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EVALUATION OF THE INTRACELLULAR ANTI-MYCOBACTERIAL ACTIVITY OF A CLOFAZIMINE NANOPARTICLE FORMULATION IN MACROPHAGES

by

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DECLARATION

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

Signed: ……. Date: ……..

DEDICATION

I dedicate this work to my daughter Bokamoso, my brother and mother whom I love very much and in the memory of my late father Molefi Motheo.

ACKNOWLEDGEMENTS

The honour and glory be to God Almighty for under His wings I found rest, peace of mind, wisdom, hope and perseverance throughout the hardships I went through in this study. For without Him I wouldn't have seen the light through to the end of this journey.

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cells
BCG	Bacille Calmette-Guérin
CHO	Chinese hamster ovary
CD	Cluster of differentiation
CFU	Colony-forming units
CLF	Clofazimine
CR	Complement receptors
CpG-DNA	Cytosine-phosphate-guanine DNA
DCs	Dendritic cells
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribose nucleic acid
Fe^{2+}	Iron
F-CLF	Free clofazimine
GM-CSF	Granulocyte-macrophage colony stimulating factor
HSP	Heat shock proteins
HIV	Human immunodeficiency virus
H_2O_2	Hydrogen peroxide
INF-γ	Interferon-gamma
iNOS	Inducible nitric oxide synthase
IL-	Interleukin
K^+	Potassium

KCN	Potassium cyanide
LAM	Lipoarabinomannan
L-CLF	Liposomal clofazimine
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAC	*Mycobacterium avium* - *M. intracellulare* complex
MCP-1	Monocyte chemotactic protein-1
MDR	Multi-drug resistant
MIP	Macrophage inflammatory protein
M. tuberculosis	*Mycobacterium tuberculosis*
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
NK cells	Natural killer cells
NO	Nitric oxide
OBr^-	Hypobromite
OCl^-	Hypochlorite
•OH	Hydroxyl radical
PAMP	Pathogen-associated molecular patterns
PAS	Para-aminosalicylic acid
PBS	Phosphate buffered saline solution
PIM	Mannosylated phosphatidylinositol
PLA_2	Phospholipase A_2
PRRs	Pattern recognition receptors
PGL-1	Phenolic glycolipid-1

RANTES	Regulated on activation normal T cell expressed and secreted
RNA	Ribonucleic acid
ROI/RNI	Reactive oxygen/reactive nitrogen intermediates
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscope
SEM	Standard error of the mean
SR-A	Scavenger receptor-A
TB	Tuberculosis
TGF-β	Transforming growth factor-beta
Th-	T helper lymphocytes
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha
XDR	Extensively drug-resistant

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SUMMARY

Tuberculosis is a disease caused by the Gram-positive acid fast bacillus, *Mycobacterium tuberculosis* (*M. tuberculosis*). This intracellular pathogen has infected one-third of the world's population and accounted for over two million deaths per year. The long-term persistence of *M. tuberculosis*, TB-human immunodeficiency virus coinfections, and the emergence of multidrug resistant (MDR) and extensively resistant (XDR) *M. tuberculosis* strains are the main factors contributing to the high global burden of this disease. Management of TB remains a great challenge despite the use of BCG vaccination and the availability of anti-tuberculous therapies, many of which are usually administered for longer periods. Hence ongoing research is aimed at developing new, efficacious, orally available drugs with the ability of shortening and simplifying the long and complex treatments of both drug-sensitive and drug-resistant tuberculosis. The success of such developments will be of great value if emphasis is based on re-formulation of the older drugs for the treatment of TB, such as clofazimine.

Previous studies on clofazimine demonstrated that it was highly effective against *M. tuberculosis in vitro*, as well as in murine models of experimental, disseminated TB, but was of limited, if any, value in the treatment of TB in humans. This is probably due to the pharmacokinetic properties of this riminophenazine antimicrobial agent, which appears to have poor penetration into the airways. A renewed interest in clofazimine as an anti-TB agent was elicited by the emergence of MDR and extensively drug-resistant (XDR) TB, together with advances in technology for the delivery of lipophilic drugs to target organs. The primary objective of the laboratory research described in this dissertation was to compare the activities of a conventional preparation and a novel nanoparticle formulation of clofazimine against intracellular *M. tuberculosis* concealed in human monocytes/ macrophages.

In the present study, a procedure was established for isolating monocytes from human peripheral blood and subsequently maturing these cells into homogenous populations of monocyte-derived macrophages. Exposure of the monocytes to the cytokine growth factors, IL-3 and GM-CSF, over a 7 day incubation resulted in a significant increase in the level of expression of $CD14^+$/ $CD16^+$ markers showing accelerated differentiation. Importantly, this cell population was highly homogeneous and accounted for >95% of the total cell population.

These findings confirmed that the modified procedure results in the acquisition of synchronised human monocyte-derived macrophages, in adequate numbers. The monocyte-derived macrophages were then infected with H37Rv *M. tuberculosis* laboratory strain at a 1:10 macrophage: bacteria multiplicity of infection. Concentrations ranging from 0.15 - 2.5 µg/ml for both the native and spray-dried clofazimine preparations together with corresponding drug-free controls were used to treat the infected cells for 48 hours. The intracellular bioactivities of the two preparations were determined by quantifying the number of surviving bacteria inside macrophages following treatment. Findings demonstrated comparable efficacy, with dose-dependent response inhibition of colony formation when *M. tuberculosis* was exposed to either of the clofazimine preparations, with significant inhibition of growth at 1.25 µ/ml, with no detectable colonies at 2µg/ml. No statistically significant differences were found between the treatments with both preparations. These observations demonstrate that the intracellular bioactivities of both clofazimine preparations are equivalent with respect to efficacy against *M. tuberculosis*. This study may therefore provide justification for future studies on the therapeutic efficacy of the spray-dried formulation of clofazimine in murine models of experimental chemotherapy.

CHAPTER 1

1.1 LITERATURE REVIEW

1.1.1 Tuberculosis as a disease

Tuberculosis (TB) is a life-threatening chronic infection caused by the Gram-positive, acid fast bacillus, *Mycobacterium tuberculosis* (*M. tuberculosis*). The organism is an obligatory aerobic, intracellular pathogen that infects the lungs (Tyagi and Sharma, 2004). One-third of the world's population is infected with this organism, leading to approximately 2 - 3 million deaths per year (WHO report, 2007). In South Africa, an estimate of 0.48 million cases was reported in 2008 and, the country is currently ranked third after India and China in ranking of the total number of annual incident cases of TB (WHO report, 2009). This high global burden of the disease is exacerbated by long-term persistence of *M. tuberculosis*, TB-human immunodeficiency virus (HIV) coinfection, and the emergence of multidrug resistant (MDR) and extensively drug-resistant (XDR) *M. tuberculosis* strains, making tuberculosis one of the leading causes of death from an infectious agent in the world, despite the use of BCG vaccination and the availability of anti-tuberculous therapy (Raviglione, 2003; Dye *et al*., 2008).

Despite this high morbidity and mortality associated with the disease, primary infections with *M. tuberculosis* are usually controlled in immuno-competent individuals. Only 5 to 10% of the infected population has a lifetime risk of developing active tuberculosis, either within one or two years after infection or thereafter. The situation is different in immuno-compromised individuals infected with HIV, whose chance of developing active disease is 10% per year (Parrish *et al*., 1998; Flynn and Chan, 2001; Corbett *et al*., 2003; Aaron *et al*., 2004).

In Africa, tuberculosis (TB) is one of the earliest occurring opportunistic infections, accounting for approximately 40% of deaths in HIV-infected individuals in this region. HIV/*M. tuberculosis* coinfection is clearly the major public health threat to developing countries in this region, predominantly affecting the under-privileged, and having the potential to devastate national health budgets (Corbett *et al*., 2003; Dye *et al*., 2008). Given the relative therapeutic efficacy of anti-retroviral drugs, there is clearly an urgent need for the development of affordable anti-TB chemotherapeutic agents which are effective against MDR/XDR strains of *M. tuberculosis*.

Without a doubt, the surveillance of *M. tuberculosis* including the nature in which this microorganism spreads, the difficulties in acquiring effective drug treatments, and a complex therapeutic regimen, form a part of the many inherent factors which still makes tuberculosis one of the major health challenges in the world (Sasindran and Torrelles, 2011). Hence ongoing research is aimed at developing new, efficacious, orally available drugs with the ability of shortening and simplifying the long and complex treatments of both drug-sensitive and drug-resistant tuberculosis (Lu *et al*., 2011). Such developments will be a great success if the value of the older drugs in the treatment of TB today is enhanced (Jawahar, 2004).

The discovery of clofazimine coincided with the emergence of the more potent anti-TB agents isoniazid and pyrazinamide in the early 1950s and rifampicin and ethambutol in the early 1960s. Early studies of clofazimine in various animal models demonstrated inconsistent therapeutic activities against experimental tuberculosis, but the drug was considered to be ineffective in the treatment of pulmonary tuberculosis (Barry and Conalty, 1965). Interest in developing clofazimine as an anti-TB agent was lost due to the unusual pharmacokinetic properties and side-effects profile of this agent, which lowered priority on the use of this drug for several decades (Drugs.com, 2011). A renewed interest in clofazimine as an anti-TB agent was elicited by the emergence of MDR and XDR-TB, together with advances in technology for the delivery of lipophilic drugs to target organs. Current studies have provided new insights into the targets and molecular mechanisms of both clofazimine-mediated antimicrobial and anti-inflammatory activity. They have also provided the drive to design and develop novel riminophenazines with improved antimicrobial efficacy and efficient delivery to target organs (Working Group on New TB Drugs, 2011; Yano *et al*., 2011).

1.1.2 Pathogenesis of tuberculosis

Central to understanding the pathogenesis of tuberculosis is the interaction between the pathogen and mononuclear phagocytes. The main route of infection is by means of aerosol transmission, whereby droplet nuclei containing one to three bacilli of particle size < 5µm, gain access to alveoli (Wells, 1955; Riley *et al*., 1959; Riley and O'Grady, 1961). The bacilli enter the airways and are engulfed by alveolar macrophages, which presumably are equipped with multiple microbicidal mechanisms, including phagolysosome fusion and a respiratory burst, that clear the host of infecting microorganisms (Sibille and Reynolds, 1990; Verhoef, 1991).

Those that survive intracellular destruction multiply and lead to disruption of the macrophages. Following this, blood monocytes and other inflammatory cells are attracted to the lungs. These monocytes will differentiate into macrophages which again readily ingest, but do not destroy, the mycobacteria. The mycobacteria grow logarithmically in the macrophages, while other blood-derived macrophages accumulate, but little tissue damage occurs. Two to six weeks after infection, T-cell immunity develops. Antigen-specific T lymphocytes then proliferate within the early lesions or tubercles, activating macrophages to kill the intracellular mycobacteria. Subsequent to this phase, the early logarithmic bacillary growth stops. The primary lesions become necrotic in the centre, which inhibits extracellular growth of mycobacteria. As a result, infection may become stationary or dormant (Van Crevel *et al*., 2002; Raja, 2004; Tyagi and Sharma, 2004).

Disease may progress and haematogenous dissemination takes place weeks, months or years afterwards, under conditions of failing immune surveillance. The solidified necrotic foci will liquefy and provide excellent conditions for extracellular growth of *M. tuberculosis*, leading to cavitations in the lungs. Cavity formation may lead to rupture of nearby bronchi, allowing the bacilli to spread through the airways to other parts of the lung and may also result in extra-pulmonary dissemination of the TB bacilli. When infection occurs in other parts of the body, such as pleura, lymphatics, bones, meninges, peritoneum, genito-urinary system or skin, the disease is referred to as extra-pulmonary TB (Raja, 2004; Dye *et al*., 2008). This type of disease is common in immuno-compromised individuals. These events are summarized in Figure 1.1 (page 4).

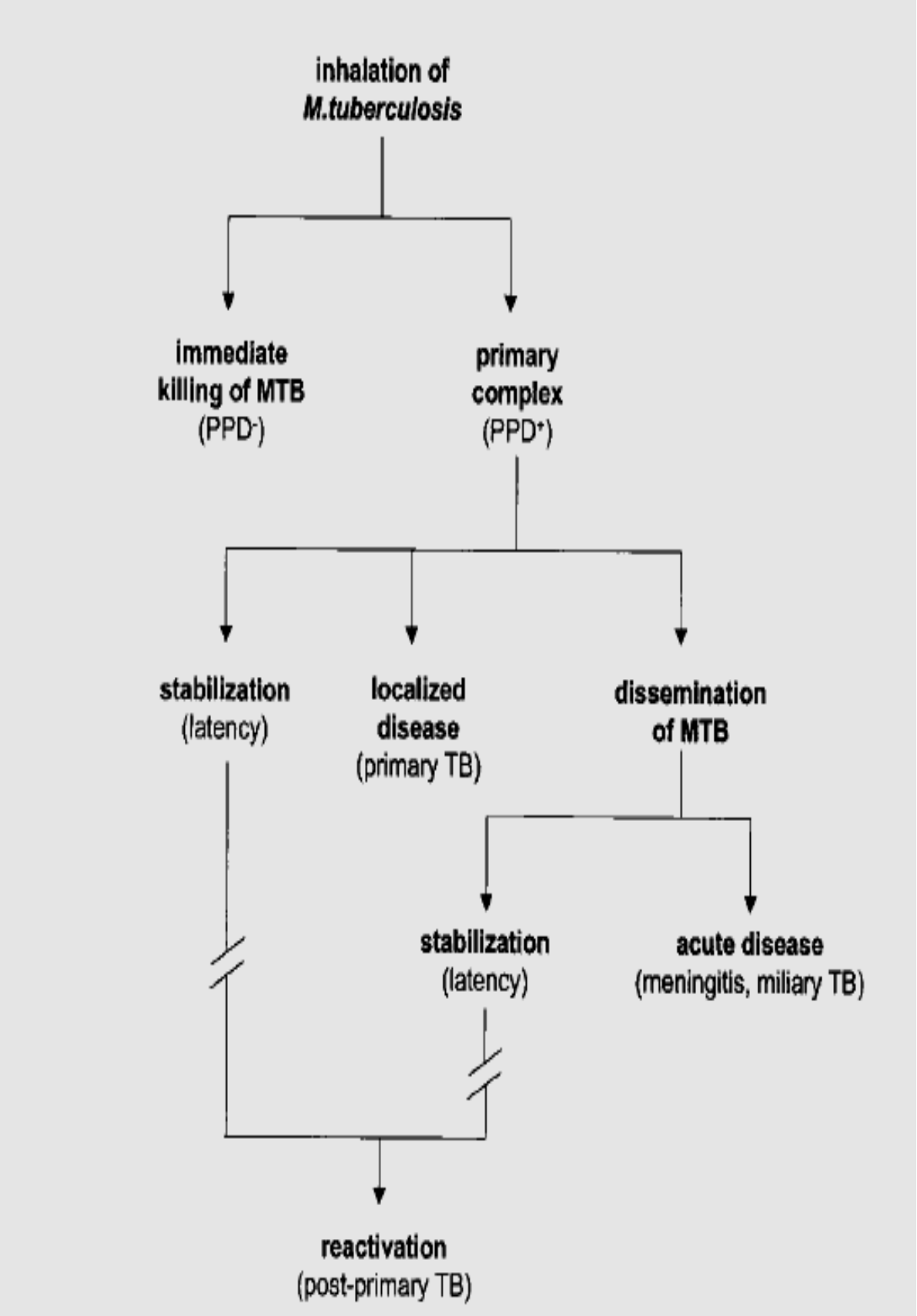


Figure 1.1. Schematic diagram of the chronological events after inhalation of *M. tuberculosis* (Van Crevel *et al*., 2002. *Clinical Microbiology Reviews*. **15(2)**:294–309).

1.1.3 Host immune mechanisms in TB

Uptake of *M. tuberculosis* by airway macrophages during the initial stages of infection involves interaction of the microbial pathogen with various surface receptors on the macrophage. These include Toll-like receptors (TLR2/ TLR4), complement receptors (CR1, CR3 and CR4), mannose receptors, cluster of differentiation (CD) 14 scavenger receptors and surfactant protein receptors (Fenton and Golenbock, 1998; Aderem and Underhill, 1999; Pasula *et al*., 1999). Signaling through TLRs results in the release of interleukin (IL)-12, which in turn promotes the release of the macrophage-activating cytokine, interferon (INF)-γ from proximal natural killer cells (Quesniaux *et al*., 2004; Korbel *et al*., 2008). IFN-γ, in turn upregulates anti-mycobacterial activities (increased production of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and nitric oxide) of the *M. tuberculosis*-infected macrophage, favouring either elimination or containment of infection. In the case of the latter scenario, containment enables activation and mobilization of adaptive immune defenses (Chan *et al*., 1992; Korbel *et al*., 2008).

1.1.3.1 Acquired immune response

Acquired immune responses to TB are mainly cell-mediated, being dependent on mature T cells. During infection, the activated T cells, both CD4+ and CD8+, migrate to the site of infection and interact with the antigen presenting cells (APC) to contain the infection and prevent reactivation (Stenger and Modlin, 1999; Harding and Boom, 2010). CD4+ T cells play a critical protective role in host defense against *M. tuberculosis*, by producing INF-γ and other cytokines, which activate macrophages. In humans, the pathogenesis of HIV infection has demonstrated that the progressive loss of CD4+ T cells greatly increases susceptibility to both acute and reactivation TB. The CD8+ T cells are also capable of secreting cytokines such as INF-γ and IL-4, and may play a role in regulating the balance of Th1 and Th2 cells at the site of infection. Th1 cells secrete IL-2 and INF-γ, and play a protective role in intracellular infections, while Th2 cells secrete IL-4, IL-5 and IL-10 and may exert a negative influence on the cell-mediated immune response (Raja, 2004).

1.1.4 Evasion of host immune response by MTB

M. tuberculosis is equipped with numerous immune evasion strategies, including modulation of antigen presentation, to avoid elimination by T cells. Proteins secreted by *M. tuberculosis* such as superoxide dismutase and catalase, neutralize antimicrobial reactive oxygen intermediates (ROI). Mycobacterial components such as sulphatides, lipoarabinomannan

(LAM) and phenolic glycolipid 1 (PGL-1) are also potent oxygen radical scavengers (Guenin-Mace *et al*., 2009). *M. tuberculosis*-infected macrophages appear to be diminished in their ability to present antigens to CD4+ T cells, which leads to persistent infection. Another mechanism by which antigen-presenting cells contribute to defective T cell proliferation and function is by production of anti-inflammatory cytokines, including TGF-β, IL-10 or IL-6 (Giacomini *et al*., 2001). In addition, it has also been reported that virulent mycobacteria were able to escape from fused phagosomes and multiply (Clemens and Horwitz, 1995; De Chastellier *et al*., 1995; De Chastellier *et al*., 2009). Activation of the macrophages is induced by bacterial components that are Toll-like receptor agonists, including lipoarabinomannan (LAM), lipoteichoic acid, muramyl dipeptide and heat shock proteins. Analysis of *M. tuberculosis*-specific responses revealed inhibition of IL-12 production, suggesting one means by which this organism survives host defenses (Nau *et al*., 2002).

1.1.5 Role of Toll-like receptors in MTB infection

The detection, recognition and response to microbial infection by the immune system depend to a considerable extent on a family of pattern-recognition receptors, Toll-like receptors (TLRs) (Takeda and Akira, 2005). These receptors which are summarized in Figure 1.2 (page 7), are expressed in many cell types such as macrophages, neutrophils, dendritic cells, and mucosal epithelial cells, although most cell types express only a select subset of these receptors (Kabelitz, 2007). They are evolutionarily conserved to recognize a variety of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), lipoprotein/lipopeptide, and flagellin, endogenous DAMPs including heat shock proteins (HSPs), calcium-binding proteins S100A8/A9, DNA and RNA viruses, fungi and protozoa and they show considerable target specificity (Akira and Takeda, 2004; Liew *et al*., 2005; Halayko and Ghavami, 2009; Wheeler *et al*., 2009).

To date, 11 mammalian TLRs have been identified, numbered 1–11 in humans and 13 in mice (Akira, 2004; Verstack *et al*., 2007). TLRs 1-9 are conserved in humans and mice, while TLR10 is present only in humans (Balaram *et al*., 2009). The C-terminal half of *Tlr10* gene in mice is substituted by a non-related sequence and the TLR10 in mice is therefore non-functional (Takeda and Akira, 2005). In contrast, in mice TLR11 is functional and silenced by a stop codon in humans, resulting in lack of its translation (Zhang *et al*., 2004; Balaram *et al*., 2009). The biological roles, the expression patterns, ligands, and modes of signaling of TLRs 10, 12, and 13 remain to be defined (Balaram *et al*., 2009; Wu *et al*., 2010). TLR2 and

TLR4 are the principal receptors for recognition of various bacterial cell wall components (Hirschfeld *et al*., 2000). TLR2 plays an important role in mediating inflammatory responses to peptidoglycan, lipoarabinomannan (LAM), lipoproteins and lipoteichoic acid (LTA) from Gram-positive bacteria (*e.g. S. pneumoniae*) and mycobacteria (Harju *et al*., 2001; Means *et al*., 2001; Knapp *et al*., 2004), and to some rare LPS species such as that from *Porphyromonas gingivalis* (Martin *et al*., 2001). TLR4 is crucial for effective host cell responses to Gram-negative bacterial lipopolysaccharide (LPS) (Poltorak *et al*., 1998; Hoshino *et al*., 1999; Hirschfeld *et al*., 2000). Furthermore, TLR4 has been shown to be involved in the recognition of endogenous ligands, such as heat shock proteins (HSP60 and HSP70), the extra domain A of fibronectins, oligosaccharides of hyaluronic acid, heparan sulfate and fibrinogen, but all these ligands require very high concentrations to activate TLR4 (Gao and Tsan, 2003).

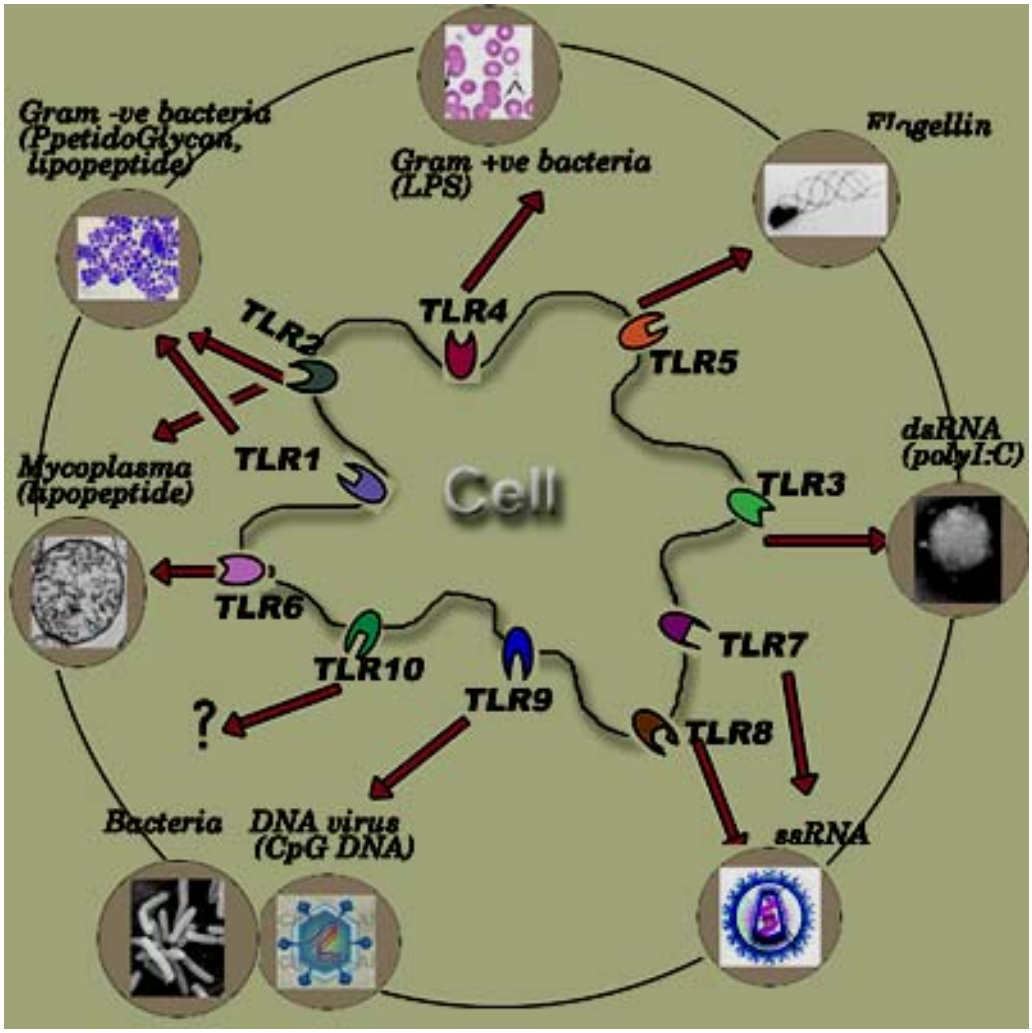


Figure 1.2. Toll-like receptors (TLRs) and their ligands (Lata and Raghava, 2008. *BMC Genomics*, **18**; 9: 180).

