009).The function of CD8$^+$ T-cells against *M. tuberculosis* infection has been rather controversial, but some of the data supports the role of these cells in protection against this pathogen (Serbina and Flynn, 2001; Lazarevic and Flynn, 2002; Vankayalapati et al., 2004).

Cytotoxic activity of $CD8^+$ T-cells consists of two separate mechanisms. One is apoptosis through the Fas-Ligand pathway, and the other by killing through perforin and granulysin (Stenger et al., 1997). Human $CD8^+$ T-cells may kill intracellular mycobacteria through the release of the antimicrobial polypeptide granulysin (Stenger et al., 1998), which is not present in murine cytotoxic T-cells. Since mice do not have this molecule, this may partially explain why $CD8^+$ T-cells are less prominent in control of infection in murine models of TB (Mogues et al., 2001).

M. tuberculosis-specific $CD8^+$ T-cells are involved in the production of cytokines such as IFN-γ and TNF (Lin et al., 1997; Lalvani, 1998). The mechanisms that control the relative cytokine-producing or cytolytic activities of $CD8^+$ T-cells during infection with *M. tuberculosis* are not yet fully understood. Sud et al. and others have reported that there are different effector functions of $CD8^+$ T-cells; however this depends on the stage of *M. tuberculosis* infection (Lazarevic et al., 2005; Sud et al., 2006).

$CD8^+$ T-cell cytotoxicity accounts for about 80%-90% of killing of infected macrophages, and close to 80% of the granulysin-mediated killing of bacteria within those macrophages. However, this activity is crucial to the control of infection. Sud et al., 2006 demonstrated that Fas-FasL apoptosis of infected macrophages induced by $CD8^+$ T-cells is significant in early infection.

1.6.3 Antigen presentation

Antigen presentation is a vital process that is required for the activation of the T-cell response against invading pathogens. Extracellular pathogens are endocytosed by specialized cells called APC and processed via an antigen presentation pathway leading to activation of $CD4^+$ T-cells (Weerdenburg et al., 2009). This results in the production of cytokines and antibodies to fight the pathogen. However, intracellular pathogens such as *M. tuberculosis* are processed via separate antigen presentation pathways that may activate cytotoxic $CD8^+$ T-cells to destroy the infected cell and the pathogen in them (Weerdenburg et al., 2009).

Macrophages and dendritic cells are the major APC. The first step in antigen presentation is when an extracellular pathogen is endocytosed by APC and is enclosed in a phagosome, which thereafter acidifies and fuses with the lysosome to form the phagolysosome as mentioned earlier. During the maturation of the phagolysosome, bacterial proteins are broken down into peptides and then transported to endocytic vesicles called MHC-II compartments (MIIC) and subsequently loaded onto MHC class II antigen-presenting molecules in the vesicles of the APC (Bryant et al., 2002).

The vesicles that contain peptide-charged MHC II molecules fuse with the plasma membrane. At the surface of these cells, MHC II molecules present these antigens to the CD4 T-cells, which are required in the immune response against infection with *M. tuberculosis* and other mycobacteria (Weerdenburg et al., 2009). Additionally, cross-presentation of peptides obtained from particulate antigens, may occur through a putative phagosome-to-cytosol mechanism, thereby recruiting the conventional MHC-I pathway (Kovacsovics-Bankowski and Rock, 1995). On the other hand, this may also occur via fusion and fission of phagosomes with endoplasmic reticulum-derived vesicles containing newly synthesized MHC I molecules (Gagnon et al., 2002; Guermonprez et al., 2003; Roy et al., 2006).

MHC class I molecules which are expressed on all nucleated cells are able to present mycobacterial-derived peptides to antigen-specific $CD8^+$ T-cells. This mechanism permits the presentation of cytosolic antigens, which can be significant as some of the mycobacteria may somehow escape the phagosome. Thirdly, non-polymorphic MHC class I molecules such as type I CD1 molecules, which are expressed by dendritic cells and macrophages, are able to present mycobacterial lipoproteins to CD1-restricted T cells (van Crevel et al., 2002).

This latter system of antigen presentation allows the activation of a larger fraction of T cells at an earlier stage of infection, before antigen -specific immunity has developed. This pathway may also engage non-polymorphic MHC class Ib molecules (Lewinsohn et al., 1998). The ability of an individual to respond to mycobacterial peptide epitopes therefore depends on the expression of class I and II MHC alleles, while alternative mechanisms for the early recognition of

mycobacterial lipids/lipoproteins also exist. Some human HLA allelic variants have been linked to susceptibility for development of TB (Goldfeld et al., 1998; Ravikumar et al., 1999). The expression of antigen-presenting molecules is a dynamic process, which is regulated by cytokines. Whereas proinflammatory cytokines, primarily IFN-γ stimulate the expression of MHC, anti-inflammatory cytokines inhibit their expression (Pancholi et al., 1993; Gercken et al., 1994). Mycobacterial antigen presentation to the immune system involves internalization, intracellular transport and proteolytic processing of mycobacterial antigens in both macrophages and dendritic cells (Kaufmann, 2006).

1.6.4 Co-stimulation

Antigen presentation can only lead to stimulation of T-cells in the presence of particular co-stimulatory signals. Co-stimulatory signals are induced by CD80 and CD86 on the APC. These molecules are expressed on dendritic cells and macrophages. They bind to CD28 and to cytotoxic lymphocyte- associated antigen (CTLA) -4 on T-cells. *M. tuberculosis* infection of dendritic cells stimulates expression of CD80, CD86 and intercellular adhesion molecules (ICAM) -1 (Henderson et al., 1997). In the absence of appropriate co-stimulatory signals, presentation of antigen may lead to increased apoptosis of T-cells (Hirsch et al., 2001)

1.6.5 Production of cytokines

Cytokines are a large group of signaling proteins. They are produced following the activation of the cells of the innate and adaptive immune systems, as well as by other cell types, and therefore act as humoral regulators which transform functions of the individual cells (Dinarello et al., 1990; Meager, 1998). Cytokines are known as key players in the host response to infection, immunological disorders and tissue injury, in an effort of the organism to defeat the insult and restore homeostasis (Bluethmann et al., 1994). They are also known as positive and negative regulators of cell replication, cell death, cell survival, differentiation and cell transformation (de Kretser et al., 1998; Kakeya et al., 2000). The effect of cytokines varies depending on the target

cell (Opal and DePalo, 2000). In healthy tissues, cytokines control the entry of the cells into the cell cycle (Kalvakolanu, 2000). Cytokines can act locally in an autocrine, juxcrine or paracrine manner, and any action is initiated through specific receptors expressed primarily on the target cell membrane (Miyajima et a1., 1992). The mechanisms by which cytokines mediate production of a signal through receptors are not fully understood. However, it is possible that during binding to the extracellular portion of the receptor the cytokines induce a conformational change of multiple receptors and as a result an activation event in the intracellular domain itself or in receptor-associated elements such that signal transduction elicits subsequent intracellular events mediated by transcription factors which promote expression of target genes (Foster, 2001).

Cytokines act on receptors that can be grouped into four families with genetic, structural and functional similarities. Some cytokine receptors are members of the immunoglobulin superfamily, some are members of the hematopoietic receptor family, others are members of the tumor necrosis factor family of receptors, and others are members of the chemokine receptor family of receptors. Cytokines are not produced by cells organized in special glands. The majority of the cytokines are not stored in the cell, their expression is strictly regulated, and they are only produced by activated cells in response to stimulation signals.

Cytokine families include inflammatory cytokines, growth factors and chemokines. The cytokines play a very crucial role in chemically-induced tissue damage repair, in the development of cancer and also in the progression and controlling of the replication of cells and programmed cell death (apoptosis), and in the modulation of immune reactions such as sensitization (Foster, 2001). Cytokines induce a number of biological activities in multiple cell types and different cytokines have been shown to have overlapping activities (Sun et al., 1999). The majority of cytokines demonstrate stimulatory or inhibitory activities and may synergise or oppose the actions of other cytokines and hormones (Matsumoto and Kanmatsuse, 2000).

The important feature of cytokine action is that a single cytokine may elicit one type of reaction under one condition, while inducing the totally opposite reaction under a different set of circumstances (Sun et al., 2000). The production of soluble cytokine receptors and anti-

inflammatory cytokines can assist in regulation of the inflammatory response during *M. tuberculosis* infection. An uncontrolled proinflammatory response can lead to extreme tissue damage, while predominance of an anti-inflammatory effect can support the outgrowth of *M. tuberculosis*. *M. tuberculosis* can evade protective immune mechanism of the host by selective induction of anti-inflammatory cytokines (van Crevel et al., 2002)

1.6.6 Cytokine production driven by *M. tuberculosis*

When *M. tuberculosis* has been phagocytosed, it leads to cell activation and the production of cytokines in a complex process of regulation and cross-regulation. This network of cytokines plays a part in the inflammatory response and also the outcome of infection due to mycobacteria.The cytokines that are mostly involved in *M. tuberculosis* infection are IL-1α, IL-1β, IL-2, IL-6, IL-12, IL-15, IL-18, IFN-γ and TNF-α, which are the proinflammatory cytokines. On the other hand, the anti-inflammatory cytokines: IL-4, IL-10 and transforming growth factor (TGF) -β antagonize the response that is initiated by the proinflammatory cytokines in order to control tissue damage (Giacomini, 2001; van Crevel et al., 2002). Furthermore, *M. tuberculosis* infection also promotes the release of chemokines such as IL-8, monocyte chemoattractant protein (MCP-1), MCP-3, macrophage inflammatory protein (MIP-1) and regulated on activation, normal T expressed and secreted (RANTES) (Raja, 2004: van Crevel et al., 2002).

1.6.6 Proinflammatory cytokines

1.6.6.1 Tumour necrosis factor-alpha

TNF-α is the prototype proinflammatory cytokine. It is induced when monocytes, macrophages and dendritic cells are stimulated by mycobacteria or mycobacterial products and is produced primarily as a type II transmembrane protein (Senaldi et al., 1996; Wajant et al., 2003). It is a strong modulator of an early inflammatory response to a number of physical, immunological and

infectious stimuli. TNF-α plays a significant role in the formation of granulomata (Senaldi et al., 1996), induces macrophage activation, and has immunoregulatory properties (Tsenova et al., 1999; Orme and Cooper, 1999). After phagocytosis of *M. tuberculosis* by macrophages, the ability of the phagolysosomal compartment to acidify depends on TNF, while maturation of the phagosome is inhibited by the TNF blockers. TNF blockers also mediate the killing of T-cells and monocytes by apoptosis, as well as by complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC), at the same time allowing expansion of immunosuppressive regulatory T-cells (Harris and Keane, 2010).

In murine models, TNF-α was found to contain the latent infection in the granuloma (Mohan et al., 2001), while paradoxically the clinical decline early in treatment is associated with a selective increase of TNF-α in plasma (Bekker et al., 1998), and rapid recovery is associated with a rapid decrease of TNF-α in plasma (Hsieh et al., 1999). In clinical studies, TNF blockers have been associated with progression of latent TB to TB disease (Keane et al., 2001; Nunez Martinez et al., 2001).

Other studies have shown that in animal models in which TNF has been neutralised, the animals were more prone to primary TB following infection with *M. tuberculosis* (Bean et al., 1999). TNF-α may support anti-TB immunity via the secretion of chemokines, upregulation of adhesion molecules and the induction of macrophage apoptosis (Lopez -Ramirez et al., 1994; Keane et al., 1997; Roach et al., 2002). TNF-α is also required for the induction of apoptosis in response to infection with *M. tuberculosis*.

1.6.6.2 Interleukin -1

IL-1 is the prototype of all pro-inflammatory cytokines. It induces the expression of a number of genes and the synthesis of variety of proteins that in turn induce acute and chronic inflammatory responses. There are two forms of IL-1 that are distinct gene products, IL-1α and IL-1β; they recognize the same cell surface receptors and share various biological activities. The effects of IL-1 are not restricted to leukocytes, but are manifested in nearly every tissue.

IL-1 belongs to a group of cytokines with overlapping biological properties, which includes TNF and IL-6. IL-1, can stimulate both T and B-lymphocytes, augment cell proliferation and also initiate or suppress gene expression of several proteins (Dinarello, 1991). IL-1β is the second most prominent proinflammatory cytokine involved in the host response to *M. tuberculosis*. Just like TNF-α, IL-1β is mainly produced by monocytes, macrophages and dendritic cells (Dahl et al., 1996). In *M. tuberculosis*-infected individuals, IL-1β is over-expressed at the site of disease (Law et al., 1996).