Receptors involved in *M. tuberculosis* pattern recognition include the mannose receptor, complement receptors (CR) CR1, CR3, and CR4 (Schlesinger, 1993), and Toll-like receptors TLR2 and TLR4. Both TLR2 and TLR4 can mediate cellular activation by *M. tuberculosis*, specifically, soluble heat-stable and protease-resistant factors via the TLR2 pathway and heat-sensitive membrane-associated factors via both receptors (Lien *et al*., 1999; Means *et al*., 1999).

It has been demonstrated that TLR2 can mediate mycobacterial lipoprotein-induced mechanisms including mycobacterial killing and apoptosis (Aliprantis *et al*. 1999; Brightbill *et al*., 1999; Underhill *et al*., 1999a; Underhill *et al*., 1999b; Thoma-Uszynski *et al*., 2000; Thoma-Uszynski *et al*., 2001). Documented *in vitro* studies have shown opposing outcomes from antigen-presenting cells (APC) following interaction of their surface TLR2 with *M. tuberculosis*. Studies that have used Chinese hamster ovary (CHO) cells as a transfection model demonstrated that the expression of TLR2 or TLR4 conferred responsiveness to both virulent and attenuated *M. tuberculosis* (Means *et al*., 1999). TLR4 plays an ambiguous role with some studies showing no effect of its deficiency (Abel *et al*., 2002), while in others it resulted in impaired clearance of tuberculosis and increased mortality (Drennan *et al*., 2004; Chaudhuri *et al*., 2005).

Other TLRs may be involved in immune recognition of *M.tuberculosis* besides TLR2 and TLR4. Usually TLR2 forms heterodimers with either TLR1 or TLR6 necessary for signal transduction (Ozinsky *et al*., 2000; Bulut *et al*., 2001), and TLR9 recognizes unmethylated bacterial or viral cytosine-phosphate-guanine DNA (CpG-DNA) (Hemmi *et al*., 2000). It appears that TLR9 also activates effective Th1 responses during *M. tuberculosis* infection in mice. Double deficiency in TLR9 and TLR2 has shown enhanced susceptibility to the mycobacteria, but this phenomenon in mice lacking either TLR2 or TLR9 alone has not been observed. It is therefore concluded that TLRs collaborate in the host to regulate responses to mycobacterial infection in the lung (Bafica *et al*., 2005).

1.1.6 Monocytes/ Macrophage

Monocytes and macrophages are critical effectors and regulators of inflammation and the innate immune response. They are regarded as the immediate arm of the immune system (Geissmann *et al*., 2010). Monocytes represent a subgroup of leukocytes originally described as a population of bone marrow-derived myeloid cells that circulate in the blood (Van Furth

and Cohn, 1968). They are equipped with chemokine receptors and adhesion receptors that mediate migration from blood to tissues during infection and also produce inflammatory cytokines and take up cells and toxic molecules. They can also differentiate into inflammatory dendritic cells (DCs) or populate tissues as macrophages during inflammation in the steady state (Serbina *et al*., 2008). The functional heterogeneity of these cells is shown in Figure 1.3 (page 10).

The macrophages become long-lived cells and develop specialized functions once they have differentiated. Their numbers are maintained by resistance to constitutive apoptosis (Murphy *et al*., 2008a), recruitment of further monocytes from blood and/or replication of local intermediates which is dependent on the prevailing stimulus and anatomical location (van oud Alblas and van Furth, 1979; Landsman and Jung, 2007).

Macrophages are prodigious phagocytic cells that clear approximately 2 x 10^{11} erythrocytes each, making a vital metabolic contribution of recycling almost 3kg of iron and haemoglobin per year for the host to reuse (Kono and Rock, 2008), while most of the phagoytosis that occurs on a daily basis by macrophages does not depend on other immune cells. The main function of macrophages is to clear the interstitial environment of extraneous cellular material which makes them function not as elite immune effector cells, but instead as common 'janitorial' cells (Zhang and Mosser, 2008).

Macrophages provide an innate, antigen-nonspecific first line of defense against infection caused by the microorganisms that enter the body by engulfing and destroying them through phagocytosis (Gordon, 1999). Bacteria are an important and highly diverse class of human pathogens which are recognized by macrophages. However, these pathogens have developed many mechanisms to avoid elimination by innate immunity, hence their ability to propagate (Pieters, 2001). Macrophages that have bound and ingested microorganisms, but have failed to destroy them, contribute to adaptive immune responses by presenting these foreign antigens to naive T cells (Gordon *et al*., 2000).

Clearly, the macrophage is both the primary target of *M. tuberculosis*, as well as being a key effector cell in the eradication of this microbial pathogen. However, the macrophage is dependent on efficient assistance from the Th1 cell to promote effective clearance of intracellular *M. tuberculosis*. Decreased numbers and/ or defective function of Th1 cells

results in chronic infection, characterised by an ineffective chronic inflammatory response in the airways, which, if not successfully treated, results in progressive pulmonary damage (Wilson *et al*., 1998; Orme and Cooper, 1999; Saunders and Cooper, 2000). This is due to the chronic release of indiscriminate toxic ROI and proteases from activated macrophages, which will also ultimately promote extra-pulmonary dissemination of *M. tuberculosis*. Effective antimicrobial chemotherapy of established *M. tuberculosis* infection requires the use of antimicrobial agents which are not only active against this microorganism, but which penetrate the infected macrophage and are bioactive intracellularly.

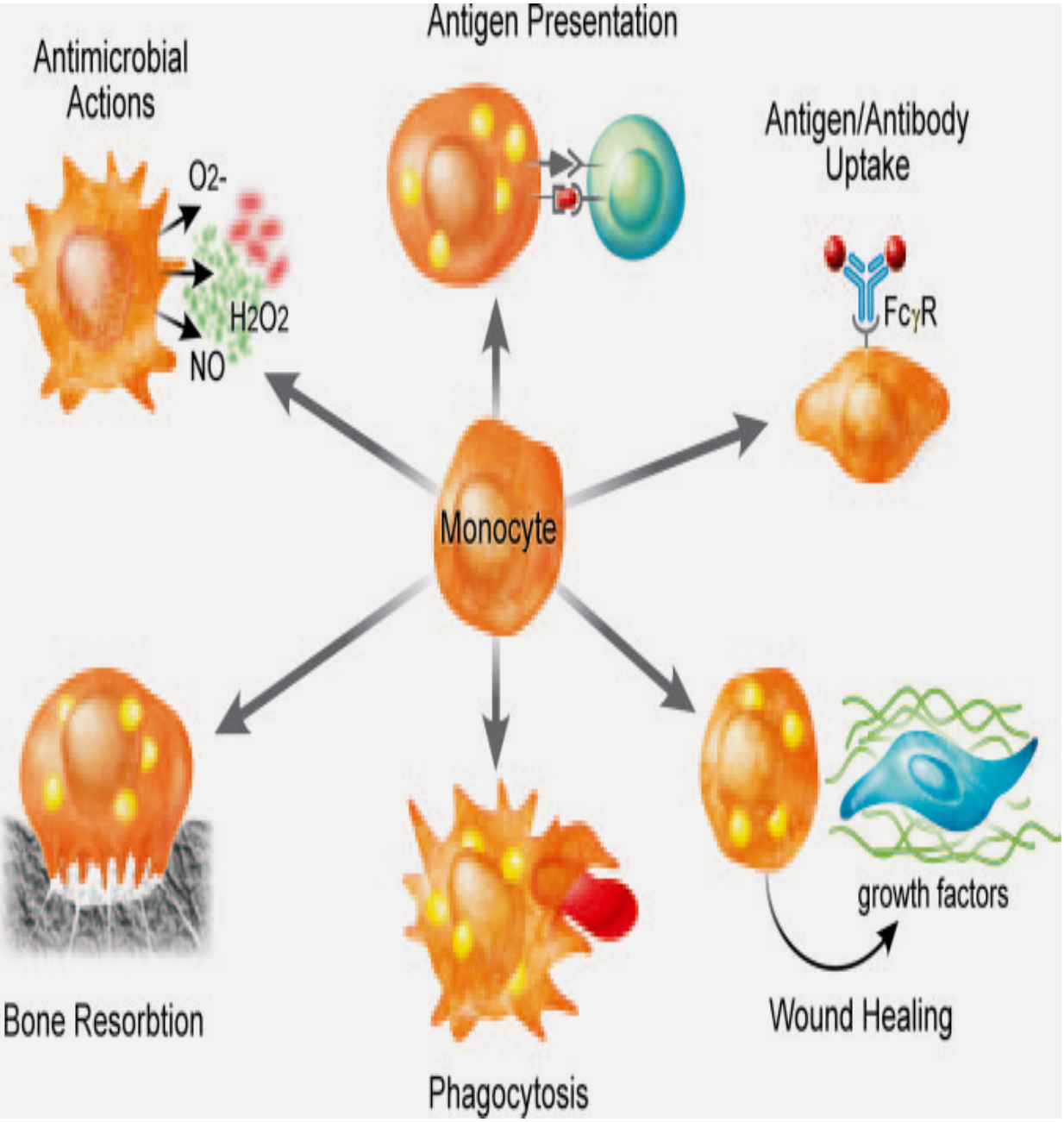


Figure 1.3. Functional heterogeneity in resident and recruited macrophages (Chawla, 2010. *Circulation Research.* **106**:1559-1569).

1.1.6.1 Adaptation of macrophages to MTB infection

Hosts identify invading microorganisms by use of the proteins called pathogen-associated molecular patterns (PAMPs), found on the cells of the innate defense system such as macrophages. They have also evolved mechanisms to identify these patterns through the action of pattern recognition receptors (PRRs) (Janeway Jr., 1989; Akira *et al*., 2006). PAMP signatures for pathogenic mycobacteria include CpG DNA, 19 kDa lipoprotein, lipoarabinomannan (LAM) and mannosylated phosphatidylinositol (PIM) (Ryffel *et al*., 2005).

As mentioned earlier, TLRs are regarded as an important class of evolutionarily conserved pathogen recognition receptors with a role that goes beyond recognizing pathogens. They are also able to trigger host defense mechanisms against invading microbes (Krutzik and Modlin, 2004). Macrophage responses include up-regulation of phagocytosis of bacteria, promotion of phagosome maturation and expression of pro-inflammatory cytokines (Blander and Medzhitov, 2004; Doyle *et al*., 2004). These responses occur upon TLR stimulation which has also been reported to promote phagosomal maturation via activation of p38 MAP kinase in a MyD88 dependent signaling relay (Blander and Medzhitov, 2004).

In recent studies, autophagy was recognized as one of the host responses to infection and also proposed as a component of innate immune mechanisms, although it was previously unappreciated (Gutierrez *et al*., 2004). Autophagy is the cellular process by which a cell degrades its own intracellular compartments, including parts of the cytoplasm, leaky mitochondria, and excessive peroxisome, into double membrane structures called autophagosomes, and promotes their delivery to lysosomes. Studies in mice have shown that stimulation of autophagy through pharmacological, physiological or immunological means results in elimination of intraphagosomal mycobacteria in infected cells (Shintani and Klionsky, 2004).

1.1.6.2 Activation of macrophages

Macrophages are activated by pathogens which are engulfed, resulting in the initiation of inflammatory responses. As mentioned above, these cells express receptors for many bacterial components, including bacterial carbohydrates (mannose and β-glucan receptors), lipids (LPS receptor) and other pathogen-derived components (Toll-like receptors (TLRs) and

scavenger receptor-A (SR-A) (Gordon and Hughes, 1997). Binding of bacteria to macrophage receptors stimulates the phagocytosis and uptake of pathogens into intracellular vesicles, where they are destroyed. Signaling through these receptors in response to bacterial components causes the secretion of 'pro-inflammatory cytokines' such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α).

Nramp1, a gene which codes for natural-resistance-associated macrophage protein (Nramp) is also involved in macrophage activation and mycobacterial killing (Blackwell *et al*., 2000). This integral membrane protein belongs to a family of metal ion transporters. These metal ions, particularly Fe^{2+}, are involved in macrophage activation and generation of toxic antimicrobial radicals (Zwilling *et al*., 1999).

A well-established feature of cellular immunity to infection with intracellular pathogens such as *M. tuberculosis* and HIV is the classical pathway of activation of macrophages. The understanding of this type of activation dates from studies in the 1960s which have shown that such pathways depend on the products of specifically activated T helper (Th1)-type lymphocytes and natural killer (NK) cells- in particular, interferon-γ (IFN-γ) and a cytokine network involving interleukin (IL)-12 and IL-18, which are produced by antigen-presenting cells (APCs) (Dalton *et al*., 1993). Other molecules which have macrophage-activating activity include tumour necrosis factor-alpha (TNF-α) and granulocyte-macrophage colony stimulating factor (GM-CSF). These molecules also promote human macrophage anti-tuberculosis activity (Denis and Ghadirian, 1990; Denis *et al*., 1990).

The concept of an alternative pathway of macrophage activation is less well defined. During this activation, IL-4 and IL-13 cytokines are produced generally in a Th2-type response, particularly in allergic, cellular and humoral responses to parasitic and extracellular pathogens. The effects of these cytokines on macrophages have been grouped together with those of IL-10, as being deactivating (Ehrt *et al*., 2001). This is associated with transition from the M1 to M2 phenotype. Pathways of macrophage activation are shown in Figure 1.4 (page 13).

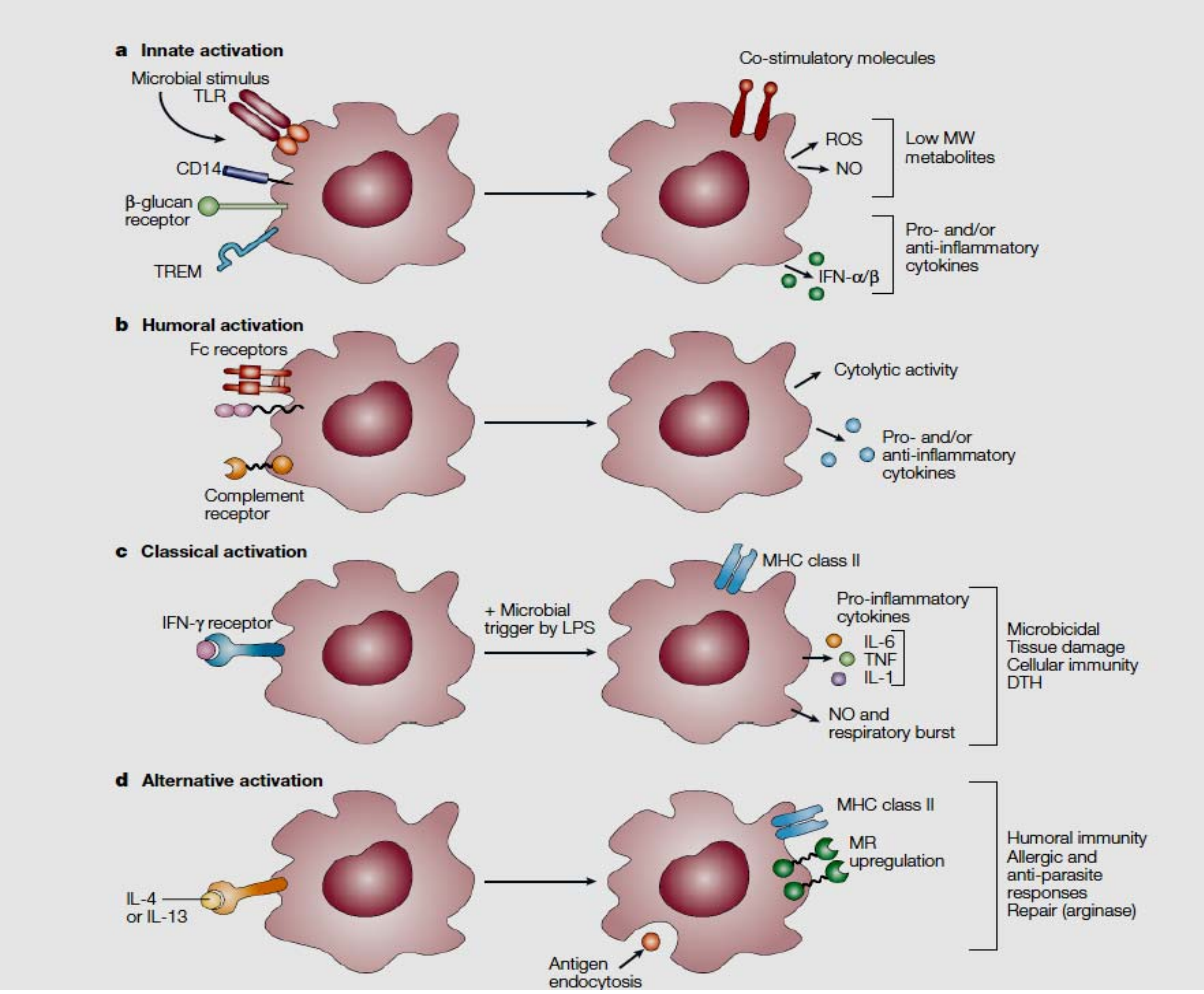


Figure 1.4. Innate and acquired immune activation of macrophages (Gordon, 2003. *Nature Reviews: Immunology*. **3**: 23-35).

1.1.6.3 Antimicrobial mechanisms of macrophages

Mechanisms of intracellular killing of *M. tuberculosis* by macrophages are not fully understood, but these cells are able to create the hostile environment of the acidified phago-lysosome containing a host of bacterial enzymes, and direct bactericidal effector functions which include generation of reactive nitrogen and oxygen intermediates (RNI and ROI) (Chan *et al*., 1992; Chan *et al.*, 1995; Nathan and Shiloh, 2000; Raja, 2004). In mouse models of TB infection, the actions of inducible nitric oxide synthase (iNOS) and the release of nitric oxide (NO) represent a powerful and necessary antimycobacterial defense mechanism (Chan *et al*., 1992; Chan *et al*., 1995; MacMicking *et al*., 1997). It has not been possible, however, to consistently demonstrate NO-mediated killing in human macrophages (Rockett *et al*.,

1998; Thoma-Uszynski *et al*., 2001), although iNOS has been detected in macrophages from human disease lesions (Nicholson *et al*., 1996).

As mentioned earlier, several studies have explored the role of autophagy, a repair mechanism of eukaryotic cells in host defense against infectious agents (Deretic *et al*., 2006). This mechanism is frequently associated with cell starvation and has been shown to eliminate intracellular bacteria through the delivery of ubiquitin-derived peptide to the mycobacterial vacuoles (Gutierrez *et al*., 2004; Alonso *et al*., 2007). Autophagy connects with apoptosis through several pathways (Deretic *et al*., 2006). It is assumed that when macrophages infected with *M. tuberculosis* fail to kill this pathogen by autophagy, then the host uses apoptosis as an alternative strategy to eliminate the intracellular bacteria (Duan *et al*., 2002).

1.1.6.4 Respiratory burst of macrophages

Macrophages have important roles in maintaining the normal physiology of tissues by secreting growth factors, contributing to the immune surveillance by killing of tumors, and as antigen presenting cells. They also play a critical role in the regulation of inflammation by initiating the process through secretion of pro-inflammatory cytokines and then resolving it through phagocytosis of apoptotic and necrotic cells. Macrophages express a functional nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase, although they are also armed with an array of non oxidant-dependent tools for killing tumor cells and microbes. The oxidative mechanisms are generally implied to be involved in the killing process, but only a few studies have clearly demonstrated a requirement for the respiratory burst in macrophage killing (Forman and Torres, 2001).

Macrophages also produce nitric oxide (NO) by a high-output form of nitric oxide synthase, iNOS2. Superoxide is generated by a multi-component, membrane-associated NADPH oxidase during the respiratory burst caused by a transient increase in oxygen consumption; the superoxide is then converted into H_2O_2 by the enzyme superoxide dismutase. Further chemical and enzymatic reactions produce a range of toxic chemicals from H_2O_2, including the hydroxyl radical (•OH), hypochlorite (OCl^-) and hypobromite (OBr^-) (Murphy *et al*., 2008b).

1.1.7 Cytokine production in MTB infection

As mentioned earlier, cytokines are members of a large family of secreted proteins that bind immune cells through specific receptors, which then signal the cell via second messengers, often tyrosine kinases, to alter gene expression and thereby cell behavior (Balaram *et al*., 2009). The production of these proteins results in the activation of an intracellular signalling cascade that mediates and regulates immunity, inflammation, and hematopoiesis (Russell, 2007). Cytokines are usually short-lived, produced locally in response to a stimulus, either acting in an autocrine, exocrine, or endocrine fashion. Interaction of *M. tuberculosis* with the TLRs on macrophages and dendritic cells leads to cell activation and production of cytokines and chemokines (Salgame, 2005; Ulrichs and Kaufmann, 2006; Russell, 2007). This cytokine network is responsible for inducing the inflammatory response and the outcome of mycobacterial infections (Van Crevel *et al*., 2002).

1.1.7.1 Pro-inflammatory cytokines

The protective immune response to *M. tuberculosis* infection relies mostly on the production of pro-inflammatory, or Th1 cytokines, which are produced mainly by Th1 lymphocytes. One of the key activators of macrophages produced by the Th1 cells is interferon gamma (IFN-γ) (Flesch and Kaufmann, 1987a; Champsi *et al*., 1994; Lake *et al*., 1994). As alluded to earlier, IFN-γ plays a protective role in *M. tuberculosis* infection (Flesch and Kaufmann, 1987b), primarily in the context of antigen-specific T-cell immunity (Andersen, 1997). It is well known that IFN-γ control of macrophage activation is associated with microbicidal and tumoricidal activity (Bloom and Bennett, 1970; David, 1973) by up-regulating lysozymal proteolytic enzymes and oxygen radicals (Hu and Ivashkiv, 2009).

Th1 cells produce additional cytokines, most notably TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF), which stimulates granulocyte and macrophage maturation (McGeachy and Cua, 2008; Ouyang *et al*., 2008). IL-3 is also a member of this large group of cytokines, and is characterised by its ability to stimulate the development of various lineages of hematopoietic cells *in vitro*. It was originally referred to as a multi-colony stimulating factor due to its interaction with immature multipotential hematopoietic progenitors as well as with lineage-committed progenitors in colony assays (Arai *et al*., 1990). IL-3 is now known to share many of its functions with the GM-CSF (Metcalf, 1991).

IL-12 is produced mainly by phagocytic cells, and phagocytosis of *M .tuberculosis* bacilli seems necessary for the production of this cytokine (Fulton *et al*., 1996; Ladel *et al*., 1997). It also has an important role in the induction of IFN-γ production (O'Neill and Greene, 1998). Early studies demonstrated that mice deficient in the p40 component of IL-12 were highly susceptible to *M. tuberculosis* infection (Flynn *et al*., 1995a). The bacterial burden was increased and was associated with a decrease in survival time probably due to the reduction of IFN-γ production (Cooper *et al*., 1997).

TNF-α is a pleiotropic cytokine produced primarily by monocytes, macrophages, and dendritic cells, as well as by Th1 cells (Henderson *et al*., 1997; Dempsey *et al*., 2003). It synergizes with IFN-γ in macrophage activation and also plays an important role in the host immune response to infection with *M. tuberculosis*. TNF-α is known to be essential for the formation of protective tuberculous granulomas, which serve to control bacterial infection (Roach *et al*., 1999). Studies on TNF-α-receptor-knockout mice showed decreased survival and disrupted granuloma formation compared to wild-type mice following infection with virulent *M. tuberculosis* (Flynn *et al*., 1995b). In addition, TNF-α-deficient mice became highly susceptible to reactivation of TB infection (Marino *et al*., 1997; Botha and Ryffel, 2003).

1.1.7.2 Anti-inflammatory cytokines

The Th2 cytokine profile involves IL-4, IL-5, IL-13, IL-10, and transforming growth factor beta (TGF-β) (Van Crevel *et al*., 2002). These cytokines typically induce humoral type responses and are usually involved in immune responses to helminths, allergies and low-dose antigens (Jancovic *et al*., 2001). These anti-inflammatory or Th2 cytokines antagonize the pro-inflammatory responses which are initiated by *M. tuberculosis* and also act as antagonists of macrophage activation (Van Crevel *et al*., 2002; Rook, 2007). Stimulation of Th2 activity and the induction of B cells to secrete IgE and IgG_1 are ascribed to IL-4, while it suppresses the production of IFN-γ (Powrie and Coffman, 1993; Lucey *et al*., 1996). Data from two studies demonstrated that IL-4 deficient mice displayed normal instead of increased susceptibility to mycobacteria, suggesting that IL-4 may be a consequence rather than the cause of development of TB (Erb *et al*., 1998; North, 1998). Another study on IL-4 KO mice showed contradictory results in which the granuloma size was increased and there was also mycobacterial outgrowth after airborne infection (Sugawara *et al*., 2000).

IL-10 is produced by macrophages and T cells after phagocytosis of *M. tuberculosis*. This cytokine possesses macrophage-deactivating properties and antagonizes pro-inflammatory cytokine responses by down-regulation of production of IFN-γ, TNF-α, and IL-12 (Gong *et al*., 1996; Fulton *et al*., 1998; Hirsch *et al*., 1999; Rojas *et al*., 1999).

TGF-β is produced by human monocytes after stimulation with *M. tuberculosis* (Toossi *et al*., 1995a). Production of this cytokine is also selectively induced by LAM from virulent mycobacteria (Dahl *et al*., 1996). TGF-β has the ability to down-modulate macrophage effector functions such as the production of ROI and RNI and is therefore thought to play a role in the pathogenesis of TB (Toossi *et al*., 1995a; Toossi *et al*., 1995b; Dahl *et al*., 1996). TGF-β and IL-10 seem to synergize within the anti-inflammatory response. IL-10 production is selectively induced by TGF-β, and both cytokines demonstrate synergy in suppressing the production IFN-γ (Othieno *et al*., 1999).

1.1.8 Chemokines

Chemokines are small 8–10 kDa chemotactic cytokines, which bind to pertussis toxin sensitive G-protein coupled receptors, inducing actin dependent processes such as membrane ruffling, pseudopod formation and assembly of adhesion complexes (Scott Algood *et al*., 2003). These molecules contribute to cell migration and localization, as well as modulating priming and differentiation of T cell responses (Bonecchi *et al*., 1998). Chemokines act both as constitutive signals, functioning to form secondary lymphoid tissues, and as inducible signals in response to physiological stress, such as inflammatory stimuli including infection (Scott Algood *et al*., 2003). It is believed that chemokines are involved in the pathogenesis of several lung diseases, such as asthma, tuberculosis, sarcoidosis, and chronic bronchitis, where they recruit leukocytes to the inflammatory site (Miotto *et al*., 2001). Functional consequences of chemokine receptor activation are not limited to locomotion as these molecules also affect granule exocytosis, gene transcription, mitogenic effects and apoptosis (Thelen, 2000).

The known chemokine system in humans is estimated to consist of approximately 50 mediators and 20 G protein–coupled receptors. Many cell types including endothelia, smooth muscle cells, stromal cells, neurons and epithelial cells express chemokine receptors, although the primary targets of chemokines are the bone marrow-derived cells (Rollins, 1997). Chemokines segregate into four families based on the differences in their structure and

function (Handel and Domaille, 1996; Luster, 1998; Gerard and Rollins, 2001; Rot and von Andrian, 2004; Cyster, 2005), C-X-C, C-C, C, and C-X3-C, depending on the number and spacing of conserved cysteine residues (Kelner *et al*., 1994; Prieschl *et al*., 1995; Bazan *et al*., 1997).

One major family is named the C-C chemokines because the first two of the four cysteine residues in these molecules are adjacent to each other. These chemokines include monocyte chemotactic protein-1 (MCP-1), also termed chemokine ligand CCL2 in the systematic nomenclature, macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), and regulated on activation normal T cell expressed and secreted (RANTES/ CCL5) (Adams and Lloyd, 1997; Proudfoot *et al*., 2003). C-C chemokines predominantly exert activating and chemotactic effects on eosinophils, lymphocytes, and monocytes (Miotto *et al*., 2001).

A second family of chemokines consists of CXC chemokines in which two cysteine residues are separated by a single amino acid. CXC chemokines include IL-8 (CXCL8), which is the prototype, and recruit neutrophils and lymphocytes to sites of acute inflammation. CXCL8 is also involved in the activation of monocytes and may direct the recruitment of these cells to vascular lesions (Gerszten *et al*., 1999; Huo *et al*., 2001).

The third family is the CX3C, which consists of only one member called fractalkine (CX3CL1) (Bazan *et al*., 1997; Pan *et al*., 1997). The CX3CL1 domain is fused to a mucin-like stalk and transmembrane and cytoplasmic regions, which forms a cell-adhesion receptor capable of arresting cells under physiologic flow conditions (Fong *et al*., 1998; Haskell *et al*., 1999). The sole member of the fourth family, lymphotactin (XCL1), has a single cysteine residue (Kelner *et al*., 1994). The different families of chemokines are shown in Table 1 (page 19).

Participation of chemokines in the control of *M. tuberculosis* infections has been investigated to a limited extent. Several studies have addressed the role of IL-8, which attracts neutrophils, T-lymphocytes, and possibly monocytes. It was demonstrated that macrophages that had phagocytosed *M. tuberculosis* or were stimulated with LAM, produced IL-8 (Zhang *et al*., 1995; Juffermans *et al*., 1999). Other chemokines that are involved in the host response to tuberculosis include monocytes chemoattractant protein-1 (MCP-1), which is produced by and acts on monocytes and macrophages and RANTES. A study on mice showed that

deficiency of MCP-1 inhibited the formation of granuloma (Lu *et al*., 1998). In studies done on patients with TB, concentrations of MCP-1 were found to be elevated in alveolar lavage fluid (Kurashima *et al*., 1997), serum (Juffermans *et al*., 1999), and pleural fluid (Mohammed *et al*., 1998). Development of *M. bovis* induced pulmonary granulomas in murine models has been associated with the expression of RANTES (Chensue *et al*., 1999). In *in vitro* studies in which human alveolar macrophages were infected with *M. tuberculosis*, monocytes, lymph node cells and BAL fluid from patients released high levels of RANTES, MCP-1 MIP1-α and IL-8 as compared to healthy controls (Kurashima *et al*., 1997; Sadek *et al*., 1998).

Chemokines
CXCL8 (Interleukin-8)
CXCL8
CCL2 (MCP-1)
CCL2, CXCL8, CCL3 (MIP1α), CCL5 (RANTES)
CCL3, CCL4 (MIP1β), CCL5
CXCL9 (Mig), CXCL10 (IP-10), CXCL11 (I-TAC)

Table 1.1. Chemokines in human tuberculosis (Peters and Ernst, 2003. *Microbes and Infection*. **5**:151–158).

1.1.9 History of chemotherapy of MTB infection

One of the greatest works on TB was performed by Robert Koch 1882, an esteemed scientist of his time. His experimental work identified the bacterium as the TB etiological agent and also led to the discovery of a TB therapeutic drug named tuberculin which was announced in 1890. This discovery allowed researchers to focus efforts on development of new and more efficient therapies to treat TB patients. Another great discovery, made in 1908, was the BCG (Bacille Calmette-Guérin) intradermal vaccine which became widely used to combat TB; this strategy relies on a prophylactic administration of live attenuated bacilli to newborns and young children to help protect them from developing severe disseminated TB (miliary TB).

One of the most remarkable achievements of the twentieth century was the development of chemotherapeutic agents for the treatment of microbial diseases. The era of the "anti-microbials" began with the discovery of the sulphonamides in the 1930's (Greenwood, 1997). From then to the current date, discoveries of many other antimicrobial agents have been made and a number of these have now become established drugs used as treatment for mycobacterial infections. These are shown in Table 2 (page 23) (Davidson and Le, 1992; Tillotson, 1996; Grange, 1997; Watt, 1997). Drugs used to target and specifically treat the causative agent became available for the first time only in 1944 when streptomycin was discovered by Selman Waksman (Waksman, 1944; Hinshaw and Feldman, 1945). This was soon followed by para-aminosalicylic acid (PAS) in 1949, isoniazid in 1952, the discovery of rifampicin in the late 1960s and the rediscovery of the antimycobacterial activity of pyrazinamide soon after. These discoveries were major breakthroughs in the treatment of tuberculosis as some of them made it feasible to shorten the duration of treatment considerably (Fox, 1981; Fox *et al*., 1999).

Observations from both animal and human studies have suggested that a patient with tuberculosis can harbour four hypothetical populations of organisms (Parrish *et al*., 1998). The currently available anti-tuberculosis drugs are proposed to have three major actions (Mitchison, 1979). Firstly, their bactericidal action, which is the ability to kill actively growing bacilli rapidly, and often this ability, is assessed by the decrease in quantitative sputum culture bacillary count in the first few days of treatment. Secondly, their sterilising action, which is the ability to kill persisters under acid inhibition or with spurts of metabolism, and this is revealed by the ability to prevent relapse or its proxy marker, such as

2-month sputum culture conversion rate (Mitchison, 1993). And thirdly is their ability to prevent the emergence of bacillary resistance to drugs.

In order to treat tuberculosis successfully, prolonged administration of a minimum of three drugs to which the organisms are susceptible, and at least one drug which is bactericidal, is required. It was evident from early clinical trials that monotherapy led to the spontaneous emergence of drug resistance in a small number of tubercle bacilli, with even the most potent bactericidal drugs and this also led to failure of the treatment (Centers for Disease Control, 1994; Department of Health and Human Services, 1994; De Cock *et al*., 1995). From the late 1960s, chemotherapy involved the administration of a combination of multiple drugs as this appeared more effective for treating tuberculosis (Mitchison, 2005).

Antibiotics against TB can be classified into two lines of combination treatment namely, the first line drugs, i.e. isoniazid, rifampicin, pyrazinamide, ethambutol, and streptomycin, while the second line drugs which are dictated by the development of drug resistance to the first line agents, but these drugs have less efficacy and greater toxicity (British Medical Association and Royal Pharmaceutical Society of Great Britain, 2006).

The following are considered the most effective and extensively used drugs at this time for the treatment of tuberculosis. Isoniazid is the most potent bactericidal drug available, killing more than 90% of bacilli within 7 days and is particularly effective against actively growing organisms (Dean *et al*., 2002). It is a relatively non-toxic, easily administered, and inexpensive drug which is also effective in preventing the emergence of drug resistance. The mechanism of action for this drug is complex and includes the inhibition of mycolic acid synthesis (Davis, 2007). Hepatotoxicity is one of the frequently reported adverse effects of isoniazid. The increase in probability of this side effect is associated with patients with a history of excessive alcohol consumption or hepatitis infection (Watts and Lifeso, 1996).

Rifampicin is a bactericidal drug with a potent sterilizing effect and the ability to prevent emergence of drug resistance. The mechanism of action for this drug involves the inhibition of DNA-dependent RNA polymerase. The most common adverse reaction is gastrointestinal upset, but mild jaundice may also occur (Watts and Lifeso, 1996; Dean *et al*., 2002).

Pyrazinamide, although bactericidal for *M. tuberculosis* as well, is used mostly for its sterilising effect, and its efficacy for killing bacilli sequestered by macrophages in an acid environment. The exact mechanism of action for this drug is uncertain, but inhibition of fatty acid synthesis in *M. tuberculosis* has been reported. Side effects include hepatotoxicity, gout, and labile blood sugar measurements in diabetes (Watts and Lifeso, 1996; Dean *et al*., 2002).

Ethambutol is a less potent, bacteriostatic drug. This drug works by inhibiting incorporation of mycolic acids into cell wall and interfering with an enzyme involved in the synthesis and stabilisation of RNA. The most frequent and serious adverse effect is retrobulbar neuritis, with symptoms including blurred vision, central scomata, and color blindness (Watts and Lifeso, 1996; Dean *et al*., 2002; Davis, 2007).

Streptomycin is also a less potent bactericidal drug. This antibiotic can play a role in HIV-infected patients who show an increased risk of drug resistance (Dean *et al*., 2002). The mode of action of this drug has been mostly studied in *E. coli*. The drug's mechanism of action in mycobacteria is comparable to that in *E. coli*, with early data pointing to the inhibition of protein synthesis by this antibiotic (Winder, 1982). The most common serious side effect is ototoxicity, which usually results in vertigo and patients may also develop pruritis and skin rash (Watts and Lifeso, 1996; WHO, 2003).

Drugs used in the second line combinations include fluoroquinolones such as sparfloxacin, ofloxacin, levofloxacin, moxifloxacin and ciprofloxacin (O'Brien and Vernon, 1998; Davis, 2007) and three most potent injectables (second-generation aminoglycosides such as kanamycin and amikacin, and cyclic polypeptides such as capreomycin) (Shah *et al*., 2007). Other drugs that are useful and included in this group are para-aminosalicylic acid, which is involved in the inhibition of folate metabolism, cycloserine and ethionamide (Davis, 2007). Second line drugs are more toxic and their level of tolerance in patients is less compared to the first line agents, hence recommendation for their use should be through consultation with a specialist in the treatment of tuberculosis. These drugs are mostly used in MDR-TB or XDR-TB cases (Watts and Lifeso, 1996).

Broad Spectrum Agents
Cycloserine
Fluoroquinolones
Macrolides
Rifamycins
Streptomycin
Narrow Spectrum (anti-mycobacterial) Agents
Capreomycin
Clofazimine
Dapsone
Ethambutol
Ethionamide
Isoniazid
Isoxyl/Thiocarlide
Para-aminosalicylic acid
Pyrazinamide
Thiacetazone

Table 1.2. Drugs used or under investigation at that time for the therapy of mycobacterial infections (Chopra and Brennan, 1998. *Tubercle and Lung Disease*. **78(2):** 89-98.

The most extensively used chemotherapeutic strategy for pulmonary TB in adults or children involves taking rifampicin, isoniazid, pyrazinamide and ethambutol for 2 months followed by a further 4 months of rifampicin and isoniazid (Fox and Mitchison, 1976; Neff, 2003; National Collaborating Centre for Chronic Conditions, 2006). Results from both trial and routine clinical use of this standard regime have shown to have cure and completion rates of more than 95% and relapse rates of 0–3% (Ormerod and Horsfield, 1987; Ormerod *et al*., 1991). Regimens of shorter periods have been associated with higher relapse rates (Gelband, 2000).

The worldwide emergence of MDR and XDR strains of *M. tuberculosis* has posed a serious concern about the continued ability to contain this disease (Barry 3rd and Blanchard, 2010). The magnitude of both MDR and XDR-TB results from their poor response to available therapeutic regimens. Drugs that are used as treatment are less effective, more toxic and much more expensive compared to those used in treatment of patients with drug susceptible tuberculosis (Iseman, 1993). Hence, in more recent years efforts have been directed at development of tuberculosis control programmes and multidrug regimens aimed at preventing the spread of tuberculosis, MDR and XDR *M. tuberculosis* infections (Bass *et al*., 1994; Bloch *et al*., 1994; Mitchison, 1998). Most activity has been centred within academic and governmental laboratories, as opposed to the biotechnology or large pharmaceutical industries (Barry 3rd, 2001; Spigelman, 2007; Ma and Lienhardt, 2009). Furthermore, there has been resurgence in interest in identifying new formulations that make use of drug-loaded microspheres (Quenelle *et al*., 2001) and newer drug combinations in order to increase their therapeutic potency and to reduce the toxicity of these anti-mycobacterial agents (Cavalieri *et al*., 1995; Abate and Hoffner, 1997; Wiid *et al*., 1999).

1.1.10 Clofazimine

The clofazimine development program began with large-scale screening of substances extracted from lichens such as usnic acid, roccellic acid, and diploicin. Diploicin, was the first organic chlorinated compound that occurred in nature which was shown to have inhibitory effects against *M. tuberculosis in vitro* at 1/100000 dilutions, but was inactive in animal models, and this prevented further development of this compound. However, molecular structure/ function studies identified a compound, termed B283, which demonstrated *in vivo* activities against leprosy and urinary tuberculosis, but with evidence of toxicity (Lane, 1951; Allday and Barnes, 1952). One chlorinated derivative of B283, known

as clofazimine or B663 demonstrated high activity against *M. tuberculosis* in murine models of experimental infection (Barry and Conalty, 1965), but very limited clinical trials in humans produced poor results.

Clofazimine is a riminophenazine antibiotic that was first synthesized in 1954 as an anti-tuberculosis lichen-derived compound. It was thought that this drug was ineffective against mycobacterial infections until, in 1959, Chang demonstrated its effectiveness against leprosy. In 1969 after clinical trials, the product was launched as Lamprene or B663 (Clofazimine, 2008). This drug has also found diverse clinical applications in several infectious and non-infectious diseases, such as atypical mycobacterial infections, rhinoscleroma, pyoderma gangrenosum (Michaelsson *et al*., 1976; Kaplan *et al*., 1992), necrobiosis lipoidica, severe acne, pustular psoriasis, and discoid lupus erythematosus (Markey and Barnes, 1974). Clofazimine acquired new prominence as a component of therapy in the treatment of *Mycobacterium avium* infections in AIDS patients (Agins *et al*., 1989), although its efficacy is questionable.

Although clofazimine is highly effective against *M. tuberculosis in vitro*, as well as in murine models of experimental, disseminated TB, it is of limited, if any, value in the treatment of TB in humans. This is probably due to the pharmacokinetic properties of this riminophenazine antimicrobial agent, which appears to have poor penetration into the airways. Consequently, current efforts are focused on the development of clofazimine nanoparticles, which can be delivered directly into the airways.

1.1.10.1 Structure and Pharmacology

Clofazimine [3-(*p*-chloroanilino)-10-(*p*-chlorophenyl)-2,10-dihydro-2-(isopropylimino)-phenazine], the molecular structure of which is shown in Figure 1.5 (page 30), belongs to a group of phenazines consisting of substituents on the N_2, N_3, and C_7-atoms, which were termed riminophenazines by Barry and co-workers (Barry *et al*., 1957; British Pharmacopoiea Commission, 1988; Dollery, 1991). This compound is practically insoluble in water and other non-acidic aqueous solutions (Morrison and Marley, 1976[a; b]), but is soluble in dilute acetic acid, and in small parts in chloroform, ethanol, and ether (Clofazimine, 2008). Clofazimine is highly lipophilic, and can slowly build up in the plasma and then concentrate mainly in the cells of the monocyte/ macrophage system, and fat cells in adipose tissues (Conalty and Jackson, 1962; Conalty, 1966; Vischer, 1969; Conalty *et al*.,

1971). It has been estimated that clofazimine needs 70 days to reach a steady-state plasma concentration and this correlates with the long time taken to achieve clinical efficacy (Banerjee *et al*., 1974; Venkatesan, 1989).

The crystalline form of clofazimine has been detected in bone, muscle, skin, heart, eye, galbladder, and nervous tissues with the highest levels found in the spleen, liver, lung, and mesentery (Vischer, 1969; Mansfield, 1974; Desikan *et al*., 1975; Desikan and Balakrishnan, 1976; Kumar *et al*., 1987). Skin is the most common organ adversely affected by this drug, where an orange-pink discolouration can be seen. This pigmentation disappears a few months after treatment has ceased (Garrelts, 1991). The second most common cutaneous side effect is ichthyosis (scaly patches) which progresses from general dryness of the skin and xerosis (Caver, 1982). Only small traces of clofazimine can be detected in urine as most of it is excreted through the faeces. Another clinically significant method of elimination is reported to occur through sebum, perspiration, tears, semen and milk (Knable, 2001; Reddy *et al*., 1999). Since up to 99% of clofazimine can be found in many tissues, it is believed that this drug can remain in the body for years after administration has ceased (Banerjee *et al*., 1974).

1.1.10.2 Immunomodulatory Actions

The mode of action of clofazimine remains uncertain, although some reports have demonstrated that it mainly alters the function of monocytes and macrophages. It also plays a role in inhibiting of mobility of neutrophils, as well as lymphocyte proliferation, both activities being dose-dependent (Knable, 2001). Clofazimine has also been reported to stimulate oxygen consumption and generation of superoxide by neutrophils, as well as the activity of phospholipase A_2 (PLA_2), resulting in the release of lysophosphatidylcholine and arachidonic acid from the neutrophil membrane. This mechanism is said to be similar to that of interferon gamma (IFN-α), which also increases the activity of PLA_2, the enzymatic hydrolysis products being toxic to Gram-positive organisms and mycobacteria (Arbiser and Moschella, 1995; López-Barcenas *et al*., 2005).

A study conducted by Sarracent and co-workers demonstrated that treatment of macrophages with clofazimine induced an increase in the levels of lysosomal enzymes, which correlated with morphological changes characterized by an increase in cell size and granularity. This was proposed to underpin the increased capability of macrophages to kill intracellular parasites (Sarracent and Finlay, 1982). Another study demonstrated that a 25 Kd

glycolipoprotein derived from *M. tuberculosis* was capable of inhibiting the intracellular killing ability of phagocytic cells. Clofazimine and B669 were reported to reverse the immunosuppressive activity of this mycobacterial glycolipoprotein (Wadee *et al*., 1987; Wadee *et al*., 1988; Wadee *et al*., 1995).

Other studies have reported that clofazimine stabilizes lysosomal membranes of macrophages and induces the inhibition of *Mycobacterium leprae* metabolism in mouse peritoneal macrophages (Sarracent and Finlay, 1982; Ramasesh *et al*., 1989). A study by van Rensburg *et al* also demonstrated that clofazimine has anti-proliferative effects on various cancer cell lines. Constant exposure to α-tocopherol, an antioxidant, was needed to antagonize the anti-proliferative effect of clofazimine (Van Rensburg *et al*., 1993).

1.1.10.3 Antibacterial Actions

Even though clofazimine was described in 1957, the molecular/ biochemical mechanism of its antimicrobial activity remains uncertain (Barry *et al*., 1957). However, the outer membrane has been reported to be the probable primary site of action for this drug (De Bruyn *et al*., 1996; Oliva *et al*., 2004; O'Neill *et al*., 2004; Shen *et al*., 2010; Yano *et al*., 2011). This drug has a highly lipophilic nature and a redox potential of -0.18 V at pH 7. It has therefore been proposed that intracellular generation of hydrogen peroxide by redox cycling mechanisms may contribute to the antimicrobial activity of clofazimine by a mechanism that involves oxidation of reduced clofazimine, leading to generation of the antimicrobial reactive oxygen species and superoxide and hydrogen peroxide. However, it has also been reported that clofazimine is capable of blocking the template function of DNA by binding to the guanine bases of DNA, which leads to inhibition of bacterial proliferation (Morrison and Marley, 1976a). The increase in the guanine and cytosine content of microbial DNA relative to that of human DNA may explain the selective inhibitory effects of clofazimine on the proliferation of microbial cells.

Van Rensburg and co-workers also investigated the antimicrobial spectrum of clofazimine and its analog B669, as well as the biochemical mechanism of the antimicrobial activities of these agents. Their data demonstrated that exposure of Gram-positive bacteria to these riminophenazines, may disrupt the cell membrane structure, making the integral phospholipids more susceptible to attack by PLA_2. These agents were shown to have selective antimicrobial activities against Gram-positive bacteria, whereas Gram-negative microorganisms were uniformly resistant (Van Rensburg *et al*., 1992).

Clofazimine has a high anti-tuberculosis activity, which was demonstrated by a low MIC value for the H37Rv strain and a high efficacy against clinical isolates, including MDR-TB strains (Reddy *et al*., 1999; De Logu *et al*., 2002; Cholo *et al*., 2006). Nevertheless, some MDR-TB strains are resistant to clofazimine. The drug is suggested to interfere with the K^+ transporters of *M. tuberculosis* either directly, or by membrane destabilizing mechanisms (Steel *et al*., 1999; Cholo *et al*., 2006). It has a high redox potential, which may result in intracellular generation of hydrogen peroxide and may also operate by interfering with electron transport mechanisms (Niwa *et al*., 1984). In addition to antimicrobial mechanisms, it has some anti-inflammatory and pro-oxidative activities as mentioned earlier (Durandt *et al*., 1996).