1.6.6.3 Interleukin-6

IL-6 is produced early during mycobacterial infection and also at the site of infection. It has both proinflammatory and anti-inflammatory properties (van Heyningen et al., 1997; Hoheisel et al., 1998). IL-6 can be damaging during mycobacterial infection as it antagonises the production of TNF- α and IL-1β, and promotes the growth of *M. avium* complex (Schindler et al., 1990).

1.6.6.4 Interleukin-12

IL-12 (IL-12p70) is a heterodimeric cytokine that consists of two subunits: p35 and p40 (Me´ndez-Samperio, 2009). IL-12 is produced by monocytes, macrophages and dendritic cells and its production is essential during *M. tuberculosis* infection. IL-12 promotes Th1 immune responses and enhances cell-mediated immunity against airways infections caused by mycobacteria, viruses, parasites and fungi. IL-12 induces the production of IFN-γ from CD4 T-cells, specifically Th1 effectors, and is important in protection against bacterial infection (Kang and Kim, 2006).

IL-12 is also involved in the formation of the granuloma by promoting the Th1 response and inducing IFN-γ positive CD4 T-cells (Seder et al., 1993). Some studies in mice have shown that neutralization of IL-12 by monoclonal antibodies results in a reduction in granuloma integrity and slowing of the capacity of the mice to control the growth of *M. tuberculosis* (Cooper et al., 1995). Later studies in humans have corroborated the importance of IL-12 in granuloma maintenance, because specific mutations in either the IL-12 p40 or IL-12Rβ1 (IL-12 receptor) genes confirmed reduced levels of IL-12 and IL-23 resulting in reduced Th1 cell responses and decreased IFN-γ in subjects with susceptibility to mycobacterial infections (Remus et al., 2001; Fieschi and Casanova, 2003)

In a study done by Metzger et al., 1997, they showed that IL-12 induces immunoglobulin isotype switching by acting on B cells both directly and indirectly through T cell-derived IFN-γ. As a result, there was enhanced production of IgG2a antibodies and inhibition of IgE and IgG1 synthesis. In patients with TB, IL-12 has been found in lung infiltrates, in pleural, in granulomas and in lymphadenitis. At the site of infection there is an increase in the expression of IL-12 receptors on T-cells. TB patients that have mutations in genes encoding IL-12p40 and IL-12R as mentioned above have displayed a reduced capacity to produce IFN-γ (De Jong et al., 1998; Ottenhoff et al., 1998).

1.6.6.5 Interleukin-18

In addition to IL-12, there is another cytokine that is of importance in the IFN-γ axis and that is IL-18. IL-18 is a novel pro-inflammatory cytokine that shares many characteristics with IL-1 (Dinarello et al., 1998). IL-18 was first discovered as an IFN-γ-inducing factor, synergistic with IL-12 (O'Neill et al., 1998). It has also been reported that IL-18 stimulates the production of other proinflammatory cytokines, chemokines and transcription factors (Puren et al., 1998; Netea et al., 2000). The *M. tuberculosis*-associated production of IL-18 by peripheral blood mononuclear cells is decreased in TB patients and this reduction may be responsible for reduced IFN-γ production (Vankayalapati et al., 2000).

1.6.6.6 Interferon- gamma

IFN-γ is also important in the formation of granulomas (Flynn et al., 1993). Studies on IFN-γ deficient mice have shown that they were incapable of developing granulomas following aerosol infection, with their lungs being infiltrated with neutrophils resulting in cellular necrosis instead of granuloma formation (Cooper at al., 1993; Pearl et al., 2001). As already mentioned above, the protective role of IFN-γ during infection with *M. tuberculosis* is well-known, mainly in the context of antigen-specific T-cell immunity and macrophage activation (Flynn et al., 1993).

Mycobacterial antigen-specific IFN-γ production *in vitro* may be used as a surrogate marker of infection with *M. tuberculosis*. In general, naïve tuberculin skin test (TST)-negative persons do not show purified protein derivative (PPD) - stimulated IFN-γ production *in vitro*. Nevertheless, in both PPD-positive and PPD-negative persons, monocytes infected with *M. tuberculosis* stimulate lymphocytes for the production of IFN-γ *in vitro* probably via IL-12 production (van Crevel et al., 2002).

1.6.6.7 Granulocyte/ Macrophage-Colony Stimulating Factor

Granulocyte/ macrophage-colony stimulating factor (GM-CSF) is a cytokine that regulates the proliferation, differentiation and also the function of macrophages and granulocytes and is derived from Th1 cells (Kedzierska et al., 2000).

1.6.7 Anti-inflammatory cytokines

Anti-inflammatory cytokines antagonize the proinflammatory response that is initiated by *M. tuberculosis*. TNF-α receptors I and II are soluble cytokine receptors; their role is to prevent the binding of cytokines to cellular receptors, blocking further signalling. There are at least three

anti-inflammatory cytokines that may inhibit the production or effect of pro-inflammatory cytokines namely: IL-4, IL-10 and TGF-β (van Crevel et al., 2002).

1.6.7.1 Interleukin-4

Harmful properties of IL-4 in intracellular infections such as TB have been ascribed to its suppression of IFN-γ production and activation of macrophages. In mice that were infected with *M. tuberculosis*, reactivation of latent infection and progressive disease are both associated with increased production of IL-4 (Hernandez-Pando et al., 1996; Howard, 1999). Similarly, over-expression of IL-4 was found to intensify tissue damage in experimental infection.

On the contrary, inhibition of IL-4 production did not seem to promote cellular immunity. However, IL-4 gene knockout mice showed normal instead of increased susceptibility to mycobacteria which suggested that IL-4 production may be a consequence rather than the cause of the development of TB (Erb et al., 1998; North, 1998).
Sanchez et al., 1994 and Schauf et al., 1993 have reported increased production of IL-4 in TB patients with cavities. However, their findings differ from those of Barnes et al., 1993 and Hernandez et al., 1994 and further research needs to be done to determine whether IL-4 causes or attenuates disease activity in human TB. Therefore the role of IL-4 in TB susceptibility is not yet fully understood.

1.6.7.2 Interleukin-10

IL-10 is an anti-inflammatory cytokine that is produced by macrophages after phagocytosis of *M. tuberculosis* and after binding of mycobacterial LAM (Dahl et al., 1996). T-lymphocytes, including *M. tuberculosis*-reactive T-cells, are also capable of producing IL-10 (Gerosa et al., 1999; Boussiotis et al., 2000). In TB patients, the expression of IL-10 mRNA has been shown in

circulating mononuclear cells, at the site of disease in pleural fluid, and also in alveolar lavage fluid (Barnes et al., 1993; Gerosa et al., 1999). IL-10 opposes the pro-inflammatory cytokine response by down-regulating the production of IFN-γ, TNF-α and IL-12 (Gong et al., 1996; Fulton et al., 1998).

IL-10 may block the protective immune response by inhibiting the production of IL-12. Since the pro-inflammatory cytokines are essential for their protection against TB, IL-10 would be expected to interfere with the host immune defence against *M tuberculosis*. Indeed, studies using IL-10 transgenic mice infected with *M. tuberculosis* showed that they developed a larger mycobacterial burden (Murray et al., 1997). Conversely, IL-10-deficient mice showed a lower bacterial burden early in *M. tuberculosis* infection (Murray et al., 1999). Studies done in humans have shown that the production of IL-10 was higher in anergic patients, both before and after successful treatment, which suggested that *M. tuberculosis*-induced IL-10 production suppresses an effective immune response (Boussiotis et al., 2000).

1.6.7.3 Transforming Growth Factor-beta

TGF-β is also an anti-inflammatory cytokine that antagonises protective immunity in TB. Mycobacterial products elicit the production of TGF-β by phagocytes. LAM from virulent strains of mycobacteria selectively induces the production of TGF-β. Similar to IL-10, TGF-β is over-produced during TB and expressed at the site of disease. During *M. tuberculosis* infection, cell-mediated immunity is suppressed by TGF-β. It also inhibits proliferation of T cells and production of IFN-γ. In macrophages, TGF-β antagonizes antigen presentation, proinflammatory cytokine production, and cellular activation. In addition, TGF-β can be involved in tissue damage and fibrosis during TB, while promoting production and deposition of macrophage-derived collagenases and damage to the collagen matrix (van Crevel et al., 2002).

1.6.8 Chemokines

The chemotactic cytokines are called chemokines. They are members of a large superfamily of low molecular weight proteins that are structurally and functionally related. Chemokines play a role in recruitment and activation of leukocytes and other cells at the inflammation site (Xu et al., 1995). There are more than 40 chemokines and 16 chemokine receptors that have been identified (Zlotnik and Yoshie, 2000, Algood et al., 2003). Chemokines include molecules such as RANTES (regulated on activation normal T expressed and secreted), MCP and lymphactin (Meager, 1998; Algood et al., 2003).

Chemokines are involved in the recruitment and the activation of the immunocompetent and inflammatory cells to the site of cell damage.Each member of the chemokine family displays four conserved cysteine residues in either of two patterns. In the C-X-C family of chemokines, their genes are found on chromosome 4, the first two conserved cysteines are separated by one amino acid, whereas in the C-C family their genes are found on chromosome 17, and the first two conserved cysteines are adjacent (Xu et al., 1995).

A number of chemokines have been examined in TB. Some of the studies have concentrated on the role of IL-8, which attracts neutrophils, and possibly monocytes. Chemokines assist in cell migration and in the subsequent formation of the granuloma. They are also essential, but undefined contributors to the inflammatory response to *M. tuberculosis* infection. The main function of chemokines is to direct cell migration and immune homeostasis in the host (Mendez-Samperio, 2008).

1.6.8.1 Interleukin-8

During phagocytosis of *M. tuberculosis* or stimulation with LAM, macrophages produce IL-8 (Zhang et al., 1995; Juffermans, 1999). Pulmonary epithelial cells also produce IL-8 in reaction to *M. tuberculosis*. IL-8 is a member of C-X-C family and is chemotactic for neutrophils and

monocytes (Xu et al., 1995). IL-8 is induced by infection of alveolar macrophages with *M. tuberculosis* and is found in the lavage fluid of patients with TB (Zhang et al., 1995). IL-8 is also found in lymph nodes and blood of TB patients (van Crevel et al., 2002).

1.6.8.2 Monocyte Chemoattractant Protein-1

MCP-1 is the second major chemokine produced by monocytes and macrophages. It is a member of the C-C subfamily of chemokines with chemoattractant activity for monocytes, T cells, mast cells and basophils (Leonard and Yoshmura, 1990; Taub and Oppenheim, 1994). Some studies have suggested that MCP-1 is a pro-inflammatory chemokine not only because of its potent chemotactic properties, but also because of its capacity to mediate degranulation of mast cells and enhancement of β integrin expression on inflammatory cells (Alam et al., 1992, Vaddi and Newton, 1994). In murine models, MCP-1 gene knockout inhibited the formation of the granuloma (Lu et al., 1998).

Previous studies have shown that the degree of MCP-1 participation varied according to immune status and was also influenced by T cells. MCP-1 contributes more to type 2 than type 1 granuloma formation. Although MCP-1 is not purely a Th2-related cytokine, it appears to be used predominantly during type 2 cytokine-mediated inflammation. Macrophages isolated from type-2 granulomas had an enhanced capacity to produce MCP-1 compared to those from type-1 granulomas. Chensue et al., 1996 also demonstrated that MPC-1 was more than just a chemoattractant. In their study they linked MCP-1 to the Th2 response by showing that IL-4 promoted MCP-1production by type 2 granuloma macrophages.

1.6.8.3 Regulated on Activation, Normal T Expressed and Secreted

A third chemokine is RANTES. It is produced by a wide variety of cells and shows promiscuous binding to multiple chemokine receptors. RANTES is a member of the C-C family which is generally chemotactic for monocytes and lymphocytes. RANTES is produced by a diversity of cells, compatible with promiscuous binding to multiple chemokine receptors. In murine models, expression of RANTES is associated with development of *M. bovis*-induced pulmonary granulomas (Chensue et al., 1999). In TB patients, RANTES has been detected in alveolar lavage fluid (Kurashima, 1997).