Lately, Yano and colleagues revived the concept of intracellular redox cycling as a mechanism of clofazimine-mediated antimicrobial activity. They reported that when clofazimine is added, at minimum inhibitory concentration (MIC), to isolated membrane fractions from *M. smegmatis* in the presence of the terminal cytochrome respiratory chain inhibitor potassium cyanide and the oxidizable cofactor, NADH, serial oxidation of NADH, reduction and oxidation of clofazimine and production of superoxide and hydrogen peroxide occurred (Yano *et al*., 2011). As reported by Cholo *et al*. (2011), there are, however, two possible caveats with respect to the mechanism of clofazimine-mediated antimicrobial activity described by Yano *et al* (2011). Firstly, the magnitude of production of reactive oxygen species by isolated membrane exposed to clofazimine in the absence of KCN was substantially less than that observed in the absence of the respiratory chain inhibitor. Secondly, isolated membrane fractions from *S. aureus* acted rather differently than those of *M. smegmatis,* in which oxidation of NADH was actually inhibited by clofazimine at concentrations of $\geq$ 2 mg/L, compatible with the existence of additional mechanisms of antimicrobial activity (Cholo *et al*., 2011; Yano *et al*., 2011).

1.1.11 AIMS AND OBJECTIVES

1.1.11.1 Aim of the study

The current study was designed with the aim of establishing a procedure for the isolation of monocytes from human peripheral blood and the subsequent maturation of these cells into a homogeneous population of monocyte-derived macrophages by early exposure of monocytes to the cytokine growth factors Interleukin (IL)-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF). These cells were then used to investigate the intracellular bioactivity of locally-manufactured clofazimine nanoparticles against *M. tuberculosis*.

1.1.11.2 Hypothesis

- Clofazimine nanoparticles prepared by mini-spray dry technology are more potent or equivalent to the native, solubilized clofazimine with respect to intracellular bioactivity against *M. tuberculosis* using infected, human monocyte-derived macrophages.

1.1.11.3 Objectives

The primary objectives of the present study were as follows:

i. To determine the effects of IL-3 and GM-CSF, added to adherent, isolated human blood mononuclear cells on the number of CD14-expressing cells (monocytes) and their viability following a 7-day exposure to the cytokines.

ii. To compare homogeneity (cell size/ volume, expression of surface markers of cell activation – CD14, CD16) of cell populations 7 days after exposure to the cytokines using flow cytometry.

iii. To infect mature, synchronised monocyte-derived macrophages with *M. tuberculosis* and measure the intracellular bioactivities of the two clofazimine preparations, using colony counting procedures to determine the viability of bacteria released from control and clofazimine-treated macrophages.

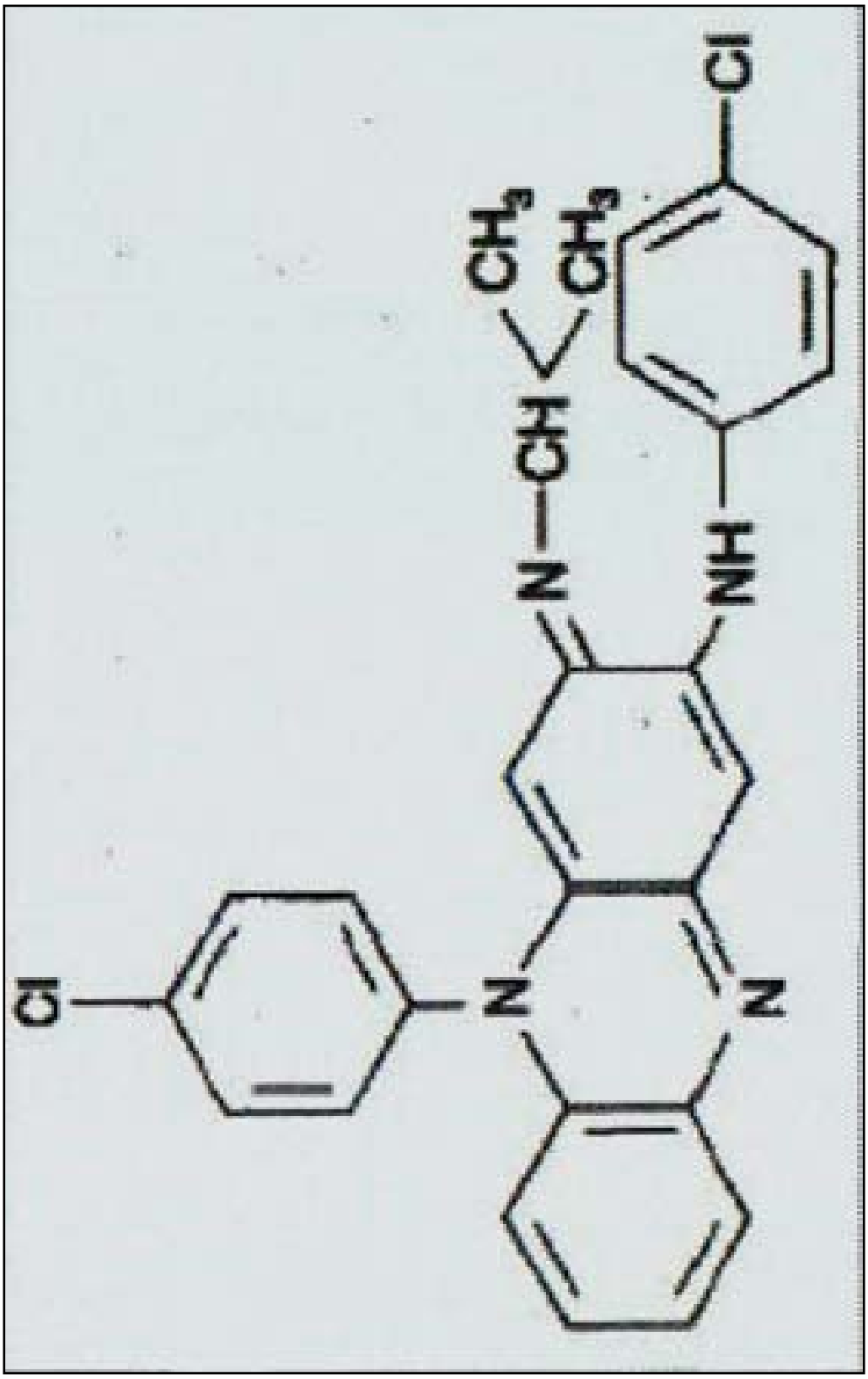


Figure 1.5. Molecular structure of clofazimine [3-(*p*-chloroanilino)-10-(*p*-chlorophenyl)-2,10-dihydro-2-(isopropylimino)-phenazine] (Gurfinkel *et al*., 2009. *Journal of Drugs in Dermatology*. **8(9)**: 846-851).

CHAPTER 2

2.1 MATERIALS AND METHODS

2.1.1 MATERIALS

2.1.1.1 Study subjects

This study was approved by the Research Ethics Committee of the Faculty of Health Sciences, University of Pretoria (Protocol S157/2010). As stipulated in protocol S157/2010, all blood donors, following prior informed consent had to undergo a blood pressure check and an overall health assessment (questionnaire) by a qualified nursing sister. The informed consent form was used only as a tool to screen the health of the donors and had no impact on the experimental design analysis and the outcome. On satisfactory completion of the informed consent/ health assessment procedures, venous blood was taken by the sister into a sterile receptacle containing preservative-free heparin (5 units/ml blood).

2.1.1.2 Chemicals and Reagents

All reagents, unless otherwise stated, were purchased from the Sigma Chemical Co. and Whitehead Scientific Pty Ltd. Media for differentiation and maintenance of macrophages including RPMI-1640 media BioWhittaker®, the Penicillin, Streptomycin and Amphotericin B (100X) (PSA) mixture, and Hanks Balanced Salt Solution with Ca^{++} (1.25 mM), Hanks Balanced Salt Solution without Ca^{++} were from Lonza (Walkerville, USA) and Highveld Biological (Pty) Ltd, Lyndhurst, RSA) respectively.

2.1.1.3 Mycobacterial Strains

All the experimental work on *M. tuberculosis* intracellular infection of human monocyte-derived macrophages in this study was performed using the *M. tuberculosis* H37Rv laboratory strain (ATCC 26518), which was provided by the Inflammation and Immunity Research Unit of the Medical Research Council, Department of Immunology, University of Pretoria, South Africa.

2.1.1.4 Growth media

The *M. tuberculosis* H37Rv laboratory strain was grown in Middlebrook 7H9 medium (Difco™, Becton Dickinson Co., MD, USA) which was prepared according to the manufacturer's instructions and supplemented with 10% Oleic Acid Dextrose Catalase (OADC) enrichment (Difco™, Becton Dickinson Co., MD, USA), 0.2% glycerol and 0.05% Tween 80.

Middlebrook 7H10 medium (Difco™, Becton Dickinson Co., MD, USA) also prepared according to the manufacturer's instructions and supplemented with 10% OADC and 0.5% glycerol was used for growing serial dilutions of lysates following infection and treatment of macrophages to allow for the appearance of the colony-forming units (cfu).

2.1.1.5 Native and spray-dried clofazimine preparations

Native (Sigma Chemicals, Co.) and spray-dried clofazimine were used for testing of intracellular activity in macrophages. The native clofazimine preparation was used to prepare the spray-dried formulation, ensuring that both preparations (native and spray-dried) are strictly comparable. The spray-dried formulation was kindly prepared by Dr Andre Germishuizen (Medicine in Need, based at MRC Building, Pretoria). The clofazimine was prepared by dissolving commercially-purchased powder in dimethyl sulfoxide (DMSO) to a concentration of 2 mg/ml and used in the assays described in the methods at final concentrations of 0.15 - 2.5 µg/ml which were prepared in double dilutions.

The spray-dried particles were prepared from clofazimine and excipient solutions to inhalable size with a Buchi Mini Spray Dryer B-290 (Flawil, Switzerland) with drying air at a pressure of 5-6 bar and a flow rate of 35 l. The clofazimine solutions were atomized by pumping at a rate of 5-7 ml/min through a 0.7 mm pressure nozzle tip located above the inlet chamber/cylinder. The inlet temperature and flow rates were optimised to result in a minimum moisture content of the dried powder. Initial inlet temperature selection was 100-140˚C. Spray dried particles were collected in 6 inch collection vessels located at the bottom of the high performance cyclone.

2.1.2 METHODS

2.1.2.1 Isolation of mononuclear leucocytes

Mononuclear leucocytes (MNL) were obtained from venous blood (5 units of preservative-free heparin /ml) of healthy adult volunteers. Monocytes were separated by collecting the cell fraction at the plasma-ficoll interphase after a standard barrier centrifugation on Histopaque®-1077 gradients at 1800 rpm for 25 minutes at room temperature. Following centrifugation, the MNL layer was aseptically decanted into separate sterile tubes and diluted 1/5 in sterile phosphate-buffered saline (PBS, 0.15M, pH 7.4). After centrifugation at 1200 rpm for 10 minutes at 4 ºC, the resultant MNL pellet was suspended in 20 ml of sterile, ice-cold, 0.83% ammonium chloride and held on ice for 10 min to haemolyse contaminating erythrocytes. Subsequent to another centrifugation step and discarding of the supernatant fluid, the cells were suspended in sterile Hanks' balanced salt solution (HBSS, indicator-free, containing 1.25 mM $CaCl_2$, pH 7.4, Highveld Biological, Johannesburg). The MNL suspension (50 µl) was added to 0.45ml of leucocyte counting fluid, placed under the cover slip of a haemocytometer and the number of MNL counted microscopically. To distinguish between the various MNL sub-populations, the cell preparation was also analysed flow cytometrically using a Beckman Coulter FC500 Flow Cytometer using the following fluorochrome-labelled monoclonal antibodies (Beckman Coulter): CD3 (FITC), CD14 (PE), CD15 (FITC) and CD19 (PE) for analysis and enumeration of total T cells, monocytes, granulocytes and B cells, respectively.

2.1.2.2 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, respectively. MNL (30 ml of a 3 x 10^7 MNL/ ml suspension in HBSS) was seeded onto sterile 75 cm^3 tissue culture flasks and incubated for 2 hours at 37 ºC/ 5% CO_2 to promote adherence of monocytes. Following incubation, each flask was gently rinsed with 50 ml of pre-warmed PBS to remove the non-adherent cells. Ten millilitres 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 then 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 then incubated for 7 days at 37 ºC/ 5% CO_2.

Following the 7-day incubation period, the tissue culture medium was discarded and each flask rinsed once with 10 ml pre-warmed PBS, followed by addition of 10 ml PBS containing the Ca^{2+}-chelating agent ethylene glycol-bis (2-aminoethylene)-N,N,N,N-tetraceticacid EGTA (2mM, final) and the flasks placed on ice with gentle agitation every 10 minutes for at least 30 minutes, to promote detachment of the cells, which were then dislodged by scraping the surface of the flask with a sterile 1.8 x 25 cm Cell Scraper (Adcock Ingram, Scientific Group). The cells were then pelleted by centrifugation, the supernatant discarded and the cell pellet resuspended in 3 ml of Ca^{2+}-free HBSS containing 2mM EGTA. The cell suspension (350µL) was then analyzed flow cytometrically using the following combinations of fluorochrome-labelled monoclonal antibodies: CD14-PE/CD16-FITC (monocytes/ macrophages) and CD3-FITC/ CD19-PE (T cells and B cells).

2.1.2.3 Preparation of macrophages for the morphological investigation using scanning electron microscope (SEM)

Following a 7 day incubation period, the medium was removed and the monolayer was washed with a phosphate buffered saline solution (PBS). The monolayer of each well containing a coverslip was fixed with 2.5% gluteraldehyde in a 0.075M phosphate buffer pH7.4. After 60 minutes the fixative was removed and each well was washed three times (15 minutes each) with 0.075M phosphate buffer. Post-fixation was then done for 60 minutes in a 1% osmium tetraoxide (OsO_4) solution. After post-fixation each well was washed three times, (15 minutes each) with 0.075M phosphate buffer to remove any remaining osmium tetraoxide. Once the washing step was complete, a dehydration step took place in which each well went through serial dehydration with ethanol, 30%, 50%, 70%, 90% and three times 100%, each dehydration step was performed for 15 minutes. The cover slips were then removed from the 24-well plates and dried, by means of CO_2 critical point drying procedures; samples were mounted, coated with gold and examined with a JEOL 840 SEM.

2.1.2.4 Preparation of macrophage monolayers for infection with *M. tuberculosis*

The cell suspension numerically adjusted to a concentration of 1 x 10^5, was added to the 1 cm diameter wells of a 48-well tissue culture plate (Costar, Corning Inc, NY, USA) followed by the addition of an excess of $CaCl_2$ (20 µL of a 100mM solution in sterile distilled water (dH_2O) = 4 mM, final in 500µl) to neutralize EGTA. The plate was then incubated for 2 hours at 37ºC/ 5% CO_2 to allow cells to attach. The volume in each well was then brought to 0.5 ml by the addition of autologous serum-supplemented RPMI 1640 tissue culture medium containing antibiotics (penicillin: streptomycin: amphotericin B, 0.1:0.25:0.1 µg/ ml. After 2 days, the antibiotic-supplemented RPMI 1640 was replaced with antibiotic-free medium.

2.1.2.5 Preparation of bacterial cultures for infection of macrophage monolayers

All work with *M. tuberculosis* was performed at the BL3 containment facility of the Tuberculosis Epidemiology and Intervention Research Unit, Medical Research Council, Pretoria. A laboratory *M. tuberculosis* H37Rv strain (ATCC 26518) was used for infection of macrophages. This bacterial strain was cultured in Middlebrook 7H9 medium (Difco™, Becton Dickinson Co., MD, USA) supplemented with 10% OADC, 0.2% glycerol, 0.05%, incubated at 37˚C and grown to mid-logarithmic phase under stirring conditions (for approximately 7 days). The culture was then transferred into 50 ml tubes and centrifuged at 3000 rpm for 15 minutes at 25 ºC. The supernatant was discarded and the pellet suspended in an equal volume of PBS (0.15M, pH 7.4), and centrifuged again. The pellet was resuspended in antibiotic-free RPMI 1640 and the OD adjusted to 0.6 at 540 nm, which is equivalent to 10^7 cfu/ml. This suspension was adjusted to yield a 10:1 bacteria:macrophage infectivity ratio.

2.1.2.6 Infection of macrophages with *M. tuberculosis* and determination of the intracellular bioactivities of the clofazimine preparations.

Following removal of the medium from the adherent monocyte-derived macrophages which had been cultured for 7 days, the wells were gently rinsed with pre-warmed RPMI 1640 (antibiotic-free supplemented with 5% autologous serum), followed by addition of 1 ml bacterial suspension in RPMI 1640 containing 1 x 10^6 cfu/ml of *M. tuberculosis* to give a 10:1 bacteria:macrophage ratio. The plates were then incubated overnight at 37ºC to allow internalization of *M. tuberculosis* by the adherent macrophages. After incubation, each of the wells was gently rinsed with 500µl of pre-warmed sterile PBS, followed by addition of 500µl RPMI 1640 containing 50 µg/ ml gentamicin and the plates incubated for a 1 hour period at

37ºC. This step was undertaken to kill non-internalized bacteria. Following this step, the wells were rinsed once with pre-warmed PBS followed by addition of 0.5 ml serum-supplemented RPMI 1640 containing 1% dimethyl sulfoxide (DMSO) solvent (control systems) or native or spray-dried clofazimine at final concentrations of 0.15 - 2.5 µg/ml clofazimine. In the therapeutic setting, clofazimine can attain maximum serum concentrations of approximately 4 µg/ml (Reddy *et al*., 1999). The tissue culture plates were then incubated for 48 hours at 37ºC/ 5% CO_2 after which the wells were gently rinsed once with pre-warmed PBS. A 100µl volume of sterile dH_2O containing 0.2% sodium dodecyl sulphate (SDS) was added to each well to lyse the infected macrophages. Serial dilutions of lysates were prepared in PBS, plated onto Middlebrook 7H10 medium (Difco™, Becton Dickinson Co., MD, USA) and incubated at 37ºC to allow for the appearance of the colony-forming units (cfu). These were scored to quantify the number of surviving intracellular bacteria. The numbers of bacteria quantified at day zero were used as zero-time sample. The growth of bacteria inside macrophages was measured by determining the increase in the number of intracellular bacteria which was calculated by dividing the quantity of intracellular bacteria at the time of lysis, by the quantity of intracellular bacteria in the zero-time sample (Pethe *et al*., 2004; Su *et al*., 2009). Results were also expressed as cfu/ml.

2.1.2.7 Statistical analysis

The results of each series of experiments are expressed as the mean value ± standard error of the mean (SEM). Levels of the statistical significance were calculated using the Mann-Whitney *U* test (non-parametric 2-tail). *P* values of ≤ 0.05 were considered significant.

CHAPTER 3

3.1 RESULTS

3.1.1 Isolation of mononuclear leucocytes

The initial yields of monocytes from the blood of 20 different human adult volunteers, as well as the numbers of contaminating cells are shown in Table 3 (page 39). The average mean percentage monocytes at this early stage of the isolation/ maturation procedures was 12.08 ± 4.9 (SEM). The average total recovery of monocytes was 7 x 10^5 cells of which 10% were monocytes (Table 3.1) from 150-200 ml blood after the seven days incubation. Assessment of the different types of mononuclear leucocytes separated from total leucocyte preparations was determined using fluorochrome-labelled monoclonal antibodies using a Beckman Coulter FC 500 flow cytometer. The majority of the cell population was the T (CD3) lymphocytes which accounted for 40-70%, while the B (CD19) lymphocytes and the monocyte/macrophage (CD 14) populations were comparable and were between 5-20% as shown respectively in Figure 3.1 [A,B and C] (page 40).

3.1.2 Separation and maturation of monocytes

With respect to the maturation strategies, the findings demonstrated that a seven-day exposure of the adherent cell population to IL-3/GM-CSF was accompanied by a significant increase in the level of expression of CD14 and CD16, as well as cell size, compatible with accelerated differentiation. Importantly, this cell population was highly homogeneous and accounted for >95% of the total cell population. These findings confirmed that the modified procedure results in the acquisition of synchronised human monocyte-derived macrophages, in adequate numbers as shown in Figure 3.2 [A,B] (page 41), which can be used for experimental infection with *M. tuberculosis in vitro*.

Further assessment of the maturation state and viability of the monocyte-derived macrophages were determined flow cytometricaly. For both cytokine and noncytokine-treated populations, > 90% of the recovered cells represented macrophages co-expressing CD14 and CD16 as shown in Figure 3.3 [A]. Cell viability was optimal when they were treated with cytokines [B] (page 42).

3.1.3 Investigation of the effects of the growth factors IL-3/GM-CSF on maturation of monocytes into macrophages the using scanning electron microscope (SEM)

The effects of the growth factors IL-3/GM-CSF on the maturation of monocytes into macrophages and their detailed morphological differentiation following 7-days incubation were investigated using the scanning electron microscope (SEM). The results of the SEM analysis for cytokine-treated macrophages are shown in Figure 3.4 (page 43) and indicate that the treated cells were highly homogenous and larger in size than the non-cytokine treated cells.

The SEM investigations on the non-cytokine treated macrophage populations indicated that the cells were highly heterogeneous, presenting with different sizes, shapes and maturation status as shown in Figure 3.5 (page 44).

3.1.4 The intracellular bioactivities of clofazimine preparations on the growth of *M. tuberculosis* in macrophages

The bioactivities of the native and spray-dried clofazimine preparations are shown in Figures 3.6-3.9 (pages 45-48). Exposure of *M. tuberculosis* to both preparations (0.15 - 2.5 µg/ml) relative to the corresponding drug-free controls demonstrated comparable efficacy, with dose-dependent response inhibition of colony formation. Significant inhibition of growth with both clofazimine preparations was demonstrated at 1.25 µg/ml and from 2.5 µg/ml, with no detectable colonies.

The percentage inhibition of the growth of *M. tuberculosis* was determined for each clofazimine preparation at each concentration and compared using the Mann-Whitney *U* test (non-parametric 2-tail) as shown in Figure 3.10 (page 49). *P* values of ≤ 0.05 were considered significant. However, no statistically significant differences were found between the treatments with respective clofazimine preparations as shown in Table 3.2 (page 50).

Donor	Total leukocytes/µl	% Monocytes (CD 14)	%Granulocytes (CD15)	% T Lymphocytes (CD3)	% B Lymphocytes (CD 19)
1	4563	6.3	2.6	71.9	5.9
2	4390	24.2	0.6	39.8	11.4
3	9368	10.4	0.5	51.8	10.3
4	5049	15.4	0.4	55.2	21.7
5	9164	8.9	0.2	61.4	18.2
6	4926	7.9	1.6	77.5	9.2
7	9651	4.4	0.2	76.1	8.3
8	11340	8.4	1.1	64.3	12.8
9	3179	15.9	0.8	41.5	7
10	5675	11.6	14.5	56.7	11.2
11	7967	10.4	3.9	57.7	0.1
12	9519	6.9	0.2	69.5	17.7
13	7839	8.3	0.3	67.9	14.7
14	6881	10.4	0.6	65.8	12.3
15	4421	17.1	2.6	47.4	8.3
16	9888	20	6.2	42.4	5.3
17	5089	14.8	1.4	58.8	7.6
18	4561	13.4	0.9	59.7	6.7
19	9278	15.4	1.2	67.6	5.9
20	8163	11.5	0.9	62.4	5.9
Average	7045.55	12.08 ±4.9 SEM	2.035	59.77	10.025

Table 3.1. Total numbers of leucocytes and monocytes isolated from human peripheral blood following barrier centrifugation assayed by flow cytometry.