1.7 MICROBIAL POTASSIUM TRANSPORTER SYSTEMS

K^+ is essential for living cells to maintain ionic homeostasis in response to environmental changes. In most bacteria such as *Escherichia coli*, K^+ is the major monovalent cellular cation. In *E. coli*, the intracellular concentrations of K^+ are maintained at a rather constant level of about 300 to 500mM and even up to 1M when the bacteria are faced with hyperosmotic media (Epstein and Schultz, 1965; Epstein, 2003).

The accumulation of K^+ in the cytosol has been ascribed to the fact that this cation is compatible with water and protein structure even at high concentrations, which may be due to its ionic radius, the magnitude of its electric field at its surface, and the structure of its hydration shell. In addition to electroneutralization, this ion also plays a significant role in a number of physiological functions (Epstein and Schultz, 1965).

There are at least two families that contribute to K^+ uptake, the Trk/Ktr/HKT and the HAK/Kup/KT systems. These families have in common that they are represented in bacteria, fungi and plants and also have close homologues in animals. These two families differ though in their relative representations. For instance the Trk/Ktr/HKT family seems to be the most ubiquitous in plants, fungi and bacteria (Schachtman and Schroeder, 1994; Uozumi et al., 2000).

These families also differ in terms of functional diversity. The Trk/Ktr/HKT family was initially thought to comprise of H^+ and K^+ symporter genes, but has now been revealed to encode a larger variety of transporters such as sodium (Na^+) and K^+ symporters, and even transporters permeable to Na^+ only (Uozumi et al., 2000), whereas the HAK/Kup/KT gene family encodes K^+ transporters (Rodrıguez-Navarro, 2000). The first member of the Trk/Ktr/HKT system to be identified at molecular level was Sc Trk 1 from *Saccharomyces cerevisiae* (Ramos et al., 1985).

Homologues were further cloned from other fungi, bacteria and even plants (Schlosser et al., 1991; Schachtman and Schroeder, 1994). They have been generically named Trk in fungi, while in bacteria they have been classified into two distinct groups namely, Trk and Ktr (Nakamura et al., 1998; Holtmann et al., 2003). In fungi, there are at least two families of K^+ transporters that are responsible for the uptake of the cation. One of these families is the Trk transporters. These transporters are encoded by *trk1 and trk-2* genes (Huson et al., 2007).

Despite the large diversity of habitats in the prokaryotes kingdom, the universality of the use of K^+ as an endocellular cation together with the quite limited number of K^+ transport systems are striking. The membrane proteins that have been identified to play a major role in transportation of K^+ are the Trk, Ktr and Kup transporters, Kdp pumps (P-type ATPase, mediating low-rate, high affinity K^+ uptake), K^+ channels, and K^+ efflux systems, including KefB and KefC (Epstein, 2003). The representation of these different types of transporters differs from species to species. Free-living archaea and bacteria usually have Trk (e.g. *E. coli*) or Ktr (e.g. Synechocystis spp.) or even both (e.g. *Vibrio alginolyticus*) and sometimes Kup or Kdp homologues in addition to or instead of the Trk or Ktr systems. Trk transporters are believed to use H^+. In *E. coli*, three types of K^+ uptake systems are displayed, Trk, Kdp and Kup (Kuo et al., 2005).

ATPase Kdp is the high-affinity K^+ uptake system which accumulates K^+ at low external K^+ concentrations; however its expression is repressed in K^+-replete cells. During normal turgor pressure *E. coli* takes up K^+ though two constitutively expressed systems, Trk (Km for K^+ uptake of 0.9 to 1.5mM) and Kup (Km for K^+ uptake of 0.37 mM). Trk has an equivalent to 100 fold higher *V*max when compared to Kup, which implies that Trk constitutes the major K^+ uptake

system under normal growth conditions. In addition, Trk relative to Kup has a relatively high specificity for K^+ (Epstein, 2003). Data supporting the hypothesis that Trk systems can mediate H^+ /K^+ symport have mainly been obtained in *E. coli* (Epstein, 2003). The main argument is that K^+ is sensitive to pH and to effectors of the proton motive force. There are at least two *trk*-genes that have been characterized in *S. cerevisiae*, namely the *Sc trk 1* and *Sc trk* 2 (Ramos et al., 1985). These two genes share 55% homology, mostly in the hydrophobic transmembrane segments. Sc Trk1 knockout mutant strains display a severe decrease in K^+ influx, retaining only the low affinity K^+ uptake activity, and as a result they are unable to grow on media with K^+ concentrations lower than 1mM.

1.7.1 The major potassium transporters of *M. tuberculosis*

M. tuberculosis, as described above, is an intracellular parasite which infects macrophages. *M. tuberculosis* uses a number of mechanisms that enable it to escape host immune defences. For this reason *M. tuberculosis* expresses a variety of genes which are crucial for adaptation and survival. Environmental factors such as concentrations of metal ions within the phagosome may influence the expression of these genes. These ions include chlorine (Cl), calcium (Ca^{2+}), potassium (K^+), manganese (Mn^{2+}), copper (Cu^{2+}), and zinc (Zn) (Wagner et al., 2005). These ions are therefore of importance in the adaptation of the organism for intracellular survival (Agranoff and Krishna, 2004).

In both prokaryotes and eukaryotes, K^+ is the most concentrated monovalent cation, reaching concentrations, as mentioned above, of between 0.1 and 1M in bacteria and close to 140mM in eukaryotic cells, in comparison to an extracellular concentration of 5mM (Steyn et al., 2003). This high level of K^+ is essential for maintenance of turgor pressure, enzyme activation, cytoplasmic pH regulation, response to stress, and gene expression (Booth, 1985; Csonka and Hanson, 1991). Intracellular bacteria require metal cations for their defense against killing by macrophages; they use these ions in the synthesis and functioning of the anti-oxidative enzymes, superoxide dismutase and catalase (Agranoff and Krishna, 2004). Even though the role of K^+

transporters in the pathogenesis and persistence of *M. tuberculosis* is not yet fully understood, the major structural differences between the K^+ transporters of *M. tuberculosis* and those of eukaryotic cells clearly emphasize the potential of these microbial cation transporters as novel targets for drugs and vaccine development (Cholo et al., 2008).

1.7.2 The protein structures of the *M. tuberculosis* potassium transporters

Most bacteria use a variety of K^+ transporters in order to maintain intracellular concentrations of K^+, which emphasizes the importance of these cations for bacterial growth. However, these differ between bacterial species. As mentioned above, *E. coli* uses three K^+ uptake systems: the Trk, Kup and Kdp transporters (Bossemeyer et al., 1989), while streptococcal species utilize the Ktrl and Ktrll systems (Kakinuma 1998). There are at least two known K^+ uptake systems that are utilized by *M. tuberculosis.* These are the Trk and Kdp systems. The Trk system is a constitutively operative, moderate-to-low affinity system, which consists of two TrkA proteins, CeoB (24kDa) and CeoC (23kDa). These proteins are encoded by the *ceoB* (684bp) and *ceoC* (663bp) genes. The *ceoBC* genes share a 49% homology to each other and are arranged as an operon in position 3009 on the chromosome. The *ceoC* gene overlaps the *ceoB* gene by one nucleotide (Cole, 1998).

The *M. tuberculosis* deduced TrkA protein sequence shares some degree of homology with those of other bacterial genera. CeoB shares about 52% and 25% amino acid identity with the TrkA of *Streptomyces coelicolor* (*S. coelicolor*) and *E. coli*. CeoC has about 24% amino acid identity to the TrkA protein of both strains. The *M. tuberculosis* TrkA proteins possess the NAD^+ binding motif which is also found in the TrkA protein of other bacterial genera such as the Gram-negative bacteria *S. coelicolor* and *Azorhizobium caulinodans*, as well as archaebacteria (Chen and Bishal, 1998; Schlösser et al., 1993; Cole, 1998). There is, however, a second K^+ uptake system utilized by *M. tuberculosis* called the Kdp. This system is an inducible, high-affinity, two components P-type, ATP-driven K^+ transporter. The Kdp consists of six proteins namely: KdpA, KdpB, KdpC, KdpD, KdpE and KdpF.

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These proteins are encoded by genes that are found at position 1148.427 on the mycobacterial chromosome and are arranged as KdpDE and KdpFABC operons. These are separated by a region of about 234bp between kdpF and kdpD and are transcribed in the opposite direction. The *kdp* genes of *M. tuberculosis* share some degree of sequence homology with the corresponding genes of *E.coli* (Cole, 1998). Nevertheless, the *E. coli kdpFABC* and *kdpDE* operons are sequentially transcribed with *kdpDE* flanking the *kdpC* at the 3' end, which is different from *M. tuberculosis* (Walderhaug et al., 1992).

1.7.3 Role of the *M. tuberculosis* potassium transporters during intracellular survival

The properties of the Trk system of *M. tuberculosis* have been partially characterised. The Trk system functions during the logarithmic stage of growth in media with a high K^+, concentration, has a low affinity for K^+ ,and is dispensable for *in vitro* growth if the Kdp system is intact (Sassett et al., 2003; Cholo et al., 2006). The Kdp system of *M. tuberculosis,* on the other hand, is repressed during the logarithmic phase of growth *in vitro* when the K^+ concentration is low as is the case with other bacteria. The Kdp system serves as a back-up when the osmolarity is low (Steyn et al., 2003). This system is expressed when the Trk system is absent or inactive, or when the concentration of K^+ is low (Cholo et al., 2006). For the Kdp system to be activated there must be activation by stimuli such as ionic and non-ionic solutes, pH, low concentration of K^+, or low growth temperature (Gowrishankar, 1985; Malli and Epstein, 1998, Steyn et al., 2003).

CHAPTER 2:

2.1 HYPOTHESIS

The Trk system of *M. tuberculosis* promotes intracellular survival and replication of the organism in macrophages, possibly by subverting vacuolar acidification and/or by modulating the cytokine environment, favouring development of the M2 phenotype.

2.2 AIM

To determine the role played by the Trk system of *M. tuberculosis* in intracellular survival during infection of macrophages, as well as in modulating macrophage cytokine production.

2.3 OBJECTIVES

- To establish the effects of knockout of the genes encoding the Trk system on the intracellular survival of *M. tuberculosis* by comparing the Wild type (WT) and *trk*-gene knockout strains with respect to their intracellular survival during macrophage infection using a procedure based on differential lysis of macrophages and enumeration of viable, intracellular bacilli in the lysates.

- To document the effects of infection of cultured human monocytes/macrophages with the WT and *trk*-gene knockout strains of *M. tuberculosis* on the cytokine secretion profiles of these cells.

CHAPTER 3

3.1 MATERIALS AND METHODS

3.1.1 MATERIALS

3.1.1.1 Chemicals and reagents

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (Germany). The cytokine kit was purchased from Bio-Rad (Switzerland).

3.1.1.2 Mycobacterial strains

In this study, two *M. tuberculosis* strains, the WT (H37Rv) and the *trk*-gene knockout, a strain that has the Trk system selectively inactivated, were used. Both of these strains were obtained from the Medical Research Council Unit for Inflammation and Immunity, Department of Immunology, Faculty of Health Sciences, University of Pretoria and Tshwane Academic Division of the National Health Laboratory Service, Pretoria, South Africa. The *trk*-gene knockout mutant was constructed using homologous recombination procedure and the details of its construction are published in Cholo et al., 2006.

3.1.1.3 Growth media

The mycobacterial strains were grown on 7H10 agar medium, which was prepared according to the manufacturer's instructions (Difco). 7H10 medium was supplemented with 10% Oleic Acid, Dextrose, Catalase (OADC) enrichment (Difco) and 0.5% glycerol.

3.1.1.4 Blood donor participants

Permission to conduct the study was granted by the Research Ethics Committee of the faculty of Health Sciences University of Pretoria. The purpose of the study was explained to the volunteers and a written consent form was completed by each volunteer prior to enrolment of the study (PROTOCOL NO: S203/2010)

The blood donors were 6 healthy adult human volunteers aged between 18 and 50 years. They were all non-smokers. Their health status was assessed with the assistance of a questionnaire. The details of the consent form and questionnaire are in Appendix A.