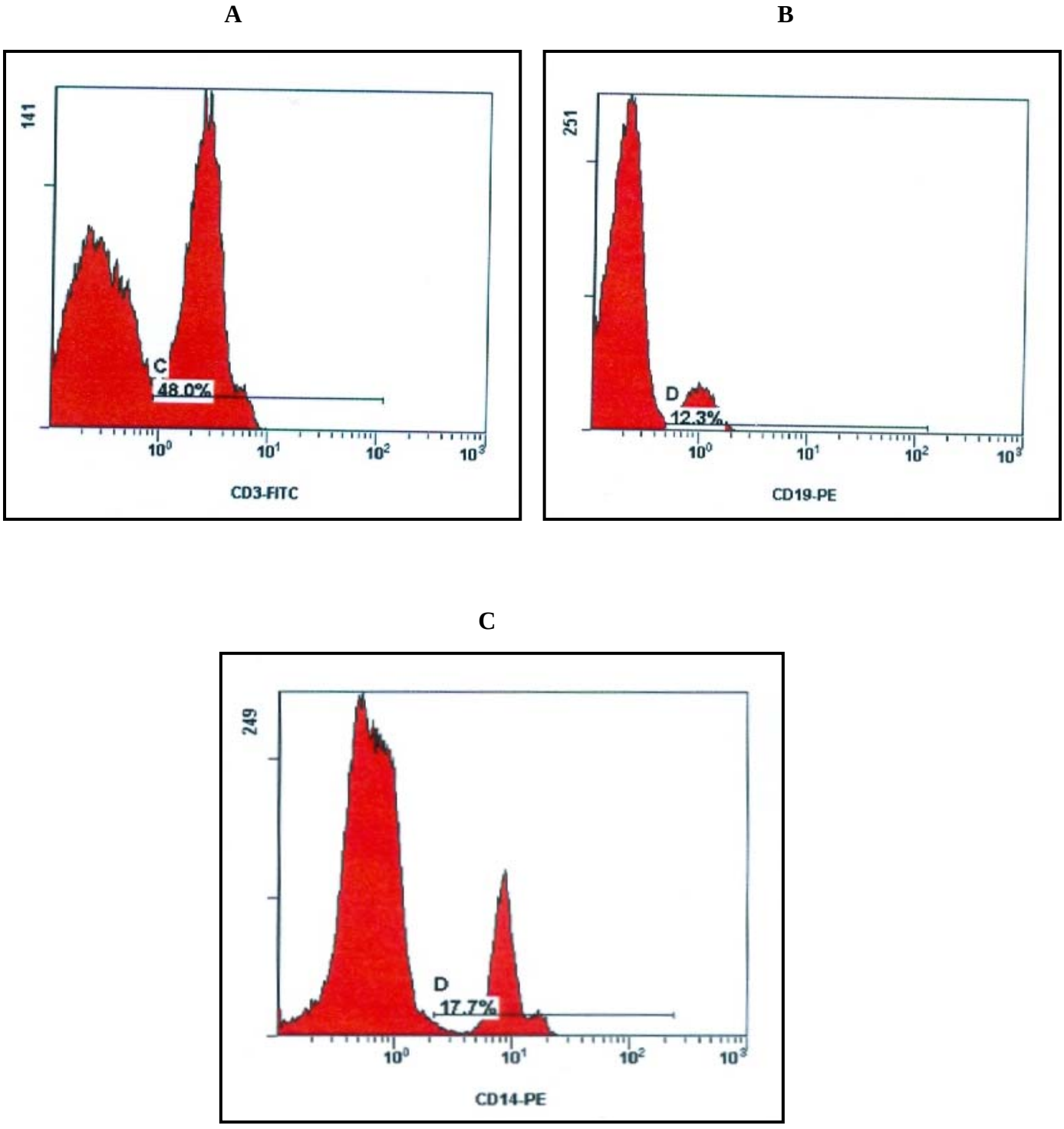
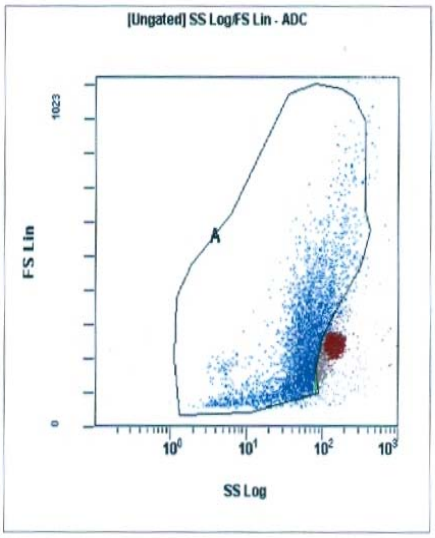


Figure 3.1. Assessment of the different types of mononuclear leucocytes separated from total leucocyte preparations was determined flow cytometricaly using fluorochrome-labelled monoclonal antibodies. The majority of the cell population was T (CD3) lymphocytes [A] which accounted for 40-70%, while the B (CD19) lymphocytes [B] and the monocyte/macrophage (CD 14) populations [C] were comparable and were between 5-20%.

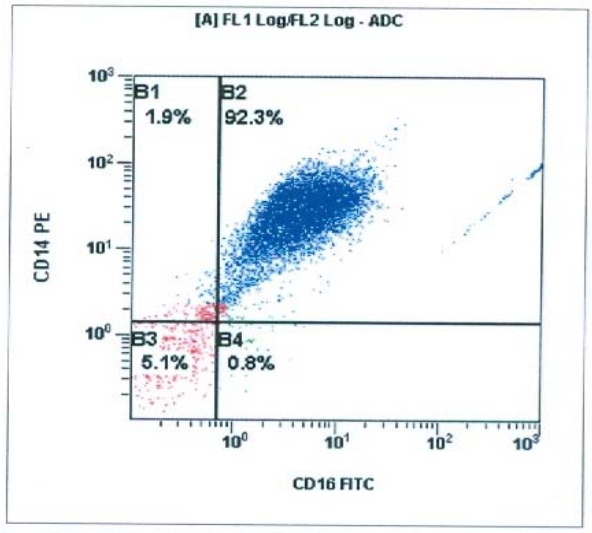
A



[Ungated] SS Log/FS Lin

Region	Cells/µL	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	2415	17109	100.00	100.00	109	244
A	1189	8420	49.21	49.21	67.3	243

B

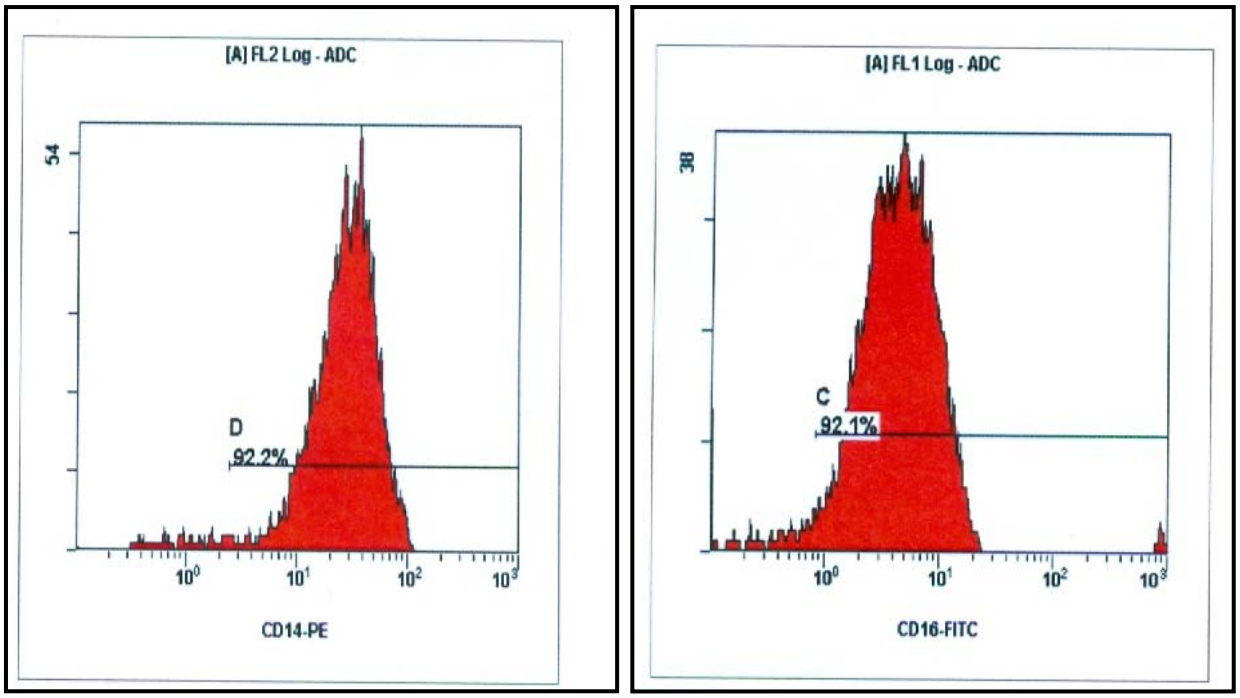


Log/FL2 Log

Cells/µL	Number	%Total	%Gated	X-Mean	Y-Mea
1355	8693	54.31	100.00	16.4	29.9
25	161	1.01	1.85	0.504	2.45
1251	8020	50.11	92.26	17.7	32.3
69	442	2.76	5.08	0.256	0.62
11	70	0.44	0.81	5.98	0.819

Figure 3.2. Assessment of the maturation state of mononuclear leucocytes into macrophages after 7 days incubation was determined flow cytometrically using fluorochrome-labelled monoclonal antibodies. The number of macrophages recovered after 7 days incubation was the same for both cytokine and non-cytokine treated populations [A]. The cell population was, however, more homogeneous when treated with cytokines [B].

A **B**



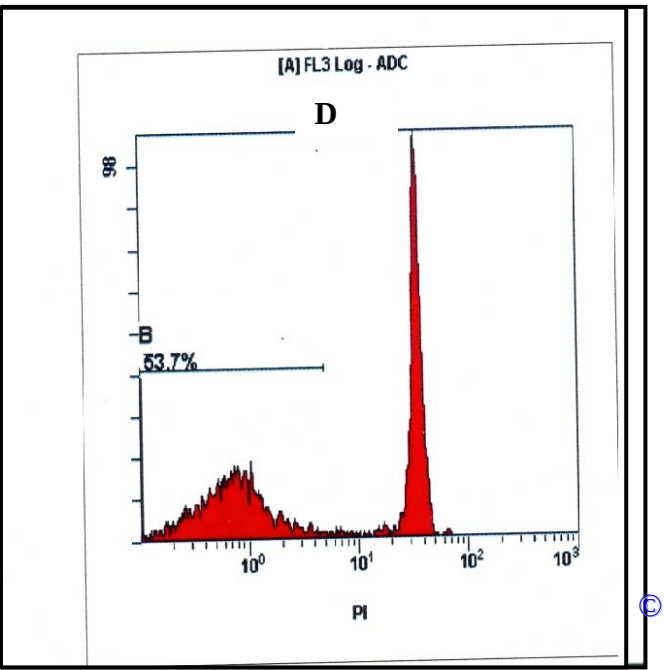
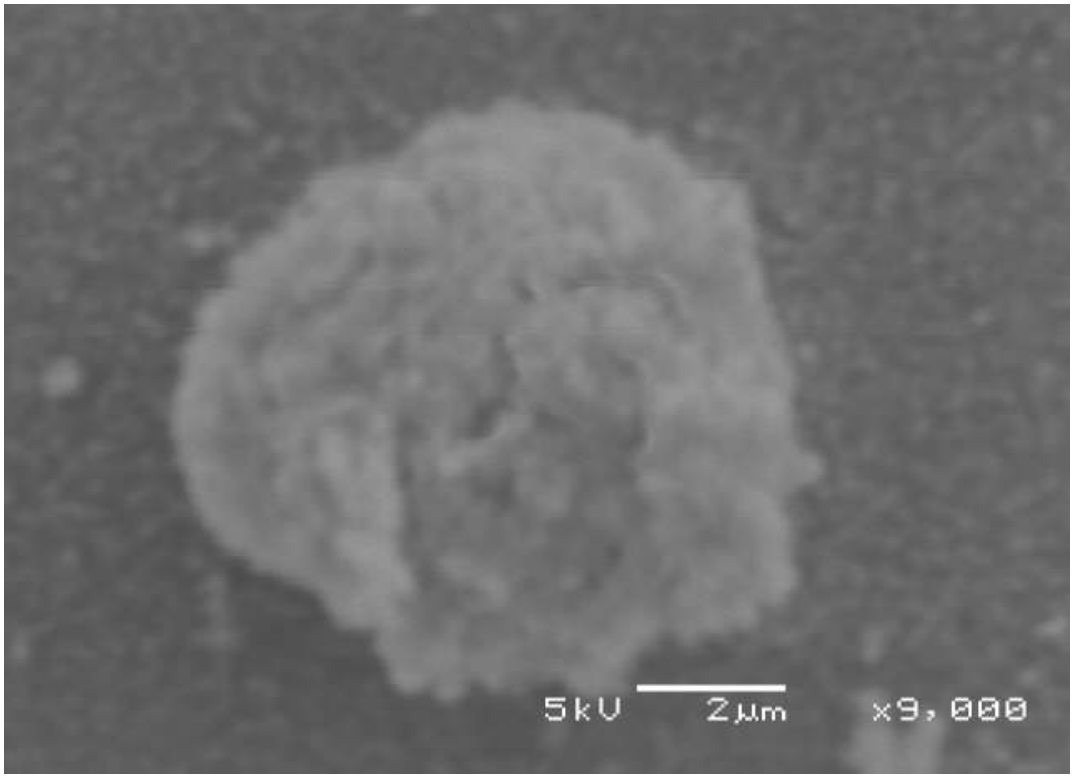


Figure 3.3. Assessment of the maturation state of monocytes into macrophages after 7 days incubation was determined flow cytometricaly using fluorochrome-labelled monoclonal antibodies. Only data for cytokine-treated cells is shown as there was no difference in the expression of CD14 [A] and CD16 [B] for cytokine and non cytokine-treated cells [A, B].

In both cell populations, >90% of the macrophages were matured. There was an improvement in viability of cells treated with cytokines [C] relative to those which were not exposed to cytokines [D].



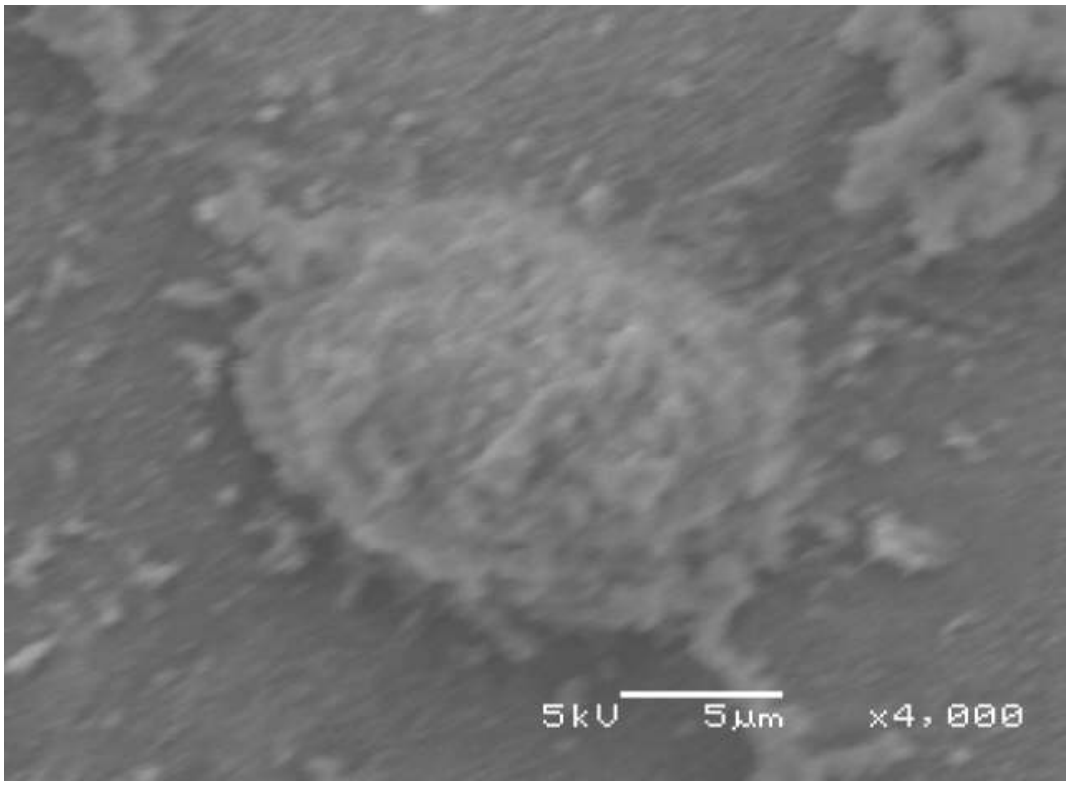
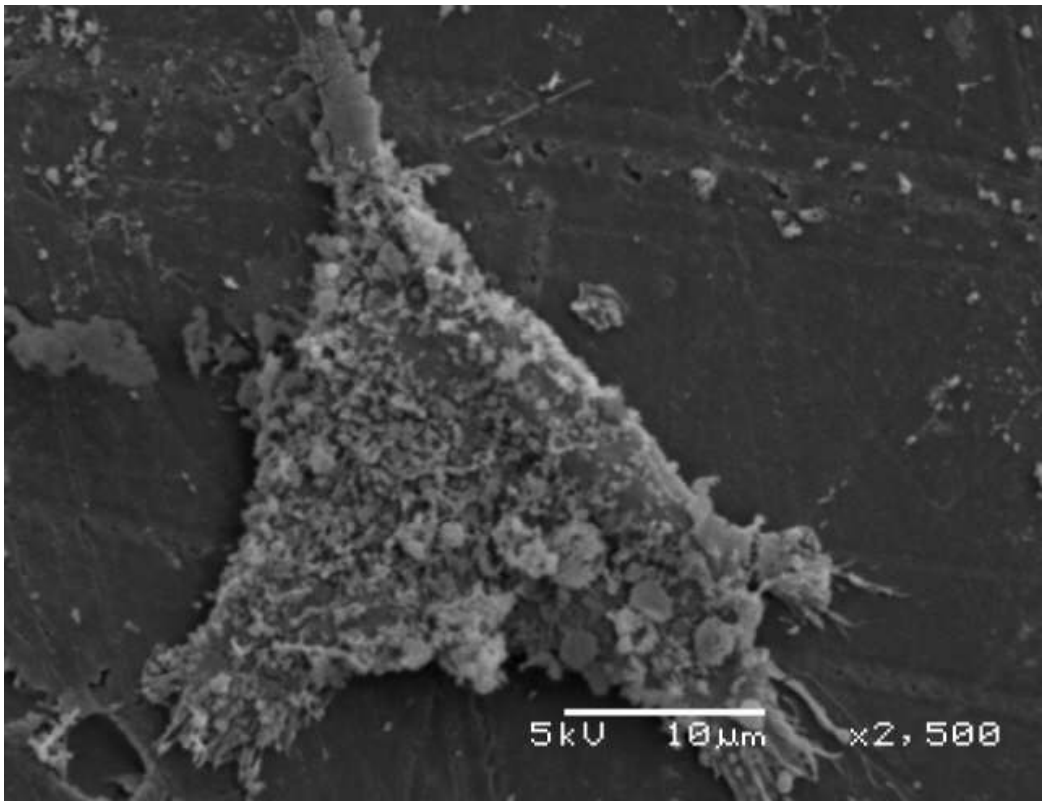


Figure 3.4. Scanning electron microscope analysis of cytokine-treated macrophages showing cells which were highly homogenous and larger in size than the non-cytokine treated cells shown on the next page.



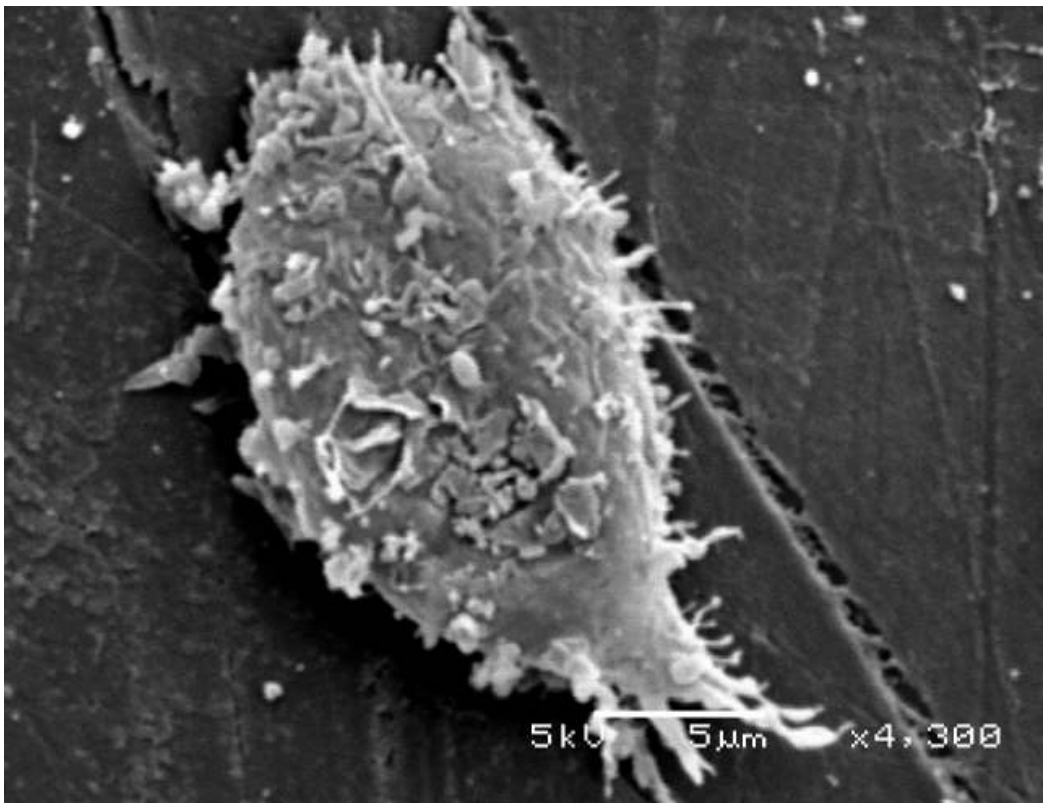


Figure 3.5. Scanning electron microscope analysis of non-cytokine treated macrophage populations showing cells which were highly heterogeneous presenting with different sizes, shapes and maturation status.

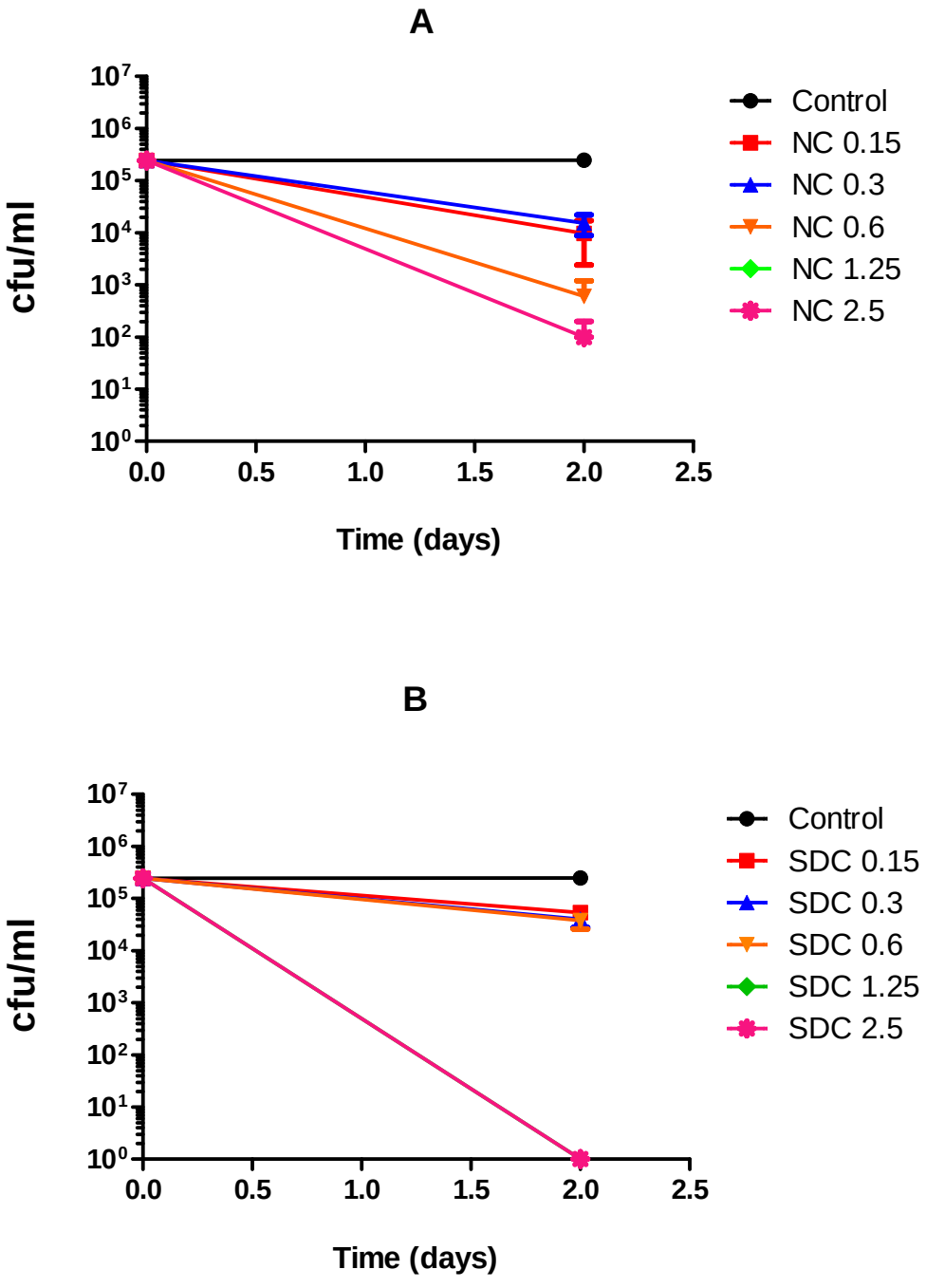


Figure 3.6. Data from experiment 1 showing the effects of varying concentrations (0.15 - 2.5µg/ml) of the native (NC) [A] and spray-dried clofazimine (SDC) [B] preparations on the survival of *M. tuberculosis* in macrophages. The results are of a single experiment represented as the mean values ± SEM of duplicate values for the drug-free control system

and each concentration of the two clofazimine preparations. The colony counts for the duplicate values for each system were very close resulting in low SEM values.