3.1.2 METHODS

3.1.2.1 Isolation of monocytes

3.1.2.1.1 Isolation of the mononuclear leukocytes from the blood

Firstly, mononuclear leucocytes (MNL) were isolated from heparinized blood using a standard barrier centrifugation procedure. Briefly, the blood was overlaid onto 15 ml of Histopaque-1077 in a sterile 50 ml test tube, and the tubes centrifuged at 1800 rpm for 25 min at 22 ºC to separate the various cellular elements of blood based on differential density. Following centrifugation, the MNL layer was aseptically decanted into separate sterile 50 ml tubes, diluted 1/5 with sterile phosphate-buffered saline (PBS, 0.15M, pH 7.4), and the tubes centrifuged at 1200 rpm for 10 min at 4 ºC. This centrifugation step resulted in pelleting of MNL, while contaminating platelets remained in the supernatant fluid, which was discarded. The cell pellets, which consisted almost entirely of MNL, contaminating erythrocytes, and a very small percentage of granulocytes, was vortexed and the cells were resuspended in 20 ml of sterile, ice-cold, 0.83% ammonium chloride (NH_4Cl) and held on ice for 10 min. This hypotonic lysis step results in removal of contaminating erythrocytes. The tubes were then centrifuged at 1200 rpm for 10 min at 4 ºC and the cell pellets resuspended in sterile Hanks' balanced salt solution (HBSS, indicator-free, containing 1.25 mM calcium chloride ($CaCl_2$), pH 7.4, Highveld Biological, Johannesburg).

3.1.2.1.2 Enumeration of total cell counts and leukocytes sub-populations

Total numbers of MNL, as well as those of contaminating granulocytes, were enumerated microscopically using a light microscope. Although useful, this procedure does not distinguish between the various MNL sub-populations (monocytes, B cells, T cells, NK cells). To achieve this distinction, the cell preparation was also analyzed flow cytometrically using a Beckman Coulter FC500 Flow Cytometer using the following fluorochrome-labelled monoclonal antibodies: CD3 (FITC), CD14 (PE), CD15 (FITC) and CD19 (PE) for analysis and enumeration of total T cells, monocytes, granulocytes and B cells, respectively (all monoclonals were from Beckman Coulter).

3.1.2.2 Separation and maturation of monocytes

Differential adherence to plastic, together with exposure to the growth factors IL-3 and GM-CSF was used to separate monocytes from other types of MNL and to promote their differentiation. MNL (30 ml of a 3 x 10^7 MNL/ml suspension in HBSS) were seeded onto a sterile 75 cm^3 tissue culture flask and incubated for 2 hours at 37 ºC in an atmosphere of 5% CO_2 to promote adherence of monocytes. Following incubation, the flask was gently rinsed three times with 20 ml volumes of pre-warm PBS to remove the non-adherent cells. Ten milliliters of tissue culture medium RPMI 1640 (BioWhittaker, Walkersville, MD, USA) supplemented with antibiotics (penicillin: streptomycin: amphotericin B, 0.1:0.25:0.1 µg/ml), 5% autologous serum and the growth factors, IL-3 and GM-CSF (both human recombinant, and each used at a final concentration of 100 ng/ml) were added to each flask. When used in serum-containing medium, these growth factors promote the differentiation of monocytes into macrophages (Suzuki et al., 2004; Shibasaki et al., 2007). The flasks were incubated for 7 days at 37 ºC/ 5% CO_2.

3.1.2.3 Harvesting of macrophages after maturation

Following the 7-day incubation period, the tissue culture medium was removed and discarded and the flask rinsed once with 10 ml PBS, followed by addition of 10 ml PBS containing the Ca^{2+}-chelating agent ethylene glycol-bis (2-aminoethylene)-N,N,N,N-tetraacetic acid (EGTA, 2mM, final) and the flask was placed on ice with gentle agitation every 10 minutes for at least 30 minutes, to promote detachment of the cells, which were dislodged by scraping the surface of the flask with a sterile 1.8 x 25 cm Cell Scraper (Adcock Ingram, Scientific Group). The dislodged cells were pelleted by centrifugation (1200 rpm, 10 minutes, 4°C), the supernatants discarded and the cell pellet resuspended in 5 ml of Ca^{2+}-free HBSS containing 2mM EGTA. The cell suspension (400 µL) was analyzed flow cytometrically using the following combinations of fluorochrome-labelled monoclonal antibodies:

- CD14-PE/CD16-FITC (monocytes/ macrophages)
- CD3-FITC/CD19-PE (T cells and B cells).
- Viability

In addition, the voltage settings of the flow cytometer were adjusted to enable detection of increased cell size ($CD14^+/CD16^+$ cells) as a marker of differentiation of monocytes into macrophages.

3.1.2.4 Preparation of macrophages for infection

Approximately 1 x 10^5 macrophages resuspended in 200 µL of HBSS Ca^{2+}- free medium were plated in 48 well tissue culture plates and calcium chloride ($CaCl_2$) added to 4mM final concentration to neutralize EGTA. These were incubated for 120 min at 37 °C/5% CO_2. The HBSS medium containing $CaCl_2$ was removed and replaced with 200 µL of RPMI 1640 containing penicillin: streptomycin: amphotericin B and 5% autologous serum and incubated for 24 hrs at 37 °C/5% CO_2. A day before the macrophages were infected with the bacteria, the RPMI 1640 containing the antibiotics was removed and replaced with antibiotic-free RPMI 1640.

3.1.2.5 Bacterial culture preparation

The bacterial cells were prepared by inoculating a frozen stock culture of each strain separately onto 7H10 agar medium and incubating at 37 °C for 3 weeks. At least 2 loop-fulls of cells were put into 50 ml tube with beads, vortexed, resuspended in PBS and centrifuged at 3500 rpm, for 15 min at 25 ºC and the supernatant discarded. The cells were resuspended in PBS and the suspension was adjusted to OD of 0.6 at 540 nm using a UV spectrophotometer. This bacterial suspension gives approximately 10^7 colony-forming units (cfu)/ml. The bacterial suspension was adjusted with RPMI to give the required multiple of infection (MOI) for each procedure.

3.1.2.6 Intracellular survival assay

The bacterial suspensions prepared as above were adjusted to 10^6 CFU /ml and added to adherent macrophages in 48 well tissue culture plates to achieve an MOI of 10:1 ratio, bacteria: macrophage. The plates were incubated for 24 hrs and thereafter rinsed with prewarmed PBS (1X) to remove extracellular bacteria. The wells were thereafter treated with 50 ug/ml gentamycin and incubated at 37ºC for at least 1 hour. The antibiotic solution was removed and the wells washed once with PBS. This was regarded as zero time after infection with the bacteria. The number of intracellular bacteria was then determined by removing the RPMI 1640 medium from the infected macrophages and lysing them with 100 µL of 0.2% sodium dodecyl sulfate (SDS) at zero, 24, 48 and 72 hrs post-infection. The lysate was serially diluted and plated onto 7H10 agar medium supplemented with 10% OADC and 0.5% glycerol and incubated at 37 ºC in the dark for 3-4 weeks for the appearance of colonies, which were then enumerated. Survival rates of the WT and Trk-knockout strains were analyzed and compared (Jun-Ming et al 2008; Sun 2009)

3.1.2.7 Quantification of cytokines

3.1.2.7.1 Collection of supernatants for the measurement of cytokines

Macrophages (1 x 10^5) were infected with the WT (H37Rv) and the *trk*-gene knockout strains of *M. tuberculosis* (10:1 number of bacteria/macrophage) as above and incubated overnight at 37°C, 5% CO_2. The supernatant was then discarded and the wells were washed with PBS (pH 7.4) and treated with 50µg/ml gentamycin for 60 min to kill any remaining extracellular organisms. The supernatant was discarded and the macrophages washed again with PBS. After washing, RPMI 1640 (antibiotic free), with 5% serum was added to the cells and incubated overnight at 37°C, 5% CO_2. The supernatant was then sampled at 0, 24, 48 and 72 hours and stored at -70°C until assayed. On the day of the assay, the supernatants were thawed and filter sterilized using 4mm diameter, 0.2 µm pore size filters.

3.1.2.7.2 Cytokine analysis

Analysis of the cytokines present in the supernatants was performed using the Bio-Plex Suspension Array System (Bio-Rad Laboratories, Inc. Hercules, Canada) and a Bio-Plex Pro™ assay kit (Bio-Rad Laboratories, Inc). The Bio-Plex Pro™ assay kit is a magnetic bead-based multiplex assay designed to measure multiple cytokines in different matrices. The assay kit used in the present study included the following cytokines: IL-1β, IL-6, IL-8, IL-10, TNF-α, and G-CSF.

The procedure for the analysis of cytokines was followed according to the manufacturers' specifications. Briefly, a four-fold serial dilution was made of the premixed standard supplied with the assay kit (Concentration range: 77755.00 – 1.48 pg/ml). The dilutions were held on ice until assayed.

A 50µl volume of either standard or sample was then added to a 96-well plate containing the magnetic beads. An antibody directed against the desired cytokine is covalently coupled to the internally dyed beads. The plate was then incubated at room temperature on an orbital shaker (300 rpm) for 30 min in the dark.

After incubation, the plate was washed 3 times using a Bio-Plex Pro wash station (Bio-Rad Laboratories, Inc.). A biotinylated detection antibody (25µl) specific to an epitope different from that of the capture antibody was then added to the reaction and the plate was incubated for a further 30 min at room temperature on an orbital shaker (300 rpm) in the dark.

The plate was then washed a further 3 times using a Bio-Plex Pro wash station followed by the addition of a streptavidin-phycoerythrin (streptavidin-PE) reporter complex (50µl) which then binds to the biotinylated detection antibodies on the bead surface. The plate was incubated again in the dark, at room temperature for 10 min on an orbital shaker (300 rpm).

After a final 3 washes, a volume of 125µl of assay buffer was added to each well and the plate was shaken at 1100 rpm for 30 seconds to ensure that the beads were resuspended. The plate was then read using the Bio-Plex Suspension Array System (Bio-Rad Laboratories, Inc) and the results were analyzed using the Bio-Plex Manager software version 4.1 (Bio-Rad Laboratories, Inc). The results are expressed as pg/ml.

Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM) and level of significance was calculated using unpaired t test and a P value ≤ 0.05 was considered significant.

CHAPTER 4

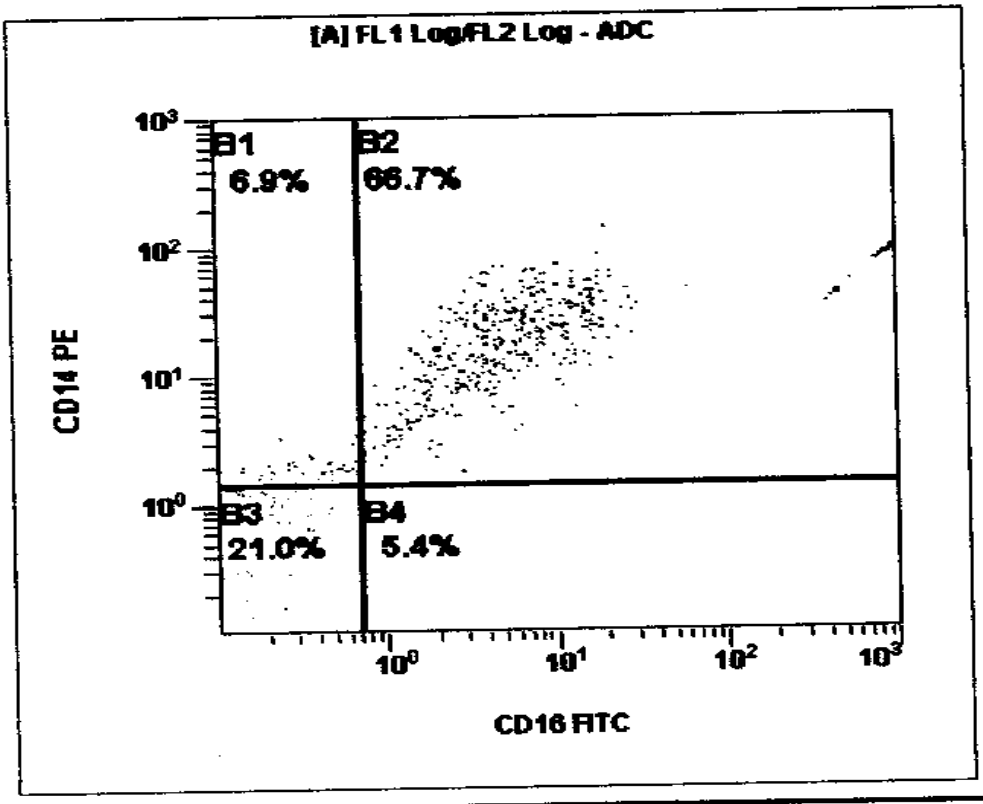
4.1 RESULTS

4.1.1 Isolation and enumeration of monocytes/macrophages

Mononuclear cells were isolated from heparinized blood, washed with PBS, and briefly suspended in 0.83% NH_4Cl to remove contaminating erythrocytes. Finally, they were resuspended in HBSS. The MNL were enumerated flow cytometrically using a Beckman Coulter FC500 Flow Cytometer (Beckman Coulter) using the following fluorochrome-labelled monoclonal antibodies: CD3 (FITC), CD14 (PE), CD15 (FITC) and CD19 (PE) for analysis and enumeration of total T-cells, monocytes, granulocytes and B-cells, respectively (all monoclonals were obtained from Beckman Coulter). Following maturation, co-expression of CD14 and CD16 (FITC) was used to characterize and enumerate the matured macrophage population. The total numbers of isolated MNL and percentages of monocytes ($CD14^+$) for each donor (n=6) following initial isolation from blood are shown in Table 4.1, while the maturation of monocytes to macrophages (CD14/CD16) is shown in Figure 4.1(Histogram), which is from a single representative experiment.

Table 4.1: Enumeration of MNL by flow cytometry.

Donors	Total flow count (cell/µl)	Monocytes ($CD14^+$)	Monocytes ($CD14^+$) %
1	6124	332	4.51
2	24785	1356	5.5
3	7593	664	8.7
4	8414	1078	12.8
5	6071	1689	27.8
6	9088	683	7.5



[A] FL1 Log/FL2 Log						
Region	Cells/µL	Number	%Total	%Gated	X-Mean	Y-Mea
ALL	183	727	14.38	100.00	65.4	26
B1	13	50	0.99	6.88	0.386	1.94
B2	122	485	9.60	66.71	97.3	38.5
B3	39	153	3.03	21.05	0.154	0.811
B4	10	39	0.77	5.36	7.21	0.809

Figure 4.1: Histogram showing flow cytometric enumeration of macrophages harvested after 7 days of incubation. In B1, 6.9% of these cells expressed the CD14 marker, in B2, 66.7 % of the cells co-expressing CD14/CD16 markers; in B3, 21% of MNL were negative for both markers; and in B4, 5.4 % of cells expressed the CD16 marker.

4.1.2 Comparison of the intracellular survival of the wild type and trk knockout strains of *M. tuberculosis*

To determine whether the Trk K^+ transporter system affects the survival of intracellular *M. tuberculosis*, macrophages were infected with the WT and *trk*-gene knockout strains for overnight (which was regarded as day 0), day 1, day 2 and day 3 after which the cells were lysed, and the viable bacteria enumerated by colony counting procedure.

Figures 4.2-4.7 comprise six separate graphs representing the results of six different experiments. The composite results for all six experiments are shown in Figure 4.8. Although minor differences were evident in the individual experiments, statistical analysis of the composite data revealed no significant differences between the WT or *trk*-gene knockout strains of *M. tuberculosis* (H37Rv) at any of the time points tested.

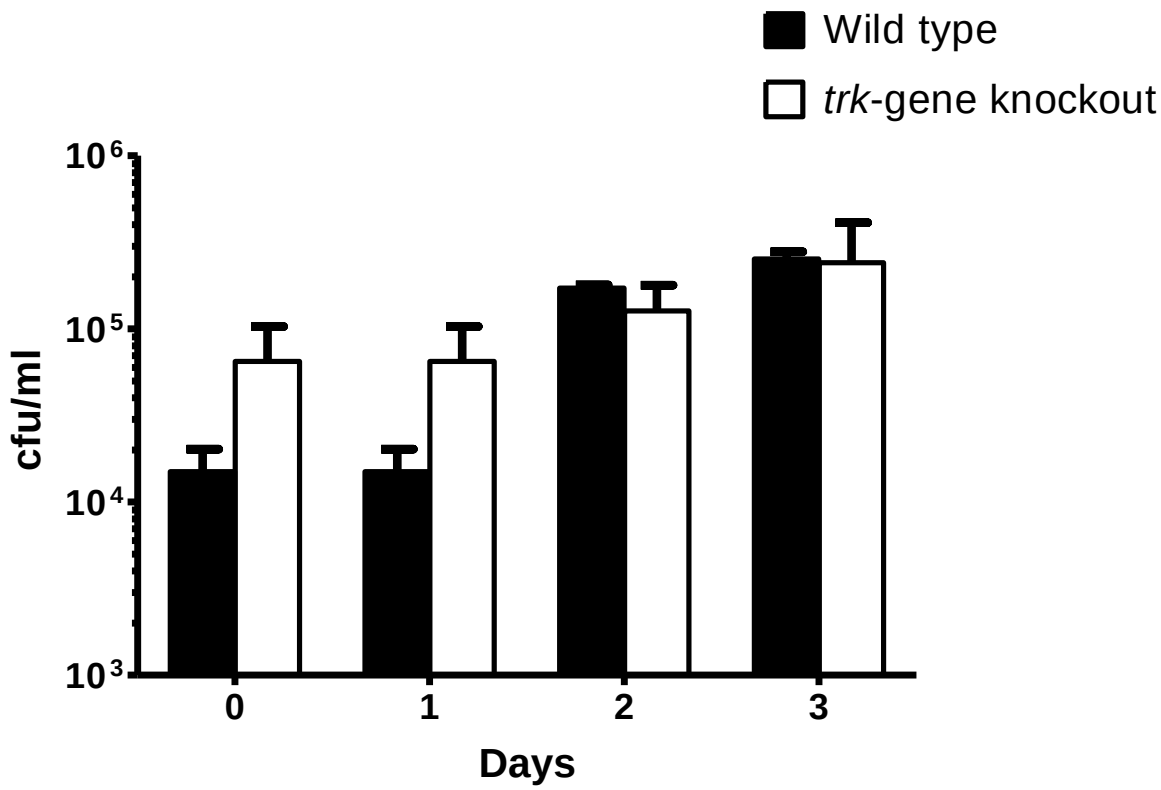


Figure 4.2: Experiment 1. Measurement of intracellular survival of the WT and the *trk*-gene knockout strains of *M. tuberculosis* (H37Rv) measured using a colony counting procedure at day 0 and at day 1, day 2, and day 3 following infection of human monocyte-derived macrophages. The results are expressed as mean values ± SEM for two replicates at each time point.

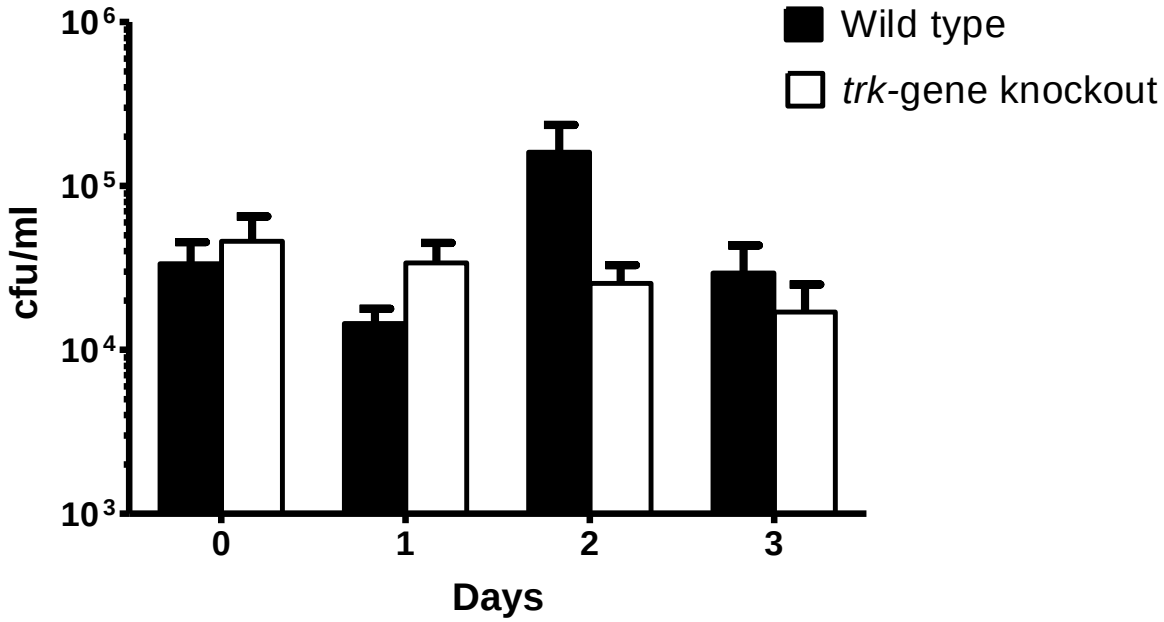


Figure 4.3: Experiment 2. Measurement of intracellular survival of the WT and the *trk*-gene knockout strains of *M. tuberculosis* (H37Rv) measured using a colony counting procedure at day 0 and at day 1, day 2, and day 3 following infection of human monocyte-derived macrophages. The results are expressed as mean values ± SEM for two replicates at each time point.

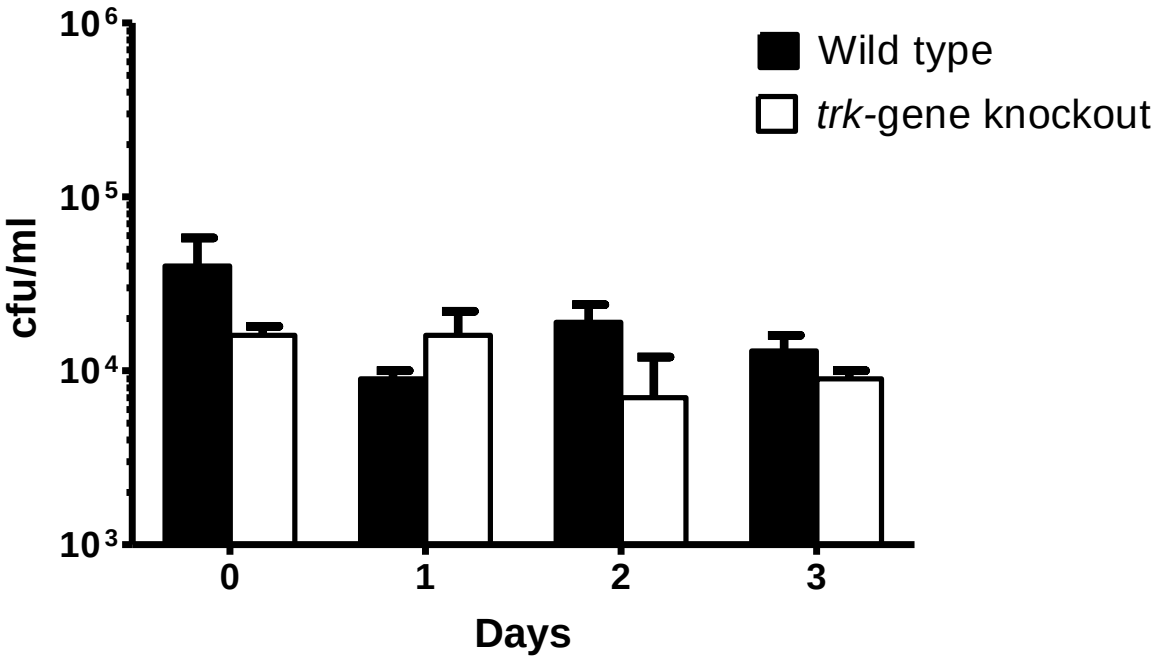


Figure 4.4: Experiment 3. Measurement of intracellular survival of the WT and the *trk*-gene knockout strains of *M. tuberculosis* (H37Rv) measured using a colony counting procedure at day 0 and at day 1, day 2, and day 3 following infection of human monocyte-derived macrophages. The results are expressed as mean values ± SEM for two replicates at each time point.