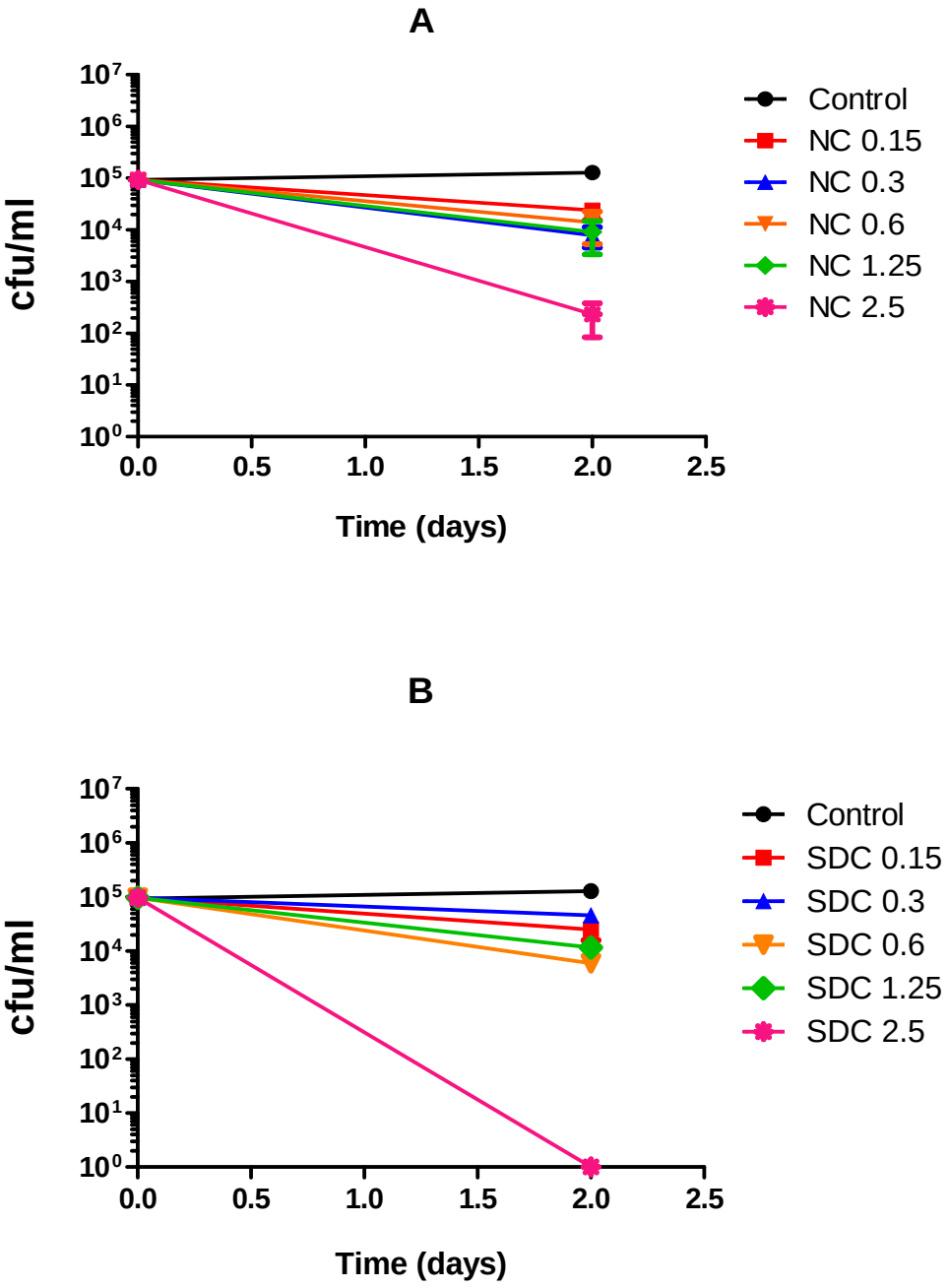


Figure 3.7. Data from experiment 2 showing the effects of varying concentrations (0.15 - 2.5 µg/ml) of the native (NC) [A] and spray-dried clofazimine (SDC) [B] preparations on the survival of *M. tuberculosis* in macrophages. The results are of a single experiment represented as the mean values ± SEM of duplicate values for the drug-free control system

and each concentration of the two clofazimine preparations. The colony counts the duplicate values for each system were very close resulting in the low SEM values.

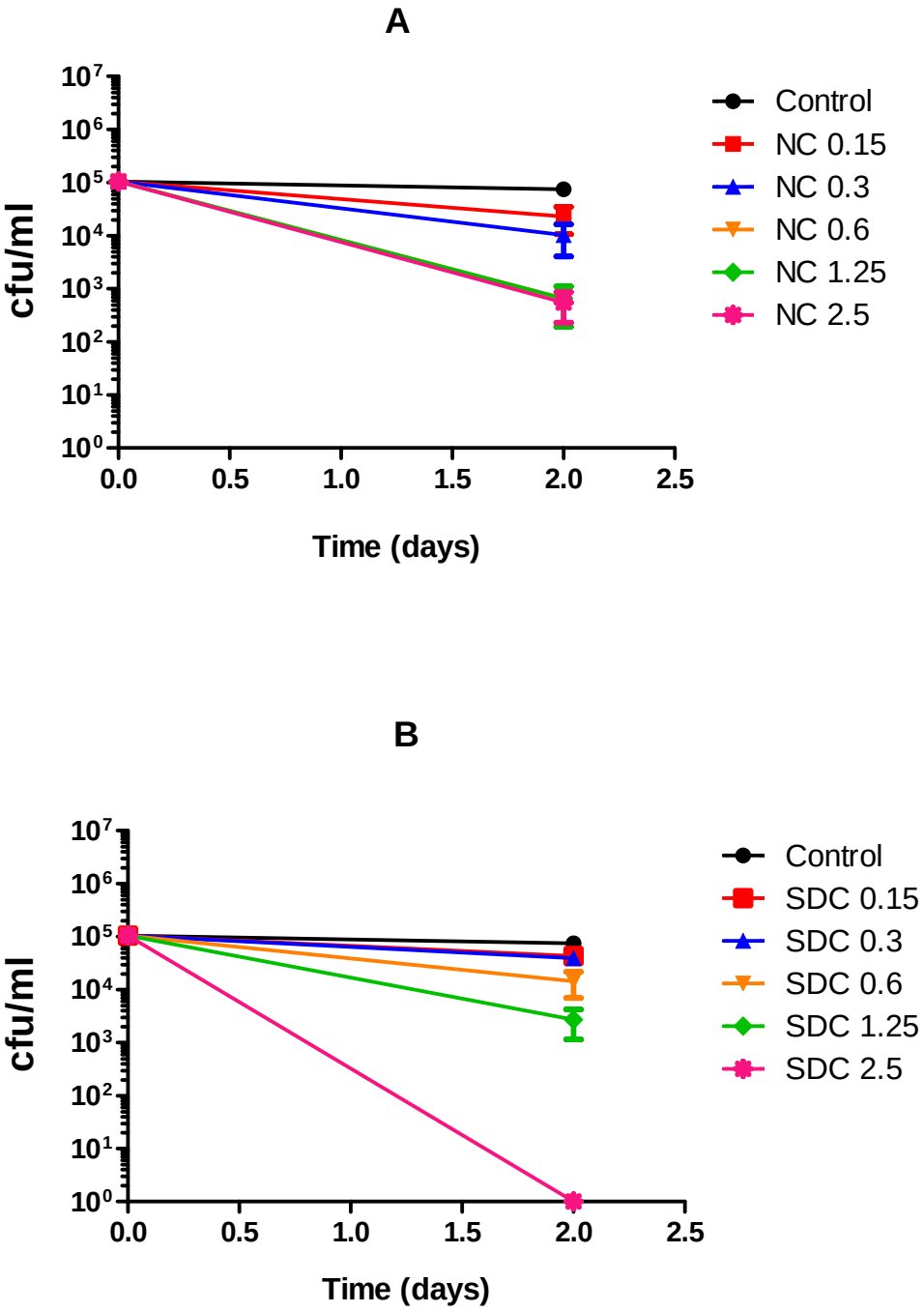


Figure 3.8. Data from experiment 3 showing the effects of varying concentrations (0.15 - 2.5 µg/ml) of the native (NC) [A] and spray-dried clofazimine (SDC) [B] preparations on the survival of *M. tuberculosis* in macrophages. The results are of a single experiment represented as the mean values ± SEM of duplicate values for the drug-free control system

and each concentration of the two clofazimine preparations. The colony counts the duplicate values for each system were very close resulting in the low SEM values.

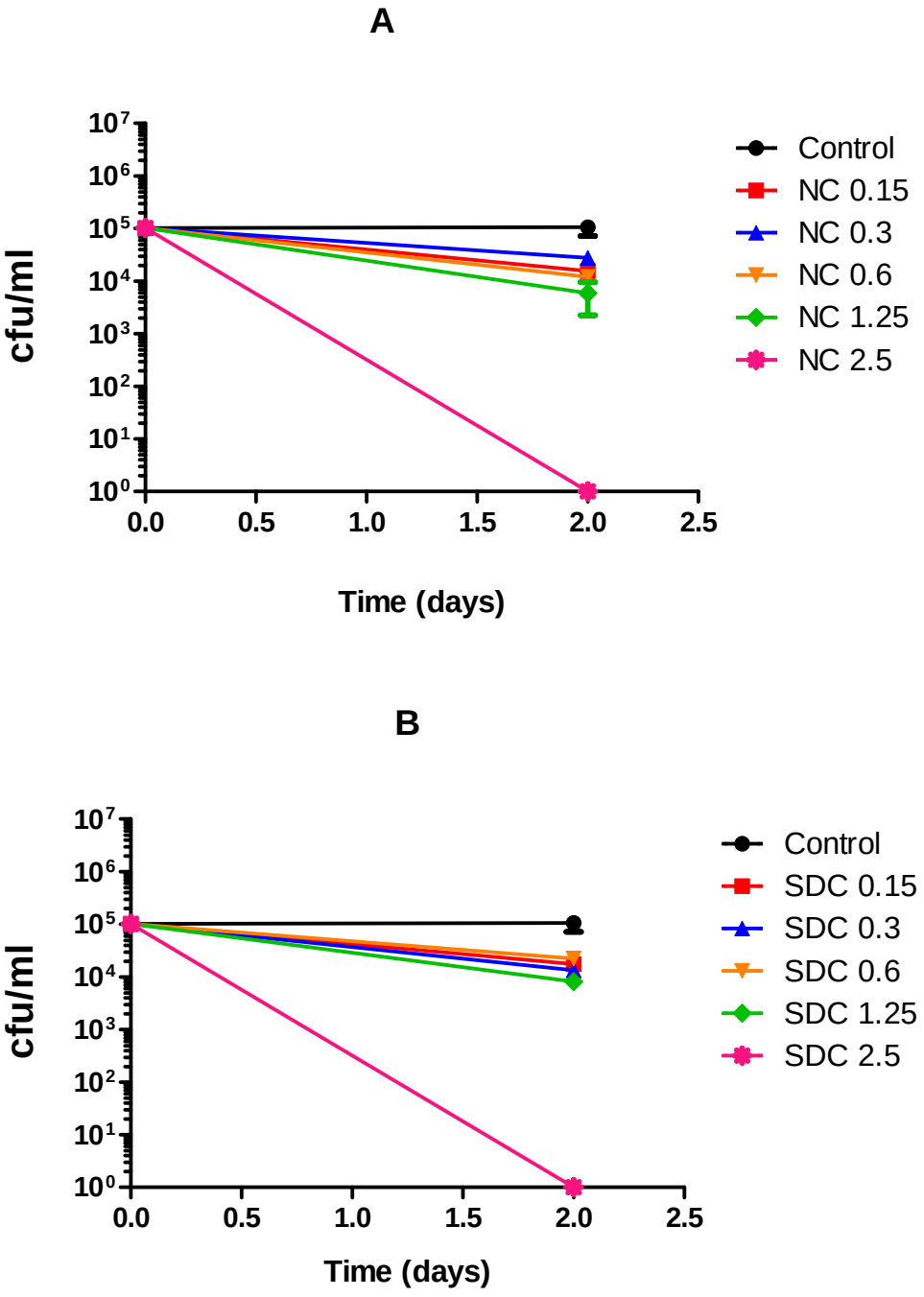


Figure 3.9. Data from experiment 4 showing the effects of varying concentrations (0.15 - 2.5 µg/ml) of the native (NC) [A] and spray-dried clofazimine (SDC) [B] preparations on the survival of *M. tuberculosis* in macrophages. The results are of a single experiment represented as the mean values ± SEM of duplicate values for the drug-free control system

and each concentration of the two clofazimine preparations. The colony counts the duplicate values for each system were very close resulting in the low SEM values.

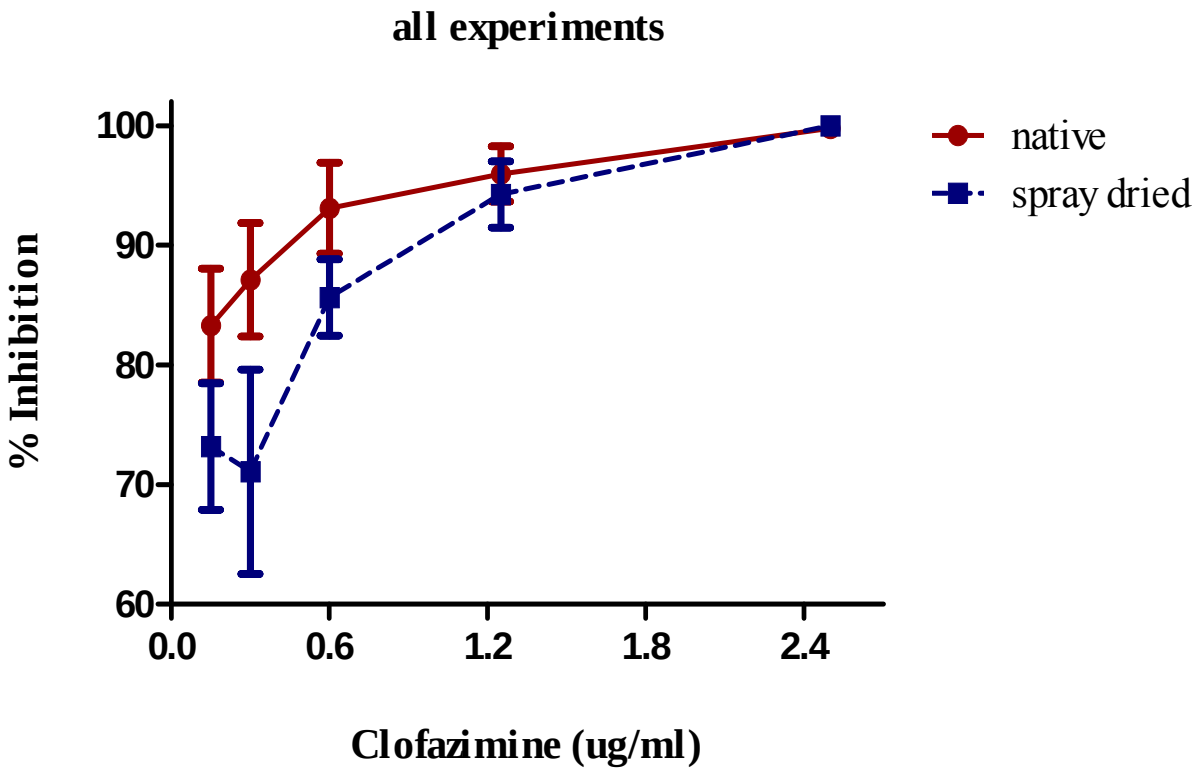


Figure 3.10. Composite results of experiments 1-4 showing the effects of varying concentrations (0.15 - 2.5 µg/ml) of the native (NC) and spray-dried clofazimine (SDC) formulations on the survival of *M. tuberculosis* in macrophages. For purposes of comparison between experiments, the results are presented as the mean percentages inhibition of bacterial growth ± SEM. No statistically significant differences were observed between the two preparations of clofazimine.

Native (NC) v/s Spray-dried (SDC) Clofazimine Concentrations (µg/ml)	*P*-values
0.15	0.8504
0.3	0.4394
0.6	0.3456
1.25	0.7325
2.5	Could not be calculated due to limited values

Table 3.2. Comparison of the levels of statistical significance between each of the concentrations of the native and spray-dried clofazimine calculated using the Mann-Whitney *U* test (non-parametric 2-tail).

CHAPTER 4

4.1 DISCUSSION

4.1.1 Development of a reliable procedure for the preparation of human monocyte-derived macrophages using growth factors IL-3/GM-CSF

Macrophages are clearly considered both the primary target of *M. tuberculosis*, as well as the key component of the first line of cellular defense against this microbial pathogen. These cells may control replication of *M. tuberculosis* by activating innate bactericidal mechanisms. This is usually observed in naturally resistant individuals and in non-resistant individuals who are infected with low doses of this pathogen (Koul *et al*., 2004; Sundaramurthy and Pieters, 2007; Liu and Modlin, 2008). However, the mononuclear phagocytes may fail to control the bacilli in susceptible individuals or those infected with high number of bacteria, and this results in survival and replication of the mycobacteria (Bhatt and Salgame, 2007). *M. tuberculosis* has developed strategies to disturb various processes within the host cells, such as the phagosome and lysosome fusion, thus avoiding the hostile environment of phagolysosomes (Armstrong and d'Arcy Hart, 1971; Fréhel *et al*., 1986); other subversive mechanisms include modulation of different signaling pathways, thereby preventing induction of bactericidal mechanisms, and promoting necrosis instead of apoptosis of infected cells (Flannagan *et al*., 2009).

Macrophages display distinct heterogeneity in their phenotypes (Mosser and Edwards, 2008). These cells develop functional diversity which results from a differentiation programme that is subject to environmental imprinting (Gordon, 2007). The selection of phenotype is further modified by exogenous stimuli such as micro-organisms. The tissue macrophage phenotype has considerable plasticity even though it has differentiated, and this is dependent on the pattern of stimulation. Macrophages mainly function in the maintenance of tissue homeostasis and responding to micro-organisms (Mosser and Edwards, 2008).

The isolation of tissue macrophages requires blood donation or collection from specific tissue by procedures such as bronchoscopy or tissue biopsy (Gordon *et al*., 2000). In addition, these procedures result in poor yields of monocytes/macrophages. Based on these factors the primary tissue macrophages cannot be readily expanded *ex vivo* and most studies have adopted the frequent use of monocytic cell lines, which are easy to acquire and reproduce as

compared to primary macrophages. However, these have varying degrees of differentiation to model macrophage function. Their differentiation state means that conclusions drawn from such experiments may not always correctly predict the behaviour of differentiated tissue macrophages (Kohro *et al*., 2004; Park *et al.*, 2007).

In order to address the above-mentioned problems posed by the two procedures, a modified method for the isolation of macrophages from healthy human blood and their differentiation into macrophages, has been developed in this study, by treating the monocytes with the cytokines, IL-3 and GM-CSF, over a period of 7 days. Both these factors promote the growth of macrophages in tissues.

Using these growth factors resulted in a homogeneous population of macrophages, which is more viable and matures faster than the non-cytokine treated cells. Approximately 95% of the cells expressed CD14 and CD16 surface markers, after a seven-day exposure to these cytokines. There was also a clear correlation of cell size with the differentiation of the cells as the cytokine-treated cells were bigger and differentiated faster than the non-cytokine treated.

Despite these positive effects, the inclusion of the cytokines did not improve the yield of the macrophages from the human blood as there was no difference in yield between the cytokine-treated and non-cytokine treated cells. Furthermore, although heterogeneous and late in maturation, the non-cytokine treated cells, were able to express the CD14 and CD16 markers to the same magnitude as the cytokine-treated cells, with over 90% of the cells expressing these markers.

In conclusion, the modified method described in this study has demonstrated that despite low yield, the macrophages derived from human blood-isolated monocytes are homogeneous and mature faster to benefit the experimental design required for many immunopathogenesis assays. Further modification of this procedure is required to improve the yield of the monocyte-derived macrophages from human blood.

4.1.2 The intracellular bioactivities of clofazimine preparations on the growth of *M. tuberculosis* in macrophages

The World Health Organization has over the past 15 years, endorsed a rigorous approach to the treatment of tuberculosis which resulted in close to 36 million people being cured of this disease (Sasindran and Torrelles, 2011). As discussed in Chapter 1, although many efforts have been made to control TB, the interaction between human immunodeficiency virus (HIV) and the tubercle bacilli poses a serious threat to the global TB control programme (Pablos-Mendez *et al*., 1998; Havlir and Barnes, 1999; Aaron *et al*., 2004; Cohen *et al*., 2006). Additionally, the emergence of multi-drug and extensively drug resistant strains of *M. tuberculosis* also forms part of the major factors, adding a burden to the TB chemotherapy (Sasindran and Torrelles, 2011). One of the ways to control this disease is by prevention strategies using the attenuated *M. bovis* Bacillus Calmette Guerin (BCG), which is the only available vaccine against TB. However, the efficacy of this vaccine has not been demonstrated in all populations, as protection, varies from 0 to 80% in individuals (Fine, 1995; Reed *et al*., 2003). Other attempts which have been followed to manage TB were through innovations in antimicrobial therapy, but appeared to be failing due to the emergence of *M. tuberculosis* resistant to the standard first-line drugs, including isoniazid and rifampin (Pablos-Mendez *et al*., 1998).

Hence, the development of improved antimycobacterial drugs that are active against the resistant strains is crucial. Creating a rational design of such drugs depends on the knowledge of the primary immune response that serves to protect most humans exposed to the pathogen, and to understand the factors that result in the failure to mount protective immunity in humans who are susceptible to tuberculosis (Flynn *et al*., 1995b). Although there is potential in candidate anti-tuberculosis drugs being tested in recent studies, there are still many obstacles that must be overcome. Problems that limit their use include, low solubility, low levels of retention or stability in the cells after uptake, or degradation before they reach target tissues. Other difficulties may be caused by failure to deliver sufficient concentrations of a drug to the site of infection due to its poor absorption properties or low penetration into cells. Additional limitations include high levels of toxicity of a potential drug, which may lead to a maximum tolerated dose resulting in tissue damage (Swenson *et al*., 1988).

Since macrophages are the primary target cells, which mycobacteria invade and colonise, sufficient concentrations of antimycobacterial drugs need to be delivered within these cellular compartments. Previous research was focused on developing liposomes containing antibiotics, which would deliver high concentrations of antimycobacterial drugs into infected macrophages, thereby improving the treatment outcomes for intracellular infections (Garrelts, 1991; Dayan Sandler *et al*., 1992; Forster *et al*., 1992).