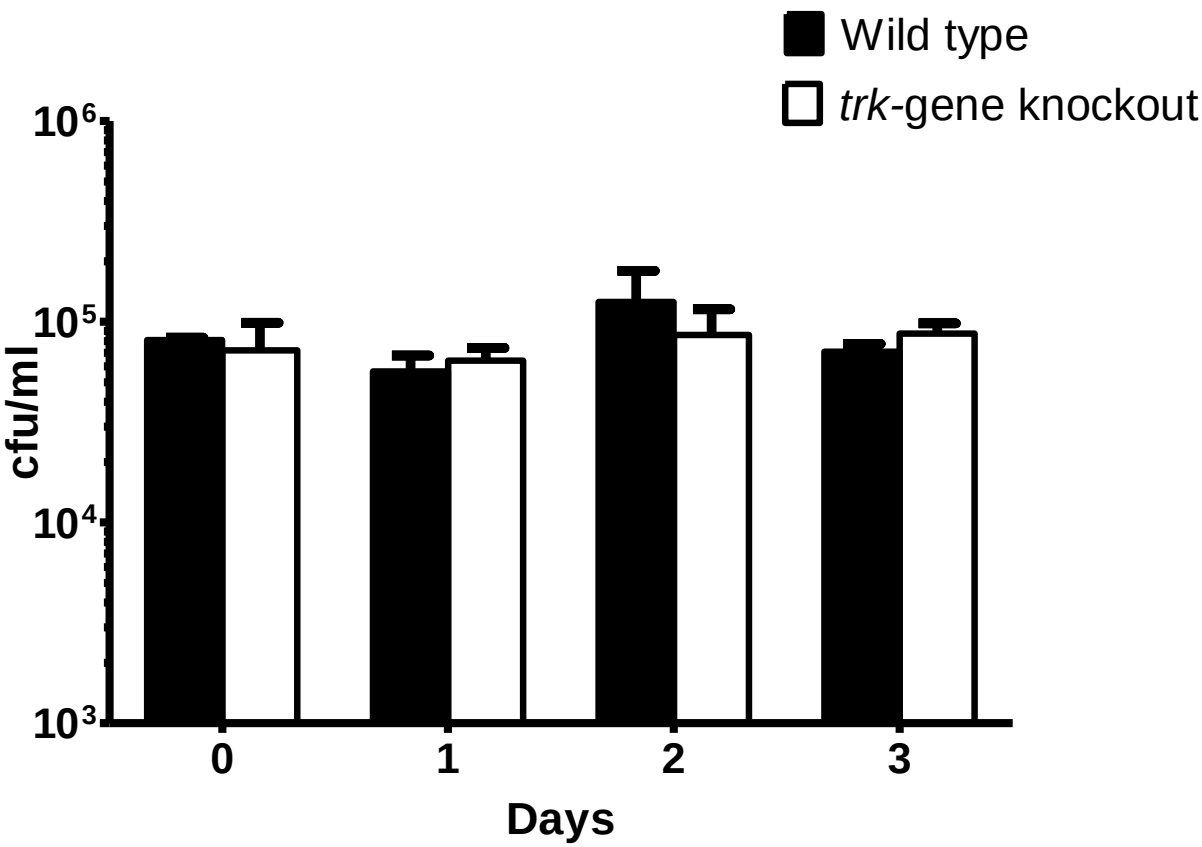


Figure 4.5: Experiment 4. Measurement of intracellular survival of the WT and the *trk*-gene knockout strains of *M. tuberculosis* (H37Rv) measured using a colony counting procedure at day 0 and at day 1, day 2, and day 3 following infection of human monocyte-derived macrophages. The results are expressed as mean values ± SEM for two replicates at each time point.

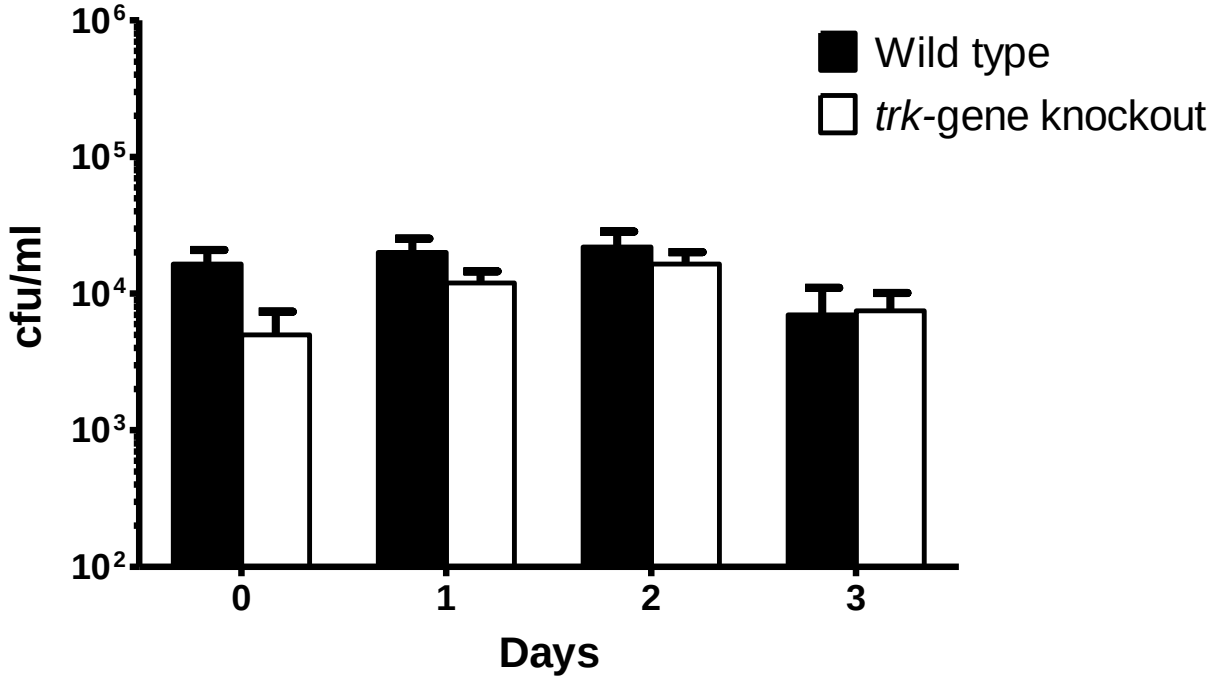


Figure 4.6: Experiment 5. Measurement of intracellular survival of the WT and the *trk*-gene knockout strains of *M. tuberculosis* (H37Rv) measured using a colony counting procedure at day 0 and at day 1, day 2, and day 3 following infection of human monocyte-derived macrophages. The results are expressed as mean values ± SEM for two replicates at each time point.

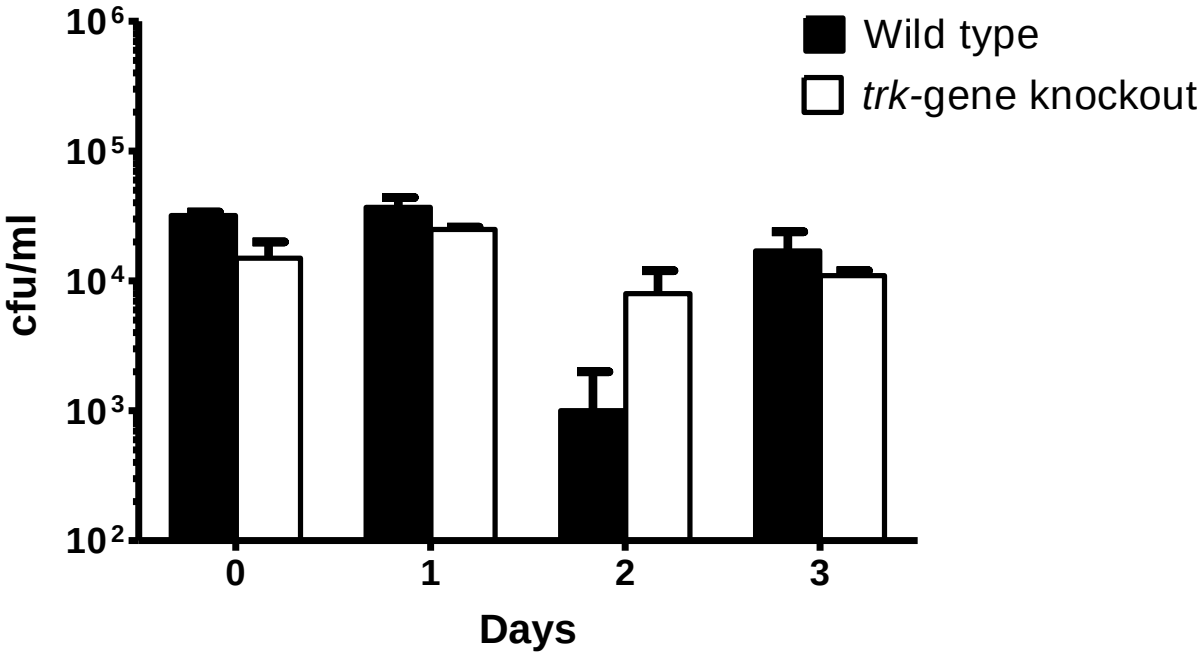


Figure 4.7: Experiment 6. Measurement of intracellular survival of the WT and the *trk*-gene knockout strains of *M. tuberculosis* (H37Rv) measured using a colony counting procedure at day 0 and at day 1, day 2, and day 3 following infection of human monocyte-derived macrophages. The results are expressed as mean values ± SEM for two replicates at each time point.

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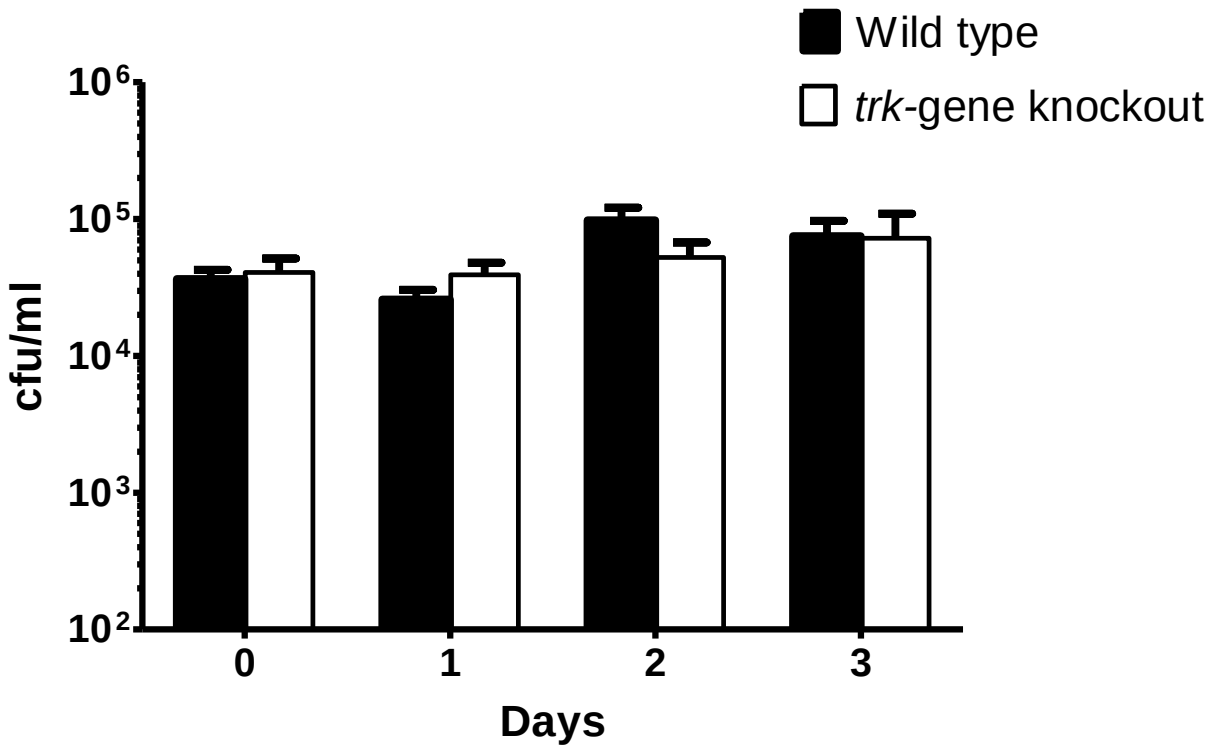

Figure 4.8: Measurement of intracellular survival of the WT and the *trk*-gene knockout strains of *M. tuberculosis* (H37Rv) measured using a colony counting procedure at day 0 and at day 1, day 2, and day 3 following infection of human monocytes-derived macrophages. The composite results for all 6 experiments are expressed as mean values ± SEM for each with two replicates at each time points.

4.1.3 Production of IL-1B, IL-6, IL-8, TNF-α and G-CSF by human monocyte-derived macrophages infected with the wild type and the trk knockout strains of *M. tuberculosis*

Macrophages infected with either the WT or the *trk*-gene knockout strains of *M. tuberculosis* were analyzed for secreted levels of the following cytokines: IL-1β, IL-6, IL-8, TNF-α and G-CSF. The results for uninfected, control macrophages are shown in Table 4.2 (day 0 and day 3), while those for macrophages infected with the WT and the *trk*-gene knockout strains of *M. tuberculosis* are shown in Tables 4.3 and 4.4 and figures 4.9-4.13 the levels of production of all cytokines by the infected macrophages, irrespective of the infecting strain, were not significantly different from that of controls with those of the control, uninfected macrophages. These results demonstrate that neither strain of *M. tuberculosis* caused macrophage activation.

Table 4.2: Time course of production of cytokines secreted by uninfected macrophages

Time (days)	IL-1β	IL-6	IL-8	TNF-α	G-CSF
0	0.257 ± 0.073	11.57 ± 0	155 ± 145.78	16.74 ± 0.855	2.41 ± 0. 319
3	1.47 ± 0.89	25.69 ± 6.893	1705 ± 460	23.65 ± 4.185	4.52 ± 0.85

Table 4.3 Time course of production of cytokines secreted by macrophages infected with the WT strain of *M. tuberculosis*

Time (Days)	IL-1β	IL-6	IL-8	TNF-α	G-CSF
0	0.3 ± 0.007	5 ± 3	34 ± 7	14 ± 1	2 ± 0.5
1	0.3 ± 0.007	7 ± 0.8	35 ±14	18 ± 2	5 ± 1
2	0.4 ± 0.2	5 ± 1	82 ± 29	18± 2	3 ± 1
3	0.4 ± 0.08	8 ± 4	121 ± 60	18 ± 2	5 ± 1

Table 4.4 Time course of production of cytokines secreted by macrophages infected with the *trk*-gene knockout strain of *M. tuberculosis*.

Time (Days)	IL-1β	IL-6	IL-8	TNF-α	G-CSF
0	0.7 ± 0.1	20± 11	65± 38	16± 1	3± 0.3
1	1± 1	4± 1	47± 13	19± 0.5	1± 0.3
2	5± 5	12± 4	126± 90	17± 1	2± 0.7
3	0.3 ± 0.08	10± 3	72± 20	15± 15	4± 0.6

The results of the three separate experiments using monocyte-derived macrophages prepared from 3 different donors are expressed as mean values ± SEM for two replicates at each time point.

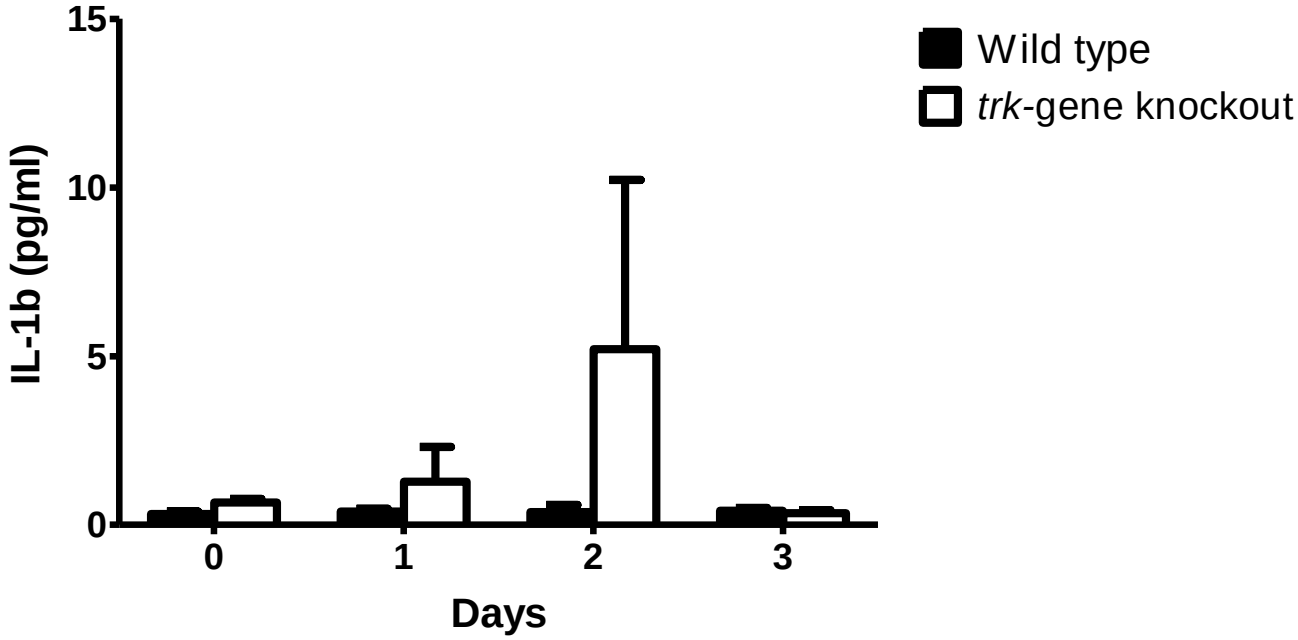

Figure 4.9: Production of IL-1β by human monocyte-derived macrophages infected with either the WT or the *trk*-gene knockout strains of *M. tuberculosis* (H37Rv) over a 3 day time course. The results of 3 separate experiments with two replicates for each system are expressed as mean values in picograms/ml supernatant ± SEM.

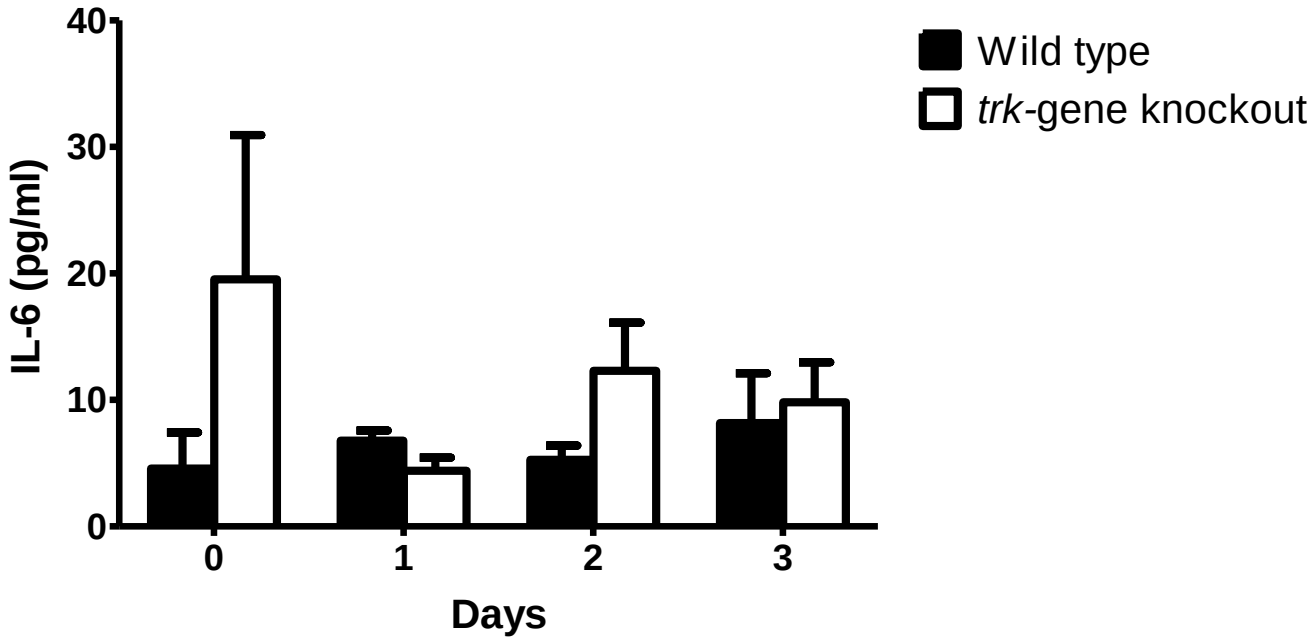

Figure 4.10: Production of IL-6 by human monocyte-derived macrophages infected with either the WT or the *trk*-gene knockout strains of *M. tuberculosis* (H37Rv) over a 3 day time course. The results of 3 separate experiments with two replicates for each system are expressed as mean values in picograms/ml supernatant ± SEM.

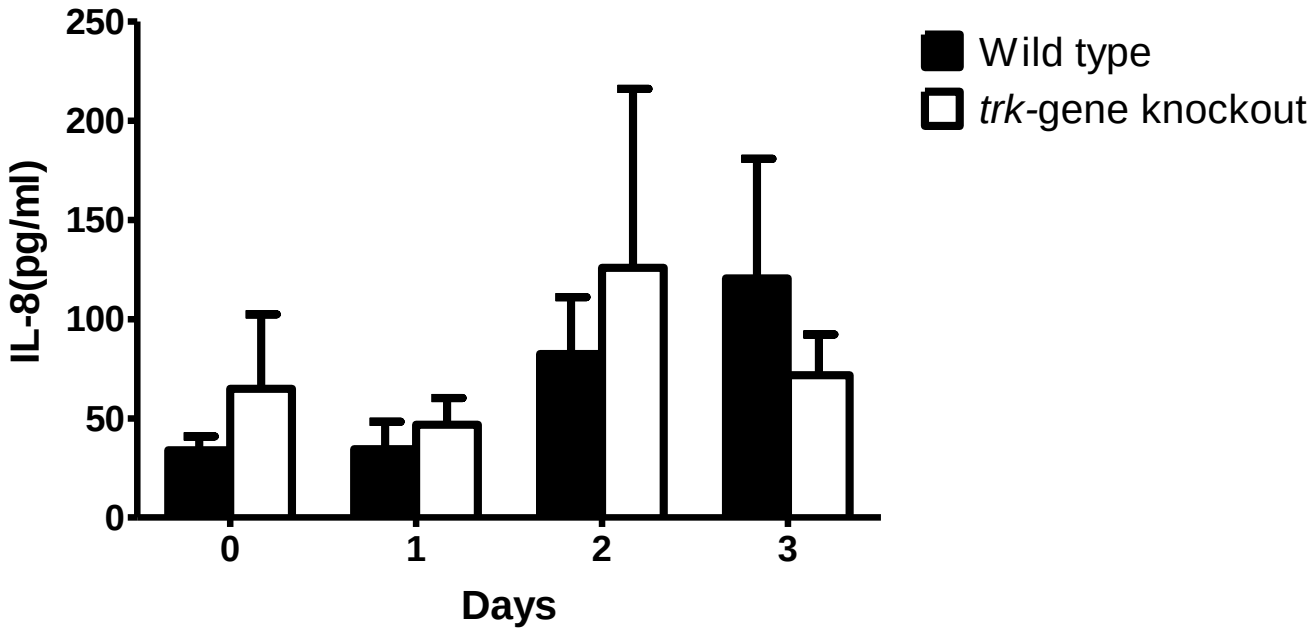

Figure 4.11: Production of IL-8 by human monocyte-derived macrophages infected with either the WT or the *trk*-gene knockout strains of *M. tuberculosis* (H37Rv) over a 3 day time course. The results of 3 separate experiments with two replicates for each system are expressed as mean values in picograms/ml supernatant ± SEM.