One such study was conducted by Adams and co-workers, who focused on the therapeutic efficacy of liposomal clofazimine (L-CLF) in mice infected with *M. tuberculosis* and demonstrated that this form of treatment was highly effective against acute and chronic infections (Adams *et al*, 1999). Observations in this study showed that the maximum tolerated dose of free clofazimine (F-CLF) was 5 mg/kg of body weight of infected mice, which was not enough to cause a significant reduction in the number of viable *M. tuberculosis* bacilli. Even this free form of clofazimine cannot be administered intravenously to patients because of its lipophilicity, the presence of organic solvents, and crystallization in the aqueous phase. In addition, it did not show improvement on the treatment outcome of the mice. However, the encapsulation of CLF in liposomes reduced its toxicity and allowed the drug to be administered intravenously and in higher doses, enhancing therapeutic efficacy (Mehta, 1996; Kansal *et al*., 1997).

Despite these improvements, there are still some limitations for using clofazimine in the chemotherapy of TB in humans. These include solubility, lipophilicity, pharmacokinetics and drug delivery in humans. In this study, the effectiveness of a nanoparticle formulation of clofazimine, was compared to the crystalline native form of the drug with respect to eradication of intracellular *M. tuberculosis* bacilli in macrophages. Both agents were comparable at each concentration tested. The outcome of this study has illustrated that this spray-dried formulation of clofazimine retains the antimycobacterial activity comparable to that of the native drug. As it has not been clearly defined in previous studies, the minimum intracellular bactericidal concentration of clofazimine has been determined in this study using human-derived macrophages.

Further improvements on clofazimine as a possible candidate drug against *M. tuberculosis* were demonstrated through the development of analogues by Lu and co-workers (2011). In this systematic molecular structure/activity study, they tested more than 500 riminophenazine

analogs for antituberculosis activity. Findings demonstrated equivalent activity compared to clofazimine against intracellular *M. tuberculosis* H37Rv, as well as panels of drug-sensitive and drug-resistant clinical isolates. Eleven of these more water-soluble analogs were dosed orally to mice and possessed shorter half-lives as compared to clofazimine. This suggested that they may accumulate less. However, nine compounds that progressed to efficacy testing showed inhibitory activity on the growth of bacteria in the lung. These agents were superior to an equivalent dose of clofazimine which had been orally administered in a murine model of acute tuberculosis over a period of 20 days (Lu *et al*., 2011).

4.2 CONCLUDING COMMENTS

As previously discussed, a renewed interest in evaluating the activity of a variety of second-line antituberculous drugs, new analogs of existing drugs, and newer drug combinations against *M. tuberculosis*, has resulted due to the emergence of multidrug-resistant (MDR) cases. The success of developing such drugs will depend on the acquisition of insights into the mechanisms involved in the intracellular survival of *M. tuberculosis* as such aspects would assist chemical biologists in prospectively selecting targets for structure-guided drug designs (Dwyer *et al*., 2009; Mitchison and Chang, 2009). One of the many challenges is the development of, affordable, non-toxic orally available drugs that would require shorter regimens for patients (Lu *et al*., 2011). Despite the limited use of clofazimine against human TB over the years, interest remains and the current study was designed with the primary objective of assessing the intracellular efficacy of a nanoparticle formulation of clofazimine which can be delivered directly into the airways. The results of the current study demonstrated impressive *in vitro* intracellular antimycobacterial activity of clofazimine using *M. tuberculosis*-infected human monocyte-derived macrophages. Although the findings with respect to a comparison of the spray-dried formulation with the native drug demonstrated no statistically significant differences with respect to inhibition of the growth of intracellular *M. tuberculosis*, they can be used to lay a foundation for further studies to probe the efficacy of the nanoparticle formulation against TB in animal models of experimental chemotherapy.

CHAPTER 5

5.1 REFERENCES

Aaron, L., Saadoun, D., Calatroni, I., Launay, O., Memain, N., Vincent, V., Marchal, G., Dupont, B., Bouchaud, O., Valeyre, D. and Lortholay, O. 2004. Tuberculosis in HIV-infected patients: a comprehensive review. *Clin Microbiol Infect*. **10:** 388–398.

Abate, G. and Hoffner, S.E. 1997. Synergistic antimycobacterial activity between ethambutol and the beta-lactam drug cefepime. *Diagnostic Microbiology and Infectious Disease*. **28:** 119-122.

Abel, B., Thieblemont, N., Quesniaux, V.J.F., Brown, N., Mpagi, J., Miyake, K., Bihl, F. and Ryfell, B. 2002. Toll-like receptor 4 expression is required to control chronic *Mycobacterium tuberculosis* infection in mice. *J Immunol*. **169:** 3155-3162.

Adams, D.A. and Lloyd, A.R. 1997. Chemokines: leucocyte recruitment and activation cytokines. *Lancet*. **349:** 490–495.

Adams, L.B., Sinha, I., Franzblau, S.G., Krahenbuhl J.L. and Mehta, R.T. 1999. Effective treatment of acute and chronic murine tuberculosis with liposome-encapsulated clofazimine. *Antimicrobial Agents and Chemotherapy*. **43(7):** 1638–1643.

Aderem, A. and Underhill, D.M. 1999. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol*. **17:** 593-623.

Agins, B.D., Berman, D.S., Spicehandler, D., El-Sadr, W., Simberkoff, M.S. and Rahal, J.J. 1989. Effect of combined therapy with ansamycin, clofazimine, ethambutol and isoniazid for *Mycobacterinum avium* infection in patients with AIDS. *J. Infect. Dis.* **159:** 784-786.

Akira, S. 2004. Toll receptor families: structure and function. *Semin. Immunol*. **16:** 1–2.

Akira, S. and Takeda, K. 2004. Toll-like receptor signalling. *Nat Rev Immunol*. **4(7):** 499–511.

Akira, S., Uematsu, S. and Takeuchi, O. 2006. Pathogen recognition and innate immunity. *Cell*. **124:** 783-801.

Aliprantis, A.O., Yang, R.B., Mark, M.R., Suggett, S., Devaux, B., Radolf, J.D., Klimpel, G.R., Godowski, P. and Zychlinsky, A. 1999. Cell activation and apoptosis by bacterial lipoproteins through Toll-like receptor-2. *Science.* **285:** 736.

Allday, E.J. and Barnes, J. 1952. Treatment of leprosy with B. 283. *Ir. J. Med. Sci.* **6(322):** 421-425.

Alonso, S., Pethe, K., Russell, D.G. and Purdy, G.E. 2007. Lysosomal killing of Mycobacterium mediated by ubiquitin-derived peptides is enhanced by autophagy. *Proc Natl Acad Sci USA*. **104:** 6031–6036.

Andersen, P. 1997. Host responses and antigens involved in protective immunity to *Mycobacterium tuberculosis*. *Scand. J. Immunol*. **45:** 115–131.

Arai, K., Lee, F., Miyajima, A., Miyatake, S., Arai, N. and Yokota, T. 1990. Cytokines: Coordinators of immune and inflamatory responses. *Annu Rev Biochem*. **59:** 783.

Arbiser, J.L. and Moschella, S.L. 1995. Clofazimine: A review of its medical uses and mechanisms of action. *J Am Acad Dermatol*. **32:** 241-247.

Armstrong, J.A. and d'Arcy Hart, P.D. 1975. Phagosome–lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. Reversal of the usual non-fusion pattern and observations on bacterial survival. *J Exp Med*. **142:** 1–16.

Bafica, A., Scanga, C.A., Feng, C.G., Leifer, C., Cheever, A. and Sher, A. 2005. TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to *Mycobacterium tuberculosis*. *J Exp Med*. **202:** 1715–1724.

Balaram, P., Kien, P.K. and Ismail, A. 2009. Toll-like receptors and cytokines in immune responses to persistent mycobacterial and Salmonella infections. *International Journal of Medical Microbiology*. **299:** 177–185.

Banerjee, D.K., Ellard, G.A., Gammon, P.T. and Waters, M.F. 1974. Some observations on the pharmacology of clofazimine B 663. *Am. J. Trop. Med. Hyg.* **23:** 1110-1119.

Barry 3rd, C.E. 2001. Preclinical candidates and targets for tuberculosis therapy. *Curr Opin Investig Drugs*. **2:** 198-201.

Barry 3rd, C.E. and Blanchard, S.J. 2010. The chemical biology of new drugs in the development for tuberculosis. *Current Opinion in Chemical Biology*. **14:** 456–466.

Barry, V.C., Belton, J.G., Conalty, M.L., Denneny, J.M., Edward, D.W., O'Sullivan, J.F., Twomey, D. and Winder, F. 1957. A new series of phenazines (rimino-compounds) with high antituberculosis activity. *Nature*. **179:** 1013-1015.

Barry, V.C. and Conalty, M.L. 1965. The antimycobacterial activity of B663. *Lepr Rev*. **36(1):** 3–7.

Bass, J.B., Farer, L.S., Hopewell, P.C., O'Brien, R., Jacobs, R.F., Ruben, F., Snider, D.E. and Thornton, G. 1994. Treatment of tuberculosis and tuberculosis infection in adults and children. American Thoracic Society and the Centers for Disease Control and Prevention. *American Journal of Respiratory and Critical Care Medicine*. **149:** 1359-1370.

Bazan, J.F., Bacon, K.B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D.R, Zlotnik, A. and Schall, T.J. 1997. A new class of membrane-bound chemokines with a CX3C motif. *Nature*. **385:** 640-644.

Beutler, B. 2004. Inferences, questions and possibilities in Toll-like receptor signalling. *Nature*. **430(6996):** 257–263.

Bhatt, K. and Salgame, P. 2007. Host innate immune response to *Mycobacterium tuberculosis. J Clin Immunol*. **27:** 347-362.

Blackwell, J.M., Searle, S., Goswami, T. and Miller, E.N. 2000. Understanding the multiple functions of *Nramp1*. *Microbes Infect*. **2:** 317–321.

Blander, J.M. and Medzhitov, R. 2004. Regulation of phagosome maturation by signals from toll-like receptors. *Science*. **304:** 1014-1018.

Bloch, A.B., Cauthen, G.M., Onorato, I.M., Dansbury, K.G., Kelly, G.D., Driver C.R. and Snider Jr, D.E. 1994. Nationwide survey of drug-resistant tuberculosis in the United States. *Journal of the American Medical Association*. **271:** 665-671.

Bloom, B.R. and Bennett, B. 1970. Macrophages and delayed-type hypersensitivity. *Semin Hematol*. **7:** 215.

Bonecchi, R., Bianchi, G., Bordignon, P.P., D'Ambrosio, D., Lang, R., Borsatti, A., Sozzani, S., Allavena, P., Gray, P.A., Mantovani, A. and Sinigaglia, F. 1998. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med*. **187(1):** 129-134.

Botha, T. and Ryffel, B. 2003. Reactivation of latent tuberculosis infection in TNF-deficient mice. *J. Immunol*. **171:** 3110–3118.

Brightbill, H.D., Libraty, D.H., Krutzik, S.R., Yang, R.B., Belisle, J.T., Bleharski, J.R., Maitland, M., Norgard, M.V., Plevy, S.E., Smale, S.T., Brennan, P.J., Bloom, B.R., Godowski, P.J. and Modlin, R.L. 1999. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science*. **285:** 732.

British Medical Association and Royal Pharmaceutical Society of Great Britain. 2006. British national formulary. **no. 51**. London: BMA, RPS.

British Pharmacopoeia Commission. 1988. *The British Pharmacopoeia*. Her Majesty's Stationary Office, London.

Bulut, Y., Faure, E., Thomas, L., Equils, O. and Arditi, M. 2001. Cooperation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and *Borrelia burgdorferi* outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling. *J. Immunol*. **167:** 987–994.

Cavalieri, S.J., Biehle, J.R. and Sanders, W.E. 1995. Synergistic activities of clarithromycin and antituberculous drugs against multidrug-resistant *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy*. **39:** 1542-1545.

Caver, C.V. 1982. Clofazimine induced ichthyosis and its treatment. *Cutis*. **29:** 341-343.

Centers for Disease Control. 1994. Guidelines for preventing the transmission of Mycobacterium tuberculosis in health-care facilities. *Morbid. and Mortal. Weekly Rep*. **43(RR-13):** 4-68.

Champsi, J.H., Bermudez, L.E. and Young, L.S. 1994. The role of cytokines in mycobacterial infection. *Biotherapy*. **7:** 187–193.

Chan, J., Tanaka, K., Carroll, D., Flynn, J. and Bloom, B.R. 1995. Effects of nitric oxide synthase inhibitors on murine infection with *Mycobacterium tuberculosis. Infect Immun*. **63:** 736-740.

Chan, J., Xing, Y., Magliozzo, R.S. and Bloom, B.R. 1992. Killing of virulent *Mycobacterium tubeculosis* by the reactive nitrogen intermediates produced by activated murine macrophages. *J Exp Med*. **175:** 1111-1122.

Chaudhuri, N., Dower, S.K., Whyte, M.K. and Sabroe, I. 2005. Toll-like receptors and chronic lung disease. *Clin Sci (Lond)* **109:** 125–33.

Chawla, A. 2010. Control of Macrophage Activation and Function by PPARs. *Circulation Research*. **106:** 1559-1569.

Chensue, S.W., Warmington, K.S., Allenspach, E.J., Lu, B., Gerard, C., Kunkel, S.L. and Lukacs N.W. 1999. Differential expression and cross-regulatory function of RANTES during mycobacterial (type1) and schistosomal (type2) antigen-elicited granulomatous inflammation. *J. Immunol*. **163:** 165–173.

Cholo, M.C., Boshoff, H.I., Steel, H.C., Cockeran, R., Matlola, N.M., Downing, K.J., Mizrahi, V. and Anderson, R.2006. Effects of clofazimine on potassium uptake by Trk-deletion mutant of *Mycobacterium tuberculosis*. *J Antimicrob Chemother*. **57:** 79-84.

Cholo, M.C., Steel, H.C., Fourie, P.B., Germishuizen, W.A. and Anderson, R. 2012. Clofazimine: current status and future prospects. *J Antimicrob Chemother*. **67(2):** 290-298.

Chopra, I. and Brennan, P. 1998. Molecular action of antimycobacterial agents. *Tubercle and Lung Disease*. **78(2):** 89-98.

Clemens, D.L. and Horwitz, M.A. 1995. Characterisation of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. *J Exp Med*. **181:** 257-270.

Clofazimine. 2008. *Tuberculosis*. **88(2):** 96–99.

Cohen, T., Lipsitch, M., Walensky, R.P. and Murray, M. 2006. Beneficial and perverse effects of isoniazid preventive therapy for latent tuberculosis infection in HIV-tuberculosis coinfected populations. *Proc Natl Acad Sci USA*. **103:** 7042–7047.

Conalty, M.L. 1966. Rimino-phenazines and the reticulo-endothelial system. *Ir. J. Med. Sci*. **6(491)**: 497-501.

Conalty, M.L. and Jackson, R.D. 1962. Uptake by reticulo-endothelial cells of the riminophenazine B663(2-P-chloroanilino-5-P-chlorophenyl-3:5-dihydro-3-isopropyliminophenazine. *Br. J. Exp. Path*. **43:** 651-654.

Conalty, M.L., Barry, V.C. and Jina, A. 1971. The antileprosy agent B663 (Clofazimine) and the reticuloendothelial system. *Int. J. Lepr. Other Mycobact. Dis.* **39(2):** 479-492.

Cooper, A.M., Magram, J., Ferrante, J. and Orme, I.M. 1997. Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis. J Exp Med*. **186:** 39-45.

Corbett, E.L., Watt, C.J., Walker, N., Maher, D., Williams, B.G., Raviglione, M.C. and Dye, C. 2003. The growing burden of tuberculosis: global trends and interactions with HIV epidemic. *Arch Intern Med*. **163(9):** 1009-1021.

Cyster, J.G. 2005. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annu Rev Immunol*. **23:** 127-159.

Dahl, K.E., Shiratsuchi, H., Hamilton, B.D., Ellner, J.J. and Toossi, Z. 1996. Selective induction of transforming growth factor-beta in human monocytes by lipoarabinomannan of *Mycobacterium tuberculosis*. *Infect. Immun*. **64:** 399–405.

Dalton, D.K., Pitts-Meek, S., Keshav, S., Figari, I.S., Bradley, A. and Stewart, T.A. 1993. Multiple defects of immune-cell function in mice with disrupted interferon-γ genes. *Science*. **259(5102):** 1739–1742.

David, J.R. 1973. Lymphocyte mediators and cellular hypersensitivity. *N Engl J Med*. **288:** 143.

Davidson, P.T. and Le, H.Q. 1992. Drug treatment of tuberculosis. *Drugs*. **43:** 651-673.

Davis, R. 2007. Tuberculosis and the intensivist. *Current Anaesthesia & Critical Care*. **18:** 76–85.

Dayan Sandler, E., Ng V.L. and Hadley, W.K. 1992. Clofazimine crystals in alveolar macrophages from a patient with acquired immunodeficiency syndrome. *Arch. Pathol. Lab. Med*. **116:** 541–543.

De Bruyn, E.E., Steel, H.C., van Rensburg, C.E.J. and Anderson, R. 1996. The riminophenazines, clofazimine and B669, inhibit potassium transport in Gram-positive bacteria by a lysophospholipid-dependent mechanism. *J Antimicrob Chemother*. **38(3):** 349–362.

De Chastellier, C., Forquet, F., Gordon, A. and Thilo, L. 2009. Mycobacterium requires an all-around closely apposing phagosome membrane to maintain the maturation block and this apposition is re-established when it rescues itself from phagolysosomes. *Cell Microbiol*. **11(8):** 1190-1207.

De Chastellier, C., Lang, T. and Thilo, L. 1995. Phagocytic processing of the macrophage endoparasite, *Mycobacterium avium*, in comparison to phagosomes which contain *Bacillus subtilis* or latex beads. *Eur J Cell Biol*. **68:** 167-182.

De Cock, K.M., Grant, A. and Porter, J.D. 1995. Preventive therapy for tuberculosis in HIV-infected persons: international recommendations, research, practice. *Lancet*. **345:** 833-836.

Dean, G.L., Edwards, S.G., Ives, N.J., Matthews, G., Fox, E.F., Navaratne, L., Fisher, M., Tayler, G.P., Miller, R., Taylor, C.B., De Ruiter, A. and Pozniak, A.L. 2002. Treatment of tuberculosis in HIV-infected persons in the era of highly active antiretroviral therapy. *AIDS*. **16:** 75–83.

De Logu, A., Onnis, V., Saddi, B., Congiu, C., Schivo, M.L. and Cocco, M.T. 2002. Activity of a new class of isonicotinoylhydrazones used alone and in combination with isoniazid, rifampicin, ethambutol, para-aminosalicylic acid and clofazimine against *Mycobacterium tuberculosis. J Antimicrob Chemother*. **49:** 275-282.

Dempsey, P.W., Doyle, S.E., He, J.Q. and Cheng, G. 2003. The signalling adaptors and pathways activated by TNF superfamily. *Cytokine Growth Factor Rev*. **14:** 193–209.

Denis, M. and Ghadirian, E. 1990. Granulocyte-macrophage colony stimulating factor (GM-CSF) restricts growth of tubercule bacilli in human macrophages. *Immunol. Lett*. **24:** 203.

Denis, M., Gregg, E.O. and Ghadirian, E. 1990. Cytokine modulation of *Mycobacterium tuberculosis* growth in human macrophages. *Int. J. Immunopharmacol*. (In Press).

Department of Health and Human Services, Centers for Disease Control and Prevention. 1994. Guidelines for preventing the transmission of Mycobacterium tuberculosis in health-care facilities. *Fed. Reg.,* **59(208):** 54242-54303.

Deretic, V., Singh, S., Master, S., Harris, J., Roberts, E., Kyei, G., Davis, A., de Haro, S., Naylor, J., Lee, H.H. and Vergne, I. 2006. *Mycobacterium tuberculosis* inhibition of phagolysosome biogenesis and autophagy as a host defence mechanism. *Cell Microbiol*. **8:** 719–727.

Desikan, K.V. and Balakrishnan, S. 1976. Tissue levels of clofazimine in case of leprosy. *Lepr. Rev*. **47:** 107-113.

Desikan, K.V., Ramanujam, K., Ramu, G. and Balakrishnan S. 1975. Autopsy findings in a case of lepromatous leprosy treated with clofazimine. *Lepr. Rev*. **46**: 181-189.

Dollery, C. 1991. *Therapeutic Drugs*, Vol. **1**, Churchill Livingstone, Edinburgh.

Dowling, D., Hamilton, C.M. and O'Neill, S.M. 2008. A comparative analysis of cytokine responses, cell surface marker expression and MAPKs in DCs matured with LPS compared with a panel of TLR ligands. *Cytokine*. **41(3):** 254–262

Doyle, S.E., O'Connell, R.M., Miranda, G.A., Vaidya, S.A., Chow, E.K., Liu, P.T., Suzuki, S., Suzuki, N., Modlin, R.L., Yeh, W.C., Lane, T.F. and Cheng, G. 2004. Toll-like receptors induce a phagocytic gene program through p38. *J. Exp. Med*. **199:** 81-90.

Drennan, M.B., Nicolle, D., Quesniaux, V.J., Jacobs, M., Allie, N., Mpagi, J., Frémond, C., Wagner, H., Kirschning, C. and Ryffel, B. 2004. Toll-like receptor 2-deficient mice succumb to *Mycobacterium tuberculosis* infection. *Am J Pathol*. **164(1):** 49-57.

Drugs.com. Clofazimine. http://www.drugs.com/cons/clofazimine. html (1 July 2011, date last accessed).

Duan, L., Gan, H., Golan, D.E. and Remold, H.G. 2002. Critical role of mitochondrial damage in determining outcome of macrophage infection with *Mycobacterium tuberculosis*. *J Immunol*. **169:** 5181–5187.

Durandt, C., van Rensburg, C.E.J., O'Sullivan, J.F. and Anderson, R. 1996. Novel riminophenazine compounds with improved anti-tumour properties. *S Afr J Sci*. **92:** 257-259.

Dwyer, D.J., Kohanski, M.A. and Collins, J.J. 2009. Role of reactive oxygen species in antibiotic action and resistance. *Curr Opin Microbiol*. **12:** 482-489.

Dye, C., Bassili, A., Bierrenbach, A.L., Broekmans, J.F., Chadha, V.K., Glaziou, P., Gopi, P.G., Hosseini, M., Kim, S.J., Manissero, D., Onozaki, I., Rieder, H.L., Scheele, S., van Leth, F., van der Werf, M. and Williams, B.G. 2008. Measuring tuberculosis burden, trends, and the impact of control programmes. *Lancet Infect Dis*. **8(4):** 233-243.

Dye, C., Scheele, S., Dolin, P., Pathania, V. and Raviglione, M.C. 1999. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO global surveillance and monitoring project. *JAMA* **282:** 677–686.

Ehrt, S., Schnappinger, D., Bekiranov S., Drenkow, J., Shi, S., Gingeras, T.R., Gaasterland, T., Schoolnik, G. and Nathana, C. 2001. Reprogramming of the macrophage transcriptome in response to interferon-γ and *Mycobacterium tuberculosis*. Signaling roles of nitric oxide synthase-2 and phagocyte oxidase. *J. Exp. Med*. **194(8):** 1123–1140.

Erb, K.J., Kirman, J., Delahunt, B., Chen, W. and Le Gros, G. 1998. IL-4, IL-5 and IL-10 are not required for the control of M. bovis-BCG infection in mice. *Immunol. Cell Biol*. **76:** 41–46.

Fenton, M.J. and Golenbock, D.T. 1998. LPS-binding proteins and receptors. *J Leukoc Biol*. **64:** 25-32.

Fine, P.E. 1995. Variation in protection by BCG: implications of and for heterologous immunity. *Lancet*. **346:** 1339–1345.

Flannagan, R.S., Cosio, G. and Grinstein, S. 2009. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nat Rev Microbiol*. **7:** 355-66.

Flesch, I. and Kaufmann, S.H.E. 1987a. Mycobacterial growth inhibition by interferon-gamma-activated bone marrow macrophages and differential susceptibility among strains of *Mycobacterium tuberculosis*. *J Immunol*. **138**: 4408-4413.

Flesch, I and Kaufmann S.H.E. 1987b. Mycobacterial growth-inhibition by interferon-gamma activated bone-marrow macrophages. *Immunobiology*. **175:** 277–8.

Flynn, J.L. and Chan, J. 2001. Immunology of tuberculosis. *Annu Rev Immunol.* **19:** 93-129.

Flynn, J., Goldstein, M., Triebold, K., Sypek, J., Wolf, S. and Bloom, B. 1995a. IL-12 increases resistance of BALB/c mice to *Mycobacterium tuberculosis* infection. *J Immunol*. **155**: 2515.