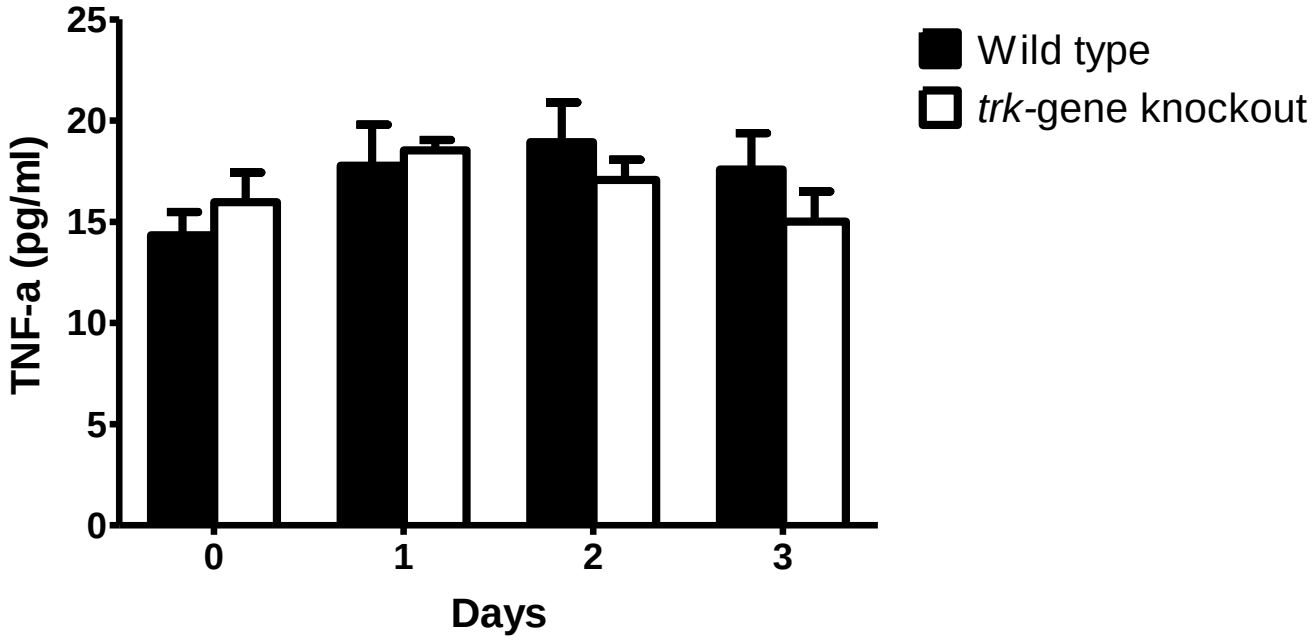

Figure 4.12: Production of TNF-α by human monocyte-derived macrophages infected with either the WT or the *trk*-gene knockout strains of *M. tuberculosis* (H37Rv) over a 3 day time course. The results of 3 separate experiments with two replicates for each system are expressed as mean values in picograms/ml supernatant ± SEM. .

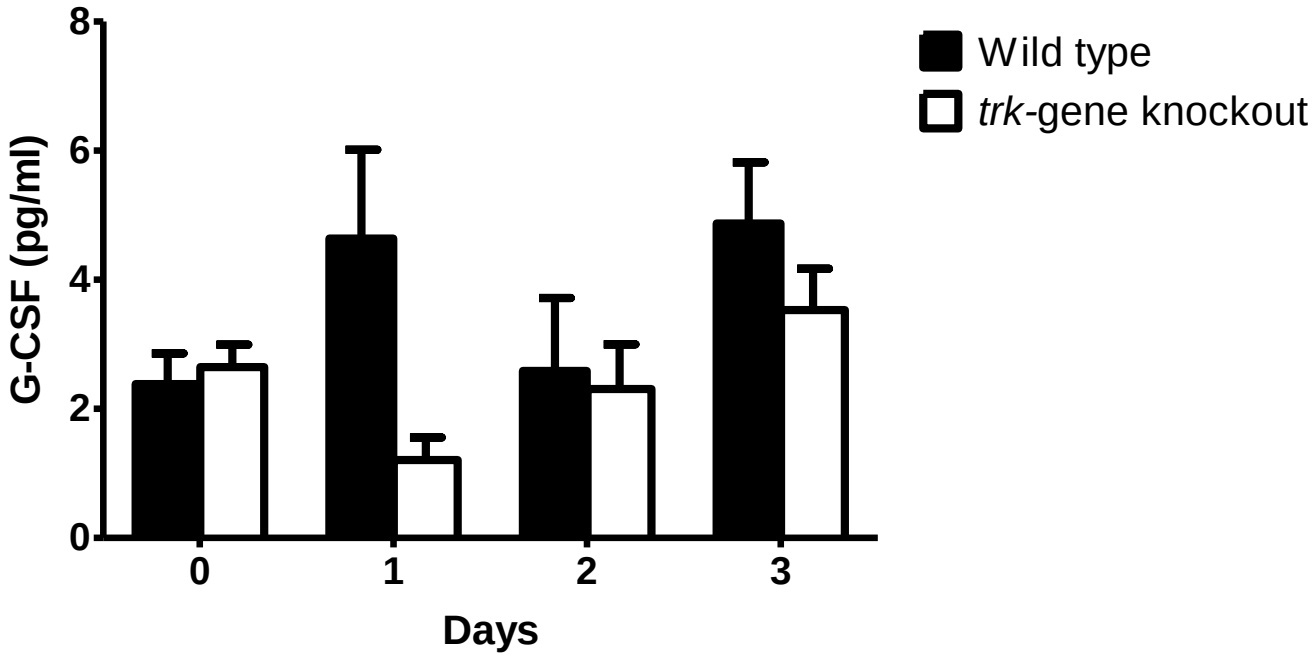

Figure 4.13: Production of G-CSF by human monocyte-derived macrophages infected with either the WT or the *trk*-gene knockout strains of *M. tuberculosis* (H37Rv) over a 3 day time course. The results of 3 separate experiments with two replicates for each system are expressed as mean values in picograms/ml supernatant ± SEM.

CHAPTER 5

5.1 DISCUSSION

5.1.1 Comparison of the intracellular survival of the wild type and *trk* gene knockout strains of *M. tuberculosis.*

Potassium is the most dominant intracellular cation, required for a number of physiological processes. There are at least two characterized K^+ transporter systems of *M. tuberculosis* namely: the Trk and the Kdp (Cole et al., 1998). Apart from maintaining the intracellular level of K^+ required to activate and maintain protein synthesis and metabolic enzymes, these K^+ transporter systems also play an important role in the regulation of cytoplasmic pH and osmolarity in bacteria (Epstein and Laimins, 1980). Given that the virulence factors are often controlled by monovalent cations (Chen et al, 2004), the objective of the research presented in this dissertation was to investigate the effect of the Trk system on the intracellular survival of *M. tuberculosis*. The Trk system is the main system responsible for the uptake of K^+ by *M. tuberculosis* when the extracellular concentration of the cation is high, and is a potential modulator of phagosomal maturation, possibly promoting microbial intracellular survival.

Similar to that of *E. coli*, the Trk system of *M. tuberculosis* is ATP-driven and requires the proton motive force, while the Kdp K^+ transporter system is driven by ATP and does not require proton motive force. The Kdp is an inducible high-affinity transporter of K^+ (Asha and Gowrishankar, 1993). This system may be induced by the loss of the turgor pressure or changes in pH of the culture (Laimins et al., 1981; Asha and Gowrishankar, 1993). When K^+ concentrations are high Kdp is repressed (Asha and Gowrishankar, 1993). The Kdp system is normally repressed and can be expressed by lowering the intracellular concentration of K^+. Therefore, this system is expected to be expressed in cells grown on medium containing very low concentrations of K^+ or when the Trk system is absent or inefficient. The Trk system accumulates K^+ until the external concentration falls below 10µM and it can also set up a concentration gradient of K^+ as high as 50,000 (Bakker and Harold, 1980). Hence, the Kdp system ought to be operative in cells grown on medium containing less than 10µM of K^+.

The hypothesis of the current study was that the Trk system of *M. tuberculosis* promotes intracellular survival and replication of the organism in macrophages, possibly by subverting vacuolar acidification and/or by modulating the cytokine environment, favouring development of the M2 phenotype. The experimental strategy involved the preparation of mature monocyte-derived macrophages *in vitro*, a multistep and extended procedure, requiring sophisticated flow cytometric analysis. These cells were then experimentally infected with either the WT or the *trk*-gene knockout strain of *M. tuberculosis* (H37Rv) and monitored for intracellular survival/ growth and cytokine secretion over a three day time course.

However, no significant changes in terms of intracellular survival of the WT and the *trk*-gene knockout strains of *M. tuberculosis* following infection of monocyte-derived macrophages were detected. This may be because *M. tuberculosis* uses a compensatory K^+ transporter such as the Kdp system, which may explain the ability of *trk* gene knockout of *M. tuberculosis* to survival intracellularly in the monocyte-derived macrophages.

5.1.2 Production of IL-1B, IL-6, IL-8, TNF-α and G-CSF by human monocyte-derived macrophages infected with the wild type and the trk gene knockout strains of *M. tuberculosis*

Macrophages represent a critical first line of defense against *M. tuberculosis.* However, in cases where macrophages activation for killing is suboptimal, *M. tuberculosis* is extremely proficient at surviving intracellularly (Bai et al., 2010). There are numerous cellular processes, which are linked to, and which precede the production of inflammatory mediators. During infection with *M. tuberculosis,* protection and pathogenesis are both mediated by cellular immune responses, involving the interaction of lymphocytes, mainly T-cells, and phagocytes of the monocyte/macrophage lineage. These interactions depend on interplay of cytokines produced by these cells.

Cytokines which are important in host defense against *M. tuberculosis* or other pathogenic mycobacteria in both experimental animals and humans include TNF-α and IFN-γ, IL-8, IL-12, IL-17, IL-18 and IL-23 (Roach et al., 2001). The study of cellular responses and the definition of target molecules are of importance when it comes to understanding the protective and the pathogenic immune mechanisms in TB, particularly for the identification of antigens that are suitable for diagnosis and the development of new vaccines (Munk and Emoto, 1995; Mustafa et al., 2002; Mustafa et al., 2006). One such possibility in the Trk system of *M. tuberculosis*

In the current study the effect of Trk knockout on the proinflammatory cytokine production was characterized by investigating the production of IL-1β, IL-6, IL-8, TNF-α and G-CSF by human monocyte-derived macrophages infected with the WT and the *trk*-gene knockout strains of *M. tuberculosis.* With respect to cytokine production and secretion by *M. tuberculosis*-infected macrophages, the levels of production of IL-1β, IL-6, IL-8, IL-10 and TNF-α were unaffected following exposure to either the wild-type or *trk* gene-knockout strains, being comparable to those of uninfected, control macrophages.

These findings demonstrate that infection of human monocyte-derived macrophages with *M. tuberculosis* for up to 3 days does not appear to cause activation of cytokine production or differentiation of the cells into either the M1 (IL-1, IL-6, TNF-α) or M2 (IL-10) phenotypes. Given that *M. tuberculosis* is a remarkably successful intracellular pathogen, these findings are not entirely surprising. Clearly, intracellular infection is a relatively subtle, passive process by which the pathogen goes undetected, at least initially, and thereafter subverts the antimicrobial mechanisms of the macrophage. Notwithstanding, discrete, passive infection of macrophages by *M. tuberculosis,* the microbial pathogen may also release suppressive proteins which modulate cytokine production (Samuel et al., 2007)

5.2 CONCLUDING COMMENTS

The findings of the current study demonstrate firstly, that the Trk K^+ transporter of *M. tuberculosis* does not appear to be necessary for intracellular survival; secondly, infection of human monocyte- derived macrophages with either the wild-type or *trk* gene-knockout strains of *M. tuberculosis* fails to activate cytokine production/secretion by these cells. The study does, however, have several limitations. These include:

- The relatively short duration of intracellular infection (3 days maximum).
- The use of an *in vitro* system of macrophage maturation/infection which does not replicate the environment of the TB granuloma. For example, the pH of the granuloma is approximately 6.4 (Li et al; 2002), while a pH of 7.4 was used throughout for the *in vitro* studies.

Future studies, for which the current study may serve as a guide, should focus on the following:

- Extended incubation times beyond the maximum 3 day period used in the current study.
- Inclusion of additional K^+ transporter gene-knockout mutants of *M. tuberculosis*, specifically the *kdp*-gene knockout and the dual *kdp*/*trk*-gene knockout.
- Inclusion of additional cytokines and surface markers representative of the M1/M2 macrophage phenotypes.
- Evaluation of the virulence of the various K^+ transport gene-knockout mutants in murine models of experimental infection.

In conclusion, the laboratory research described in this dissertation has resulted in the development of a method for the isolation and maturation of human monocyte-derived macrophages, assessment of the intracellular survival of *M. tuberculosis*, and detection of secreted cytokines by these cells. Although, the *trk*-gene knockout mutant of *M. tuberculosis* behaved similarly to the wild-type strain with respect to both intracellular survival and cytokine production, the findings may serve as a basis for future studies.

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