Flynn, J.L., Goldstein, M.M., Chan, J., Triebold, K.J., Pfeffer, K., Lowenstein, C.J., Schreiber, R., Mak, T.W. and Bloom B.R. 1995b. Tumor necrosis factor-alpha is required in

the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunity.* **2:** 561–572.

Fong, A.M., Robinson, L.A., Steeber, D.A., Tedder, T.F., Yoshie, O., Imai, T. and Patel, D.D. 1998. Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. *J Exp Med*. **188(8):** 1413-1419.

Forman, H.J. and Torres, M. 2001. Signaling by the Respiratory Burst in Macrophages. IUBMB *Life.* **51:** 365–371.

Forster, D.J., Causey, D.M. and Rao, N.A. 1992. Bull's eye retinopathy and clofazimine. *Ann. Intern. Med*. **116:** 876–877.

Fox, W. 1979. The chemotherapy of pulmonary tuberculosis: a review. *Chest*. **76:** 785-796.

Fox, W., Ellard, G.A. and Mitchison, D.A. 1999. Studies on the treatment of tuberculosis undertaken by the British Medical Research Council Tuberculosis Units, 1946-1986, with relevant subsequent publications. *Int J Tuberc Lung Dis.* **3**: S231-279.

Fox, W. and Mitchison, D.A. 1976. Short-course chemotherapy for tuberculosis. *Lancet*, **2**:13491350. An authoritative history and discussion of the development of the multidrug chemotherapy currently used in the treatment of Tuberculosis.

Fox, W. 1981. Whither short-course chemotherapy? *Br J Dis Chest*. **75:** 331-357.

Fréhel, C., de Chastellier, C., Lang, T. and Rastogi, N. 1986. Evidence for inhibition of fusion of lysosomal and prelysosomal compartments with phagosomes in macrophages infected with pathogenic *Mycobacterium avium*. *Infect Immun*. **52:** 252–262.

Fulton, S.A., Johnsen, J.M., Wolf, S.F., Sieburth, D.S. and Boom, W.H. 1996. Interleukin-12 production by human monocytes infected with *Mycobacterium tuberculosis*: role of phagocytosis. *Infect. Immun.* **64:** 2523–2531.

Fulton, S.A., Cross, J.V., Toossi, Z.T. and Boom, W.H. 1998. Regulation of interleukin-12 by interleukin-10, transforming growth factor-beta, tumor necrosis factor-alpha, and interferon-gamma in human monocytes infected with *Mycobacterium tuberculosis* H37Ra. *J. Infect. Dis*. **178:** 1105–1114.

Gandhi, N.R, Moll, A., Sturm, A.W., Pawinski, R., Govender, T., Lalloo, U., Zeller, K., Andrews, J. and Friedland, G. 2006. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet*. **368:** 1575-1580. A detailed report of an outbreak of extensively drug-resistant Tuberculosis in the KwaZulu Natal province of South Africa.

Gao, B. and Tsan, M.F. 2003. Endotoxin contamination in recombinant human heat shock protein 70 (Hsp70) preparation is responsible for the induction of tumor necrosis factor-a release by murine macrophages. *J. Biol. Chem*. **278:** 174.

Garrelts, J.C. 1991. Clofazimine: a review of its use in leprosy and *Mycobacterium avium* complex infection. *Ann Pharmacother*. **25:** 525-531.

Geissmann, F., Manz, M.G., Jung, S., Sieweke, M.H., Merad, M. and Ley, K. 2010. Development of Monocytes, Macrophages, and Dendritic Cells. *Science*. **327:** 656-661.

Gelband, H. 2000. Regimens of less than six months for treating tuberculosis. *Cochrane Database Syst Rev* **(2):** CD001362.

Gerard, C. and Rollins, B.J. 2001. Chemokines and disease. *Nat Immunol* **2:**108-115.

Gerszten, R.E., Garcia-Zepeda, E.A., Lim, Y-C., Yoshida, M., Ding, H.A., Gimbrone, M.A. Jr., Luster, A.D., Luscinskas, F.W. and Rosenzweig A. 1999. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature*. **398(6729):** 718-723.

Giacomini, E., Iona, E., Ferroni, L., Miettinen, M., Fattorini, L., Orefici, G., Julkunen, I. and Coccia, E.M. 2001. Infection of human macrophages and dendritic cells with *Mycobacterium tuberculosis* induces a differential cytokine gene expression that modulates T cell response. *J Immunol*. **166(12):** 7033-7041.

Gong, J., Zhang, M., Modlin, R.L., Linsley, P.S., Iyer, D.V., Lin, Y. and Barnes, P.F. 1996. Interleukin-10 downregulates *Mycobacterium tuberculosis* induced Th1 responses and CTLA-4 expression. *Infect Immun*. **64:** 913–918.

Gonzalez-Juarrero, M. and Orme, I.M. 2001. Characterization of murine lung dendritic cells infected with *Mycobacterium tuberculosis*. *Infect Immun*. **69:** 1127–1133.

Gordon, S. 2003. Alternative activation of macrophages. *Nature Reviews: Immunology*. **3:** 23-35.

Gordon, S. 2007. The macrophage: past, present and future. *Eur J Immunol*. **37 Suppl 1:** S9–17.

Gordon, S. and Hughes, D.A. 1997. In *Lung Macrophages and Dendritic Cells in Health & Disease* (eds Lipscomb, M. & Russell, S.) 3–31 (Marcel Dekker, New York).

Gordon, S. 1999. In *Fundamental Immunology*, ed. Paul, W. (Lippincott-Raven, New York), pp. 533–545.

Gordon, S.B., Irving, G.R., Lawson, R.A., Lee, M.E. and Read, R.C. 2000. Intracellular trafficking and killing of *Streptococcus pneumoniae* by human alveolar macrophages are influenced by opsonins. *Infect Immun.* **68:** 2286–2293.

Grange, J. Antimycobacterial agents. 1997. In: O'Grady F, Lambert HP, Finch RG, Greenwood D, eds. Antibiotic and Chemotherapy, 7th Ed. Edinburgh: Churchill Livingstone, pp 449-512.

Greenwood D. 1997. Historical introduction. In: O'Grady F, Lambert HP, Finch RG, Greenwood D, eds. Antibiotic and Chemotherapy, 7th Ed. Edinburgh: Churchill Livingstone, pp 2-9.

Guenin-Mace, L., Simeone, R. and Demangel, C. 2009. Lipids of pathogenic mycobacteria: contributions to virulence and host immune suppression. *Trans Emerging Dis*. **56**: 255-268.

Gurfinkel, P., Pina, J.C. and Ramos-e-Silva, M. 2009. Use of clofazimine in dermatology. *Journal of Drugs in Dermatology*. **8(9):** 846-851.

Gutierrez, M.G., Master, S.S., Singh, S.B., Taylor, G.A., Colombo, M.I. and Deretic, V. 2004. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell*. **119:** 753-766.

Halayko, A.J. and Ghavami, S. 2009. S100A8/A9: a mediator of severe asthma pathogenesis and morbidity? *Can J Physiol Pharmacol*. **87(10):** 743–755.

Handel, T.M. and Domaille, P.J. 1996. Heteronuclear (1H, 13C, 15N) NMR assignments and solution structure of the monocyte chemoattractant protein-1 (MCP-1) dimer. *Biochemistry*. **35:** 6569-6584.

Harding, C.V. and Boom, W.H. 2010. Regulation of antigen presentation by *Mycobacterium tuberculosis*: a role for toll-like receptors. *Nat Rev Microbiol*. **8:** 296-307.

Harju, K., Glumoff, V. and Hallman, M. 2001. Ontogeny of Toll-like receptors Tlr2 and Tlr4 in mice. *Pediatr Res* **49:** 81–83.

Haskell, C.A., Cleary, M.D. and Charo, I.F. 1999. Molecular uncoupling of fractalkine-mediated cell adhesion and signal transduction: rapid flow arrest of CX3CR1-expressing cells is independent of G-protein activation. *J Biol Chem*. **274:** 10053-10058.

Havlir, D.V. and Barnes, P.F. 1999. Current concepts: tuberculosis in patients with human immunodeficiency virus infection. *N Engl J Med.* **340:** 367–373.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S.A. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature*. **408(6813):** 740–745.

Henderson, R.A., Watkins, S.C. and Flynn J.L. 1997. Activation of human dendritic cells following infection with *Mycobacterium tuberculosis*. *J. Immunol*. **159:** 635-643.

Hinshaw, H.C. and Feldman, W.H. 1945. Streptomycin in the treatment of clinical tuberculosis; a preliminary report. *Proc Staff Meeting Mayo Clin*. **20:** 313-318.

Hirsch, C.S., Toossi, Z., Othieno, C., Johnson, J.L., Schwander, S.K., Robertson, S., Wallis, R.S., Edmonds, K., Okwera, A., Mugerwa, R., Peters, P. and Ellner, J.J. 1999. Depressed T-cell interferon-gamma responses in pulmonary tuberculosis: analysis of underlying mechanisms and modulation with therapy. *J. Infect. Dis*. **180**: 2069–2073.

Hirschfeld, M., Ma, Y., Weis, J.H., Vogel, S.N. and Weis, J.J. 2000. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J Immunol.* **165:** 618–622.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. and Akira, S. 1999. Cutting Edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps hene product. *J. Immunol.* **162**: 749.

Hu, X. and Ivashkiv, L.B. 2009. Cross-regulation of signaling pathways by interferon-gamma: implications for immune responses and autoimmune diseases. *Immunity.* **31:** 539.

Huo, Y., Weber, C., Forlow, S.B., Sperandio, M., Thatte, J., Mack, M., Jung, S., Littman, D.R. and Ley, K. 2001. The chemokine KC, but not monocytes chemoattractant protein-1, triggers monocytes arrest on early atherosclerotic endothelium. *J Clin Invest.* **108(9):** 1307-1314.

Iseman, M.D. 1993. Treatment of multidrug-resistant tuberculosis. *N Engl J Med.* **329:** 784-791.

Jancovic, D., Liu, Z. and Gause, W.C. 2001. Th1- and Th2-cell commitment during infectious diseases: asymmetry in divergent pathways. *Trends Immunol*. **22:** 450–457.

Janeway Jr., C.A. 1989. Approaching the asymptote? Evolution and revolution in immunology, Cold Spring Harb. *Symp. Quant. Biol.* **54 (Pt 1):** 1-13.

Jawahar, M.S. 2004. Current trends in chemotherapy of tuberculosis. *Indian J Med Res*. **120:** 398-417.

Juffermans, N.P., Verbon, A., van Deventer, S.J., van Deutekom, H., Belisle, J.T., Ellis, M.E., Speelman, P. and van der Poll, T. 1999. Elevated chemokine concentrations in sera of human immunodeficiency virus (HIV)-seropositive and HIV seronegative patients with tuberculosis: a possible role for mycobacterial lipoarabinomannan. *Infect. Immun*. **67:** 4295–4297.

Kabelitz, D. 2007. Expression and function of Toll-like receptors in T lymphocytes. *Curr. Opin. Immunol*. **19:** 39–45.

Kansal, R.G., Gomez-Flores, R., Sinha, I. and Mehta, R.T. 1997. Therapeutic efficacy of liposomal clofazimine against *Mycobacterium avium* complex in mice depends on size of initial inoculum and duration of infection. *Antimicrob. Agents Chemother.* **41:** 17–23.

Kaplan, B., Trau, H., Sofer, E., Feinstein, A. and Schewach-Millet, M. 1992. Treatment of *pyoderma gangrenosum* with clofazimine. *Int J Dermatol* **31(8):** 591-593.

Kelner, G.S., Kennedy, J., Bacon, K.B., Kleyensteuber, S., Largaespada, D.A., Jenkins, N.A., Copeland, N.G., Bazan, J.F., Moore, K.W. and Schall, T.J. 1994. Lymphotactin: a cytokine that represents a new class of chemokine. *Science.* **266(5189):** 1395-1399.

Knable, A.L. 2001. Miscellaneous systemic drugs. In: Wolverton SE. ed. Comprehensive dermatologic drug therapy. Philadelphia: WB Saunders.pp 445-470.

Knapp, S., Wieland, C.W., van 't Veer, C., Takeuchi, O., Akira, S., Florquin, S. and van der Poll, T. 2004. Toll-like receptor 2 plays a role in the early inflammatory response to murine pneumococcal pneumonia but does not contribute to antibacterial defense. *J Immunol.* **172(5):** 3132–3138.

Kohro, T., Tanaka, T., Murakami, T., Wada, Y., Aburatani, H., Hamakubo, T. and Kodama T. 2004. A comparison of differences in the gene expression profiles of phorbol 12-myristate 13-acetate differentiated THP-1 cells and human monocyte-derived macrophage. *J Atheroscler Thromb.* **11(2):** 88–97.

Kono, H. and Rock, K.L. 2008. How dying cells alert the immune system to danger. *Nature Rev. Immunol.* **8:** 279–289.

Korbel, D.S., Schneider, B. and Schaible, E. 2008. Innate immunity in tuberculosis: myths and truth. *Microbes Infect*. **10:** 995-1004.

Koul, A., Herget, T., Klebl, B. and Ullrich, A. 2004. Interplay between mycobacteria and host signalling pathways. *Nat Rev Microbiol.* **2:** 189-202.

Krutzik, S.R. and Modlin, R.L. 2004. The role of Toll-like receptors in combating mycobacteria. *Semin. Immunol*. **16:** 35-41.

Kumar, B., Kuar, S., Kuar, I. and Gangowar, D.N. 1987. More about clofazimine--3 years experience and review of literature. *Ind. J. Lepr.* **59(1)**: 63-74.

Kurashima, K., Mukaida, N., Fujimura, M., Yasui, M., Nakazumi, Y., Matsuda, T. and Matsushima, K. 1997. Elevated chemokine levels in bronchoalveolar lavage fluid of tuberculosis patients. *Am. J. Respir. Crit. Care Med*. **155:** 1474–1477.

Ladel, C.H., Szalay, G., Riedel, D. and Kaufmann, S.H. 1997. Interleukin- 12 secretion by *Mycobacterium tuberculosis*-infected macrophages. *Infect. Immun*. **65:** 1936-1938.

Lake, F.R., Noble, P.W., Henson, P.M. and Riches, D.W.H. 1994. Functional switching of macrophage responses to tumor-necrosis-factor-alpha (TNF-alpha) by interferons–implications for the pleiotropic activities of TNF-alpha. *J Clin Invest* **93:** 1661–1669.

Landsman, L. and Jung, S. 2007. Lung macrophages serve as obligatory intermediate between blood monocytes and alveolar macrophages. *J Immunol.* **179:** 3488–3494.

Lane, T.J.D. 1951. Chemotherapy of urinary tuberculosis. *Ir. J. Med. Sci.* **309:** 393-405.

Lata, S. and Raghava, G.P. 2008. PRRDB: A comprehensive database of Pattern-Recognition Receptors and their ligands. BMC Genomics. **18:**9:180.
http://www.imtech.res.in/raghava/prrdb/

Lien, E., Sellati, T.J., Yoshimura, A., Flo, T.H., Rawadi, G., Finberg, R.W., Carroll, J.D., Espevik, T., Ingalls, R.R., Radolf, J.D. and Golentick, D.T. 1999. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. *J. Biol. Chem.* **274:** 33419.

Liew, F.Y., Xu, D., Brint, E.K. and O'Neill, L.A. 2005. Negative regulation of toll-like receptor-mediated immune responses. *Nat Rev Immunol.* **5:** 446–458.

Liu, P.T. and Modlin, R.L. 2008. Human macrophage host defense against *Mycobacterium tuberculosis*. *Curr Opin Immunol.* **20:** 371-376.

Lu, B., Rutledge, B.J., Gu, L., Fiorillo, J., Lukacs, N.W., Kunkel, S.L., North, R., Gerard, C. and Rollins, B.J. 1998. Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J. Exp. Med.* **187:** 601–608.

Lu, Y., Zheng, M., Wang, B., Fu, L., Zhao, W., Li, P., Xu, J., Zhu, H., Jin, H., Yin, D., Huang, H., Upton, A.M. and Ma, Z. 2011. Clofazimine analogs with efficacy against experimental tuberculosis and reduced potential for accumulation. *Antimicrob. Agents and Chemotherapy*. **55 (11):** 5185-5193.

Lucey, D.R., Clerici, M. and Shearer, G.M. 1996. Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. *Clin. Microbiol. Rev*.**9:** 532–562.

Luster, A.D. 1998. Chemokines — chemotactic cytokines that mediate inflammation. *N Engl J Med.* **338:** 436-445.

López-Barcenas, A., Contreras, J., Carrillo, M., Hojyo, M.T., Arenas, R., Domínguez, L. and Vega, M.E. 2005. Dermatosis cenicienta (Eritema discrómico perstans). *Medicina Cutánea* **33(3)**: 97-102.

Ma, Z. and Lienhardt, C. 2009. Toward an optimized therapy for tuberculosis- Drugs in clinical trials and in preclinical development. *Clin Chest Med.* **30:** 755–768.

MacMicking, J.D., North, R.J., LaCourse, R., Mudgett, J.S., Shah, S.K. and Nathan, C.F. 1997. Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc Natl Acad Sci USA.* **94:** 5243-5248.

Mansfield, R.E. 1974. Tissue concentrations of clofazimine (B663) in man. *Am J Trop Med Hyg.* **23(6):** 1116-1119.

Marino, M.W., Dunn, A., Grail, D., Inglese, M., Noguchi, Y., Richards, E., Jungbluth, A., Wada, H., Moore, M., Williamson, B., Basu, S. and Old L.J. 1997. Characterization of tumor necrosis factor-deficient mice. *Proc. Natl. Acad. Sci. USA*. **94:** 8093–8098.

Markey, J.P. and Barnes, J. 1974. Clofazimine in the treatment of discoid lupus erythematosus. *Br J Dermatol.* **91:** 93-96.

Martin, M., Katz, J., Vogel, S.N. and Michalek, S.M. 2001. Differential induction of endotoxin tolerance by lipopolysaccharides derived from *Porphyromonas gingivalis* and *Escherichia coli. J Immunol.* **167:** 5278–5285.

McGeachy, M.J. and Cua, D.J. 2008. Th17 cell differentiation: the long and winding road. *Immunity.* **28:** 445–453.

Means, T.K., Jones, B.W., Schromm, A.B., Shurtleff, B.A., Smith, J.A., Keane, J., Golenbock, D.T., Vogel, S.N. and Fenton, M.J. 2001. Differential effects of a Toll-like receptor antagonist on Mycobacterium tuberculosis-induced macrophage responses. *J Immunol.* **166(6):** 4074–4082.

Means, T.K., Wang, S., Lien, E., Yoshimura, A., Golenbock, D.T. and Fenton, M.J. 1999. Human Toll-like receptors mediate cellular activation by *Mycobacterium tuberculosis*. *J. Immunol.* **163:** 3920-3927.

Mehta, R.T. 1996. Liposome encapsulation of clofazimine reduces toxicity in vitro and in vivo and improves therapeutic efficacy in the beige mouse model of disseminated *Mycobacterium avium-M. intracellulare* complex infection. *Antimicrob. Agents Chemother.* **40:** 1893–1902.

Metcalf, D. 1991. Control of granulocytes and macrophages: Molecular, cellular, and clinical aspects. *Science.* **254:** 529.

Michaelsson, G., Molin, L., Ohman, S., Gip, L., Lindström, B., Skogh, M. and Trolin, I. 1976. Clofazimine: new agent for the treatment of pyoderma gangrenosum. *Arch Dermatol.* **112(3):** 344-349.

Miotto, D., Christodoulopoulos, P., Olivenstein, R., Taha, R., Cameron, L., Tsicopoulos, A., Tonnel, A.B., Fahy, O., Lafitte, J.J., Luster, A.D., Wallaert, B., Mapp, C.E. and Hamid, Q. 2001. Expression of IFN-γ–inducible protein; monocyte chemotactic proteins 1, 3, and 4; and eotaxin in TH1- and TH2-mediated lung diseases. *J Allergy Clin Immunol.* **107(4):** 664-670.

Mitchison, D. 1979. Basic mechanisms of chemotherapy. *Chest.* **76:** Suppl., 771–781.

Mitchison, D.A. 1993. Assessment of new sterilizing drugs for treating pulmonary tuberculosis by culture at 2 months. *Am Rev Respir Dis.***147:** 1062–1063.

Mitchison, D.A. 1998. Basic concepts in the chemotherapy of tuberculosis. *In Mycobacteria. II. Chemotherapy*, (Gangadharam, P.R.J. & Jerkins, P. A., Eds), pp 15-50. Chapman & Hall, New York.

Mitchison, D.A. 2005. The diagnosis and therapy of tuberculosis during the past 100 years. *Am J Respir Crit Care Med*. **171:** 699-706.

Mitchison, D.A. and Chang, K.C. 2009. Experimental models of tuberculosis: can we trust the mouse? *Am J Respir Crit Care Med.* **180:** 201-202.

Mohammed, K.A., Nasreen, N., Ward, M.J., Mubarak, K.K., Rodriguez-Panadero, F. and Antony, V.B. 1998. Mycobacterium-mediated chemokine expression in pleural mesothelial cells: role of C-C chemokines in tuberculous pleurisy. *J. Infect. Dis*. **178:** 1450–1456.

Morrison, N.E. and Marley, G.M. 1976a. The mode of action of clofazimine: DNA binding studies. *Int. J. Lepr.* **44:** 133-135.

Morrison, N.E. and Marley, G.M. 1976b. Clofazimine binding studies with deoxyribonucleic acid. *Int J Lepr Other Mycobact Dis*. **44(4):** 475-481.

Mosser, D.M. and Edwards, J.P. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol.* **8:** 958–969.

Murphy, J., Summer, R., Wilson, A.A., Kotton, D.N. and Fine, A. 2008a. The prolonged life-span of alveolar macrophages. *Am J Respir Cell Mol Biol*. **38:** 380–385.

Murphy, K., Travers, P. and Walport, M. 2008b. Innate Immunity. In: Morales M, Masson S, eds. Janeway's Immunobiology. 7th ed. New York: Garland Science, Taylor & Francis Group, LLC.p48-49.

Nathan, C. and Shiloh, M.U. 2000. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci USA.* **97:** 8841–8848.

National Collaborating Centre for Chronic Conditions. 2006. Tuberculosis: clinical diagnosis and management of tuberculosis, and measures for its prevention and control. London: Royal College of Physicians.

Nau, G.J., Richmond, J.F.L., Schlesinger, A., Jennings E.G., Lander E.S. and Young R.A. 2002. Human macrophage activation programs induced by bacterial pathogens. *PNAS*. **99(3):** 1503-1508.

Neff, M. ATS, CDC, and IDSA update recommendations on the treatment of tuberculosis. 2003. *Am Fam Phys.* **68:** 1854, 1857–1858, 1861–1852.

Nicholson, S., da Gloria Bonecini-Almeida, M., Lapa e Silva, J.R., Nathan, C., Xie, Q.W., Mumford, R., Weidner, J.R., Calaycay, J., Geng, J., Boechat, N., Linhares, C., Rom, W. and Ho, J.L. 1996. Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J Exp Med.* **183(5):** 2293-2302.

Niwa, Y., Sakane, T., Miyachi, Y. and Ozaki, M. 1984. Oxygen metabolism in phagocytes of leprotic patients: enhanced endogenous superoxide dismutase activity and hydroxyl radical generation by clofazimine. *Journal of Clin Microbial*. **20(5):** 837-842.

North, R.J. 1998. Mice incapable of making IL-4 or IL-10 display normal resistance to infection with *Mycobacterium tuberculosis*. *Clin. Exp. Immunol*. **113:** 55–58.

O'Brien, R.J. and Vernon, A.A. 1998. New tuberculosis drug development: how can we do better? *Am J Respir Crit Care Med.* **157:** 1705–1707.

O'Neill, A.J., Miller, K., Oliva, B. and Chopra, I. 2004. Comparison of assays for detection of agents causing membrane damage in *Staphylococcus aureus*. *J Antimicrob Chemother.* **54(6):** 1127–1129.

O'Neill, L.A. and Greene, C. 1998. Signal transduction pathways activated by the IL-1 receptor family: ancient signaling machinery in mammals, insects, and plants. *J. Leukoc. Biol*. **63:** 650–657.

Oliva, B., O'Neill, A.J., Miller, K., Stubbings, W. and Chopra, I. 2004. Anti-staphylococcal activity and mode of action of clofazimine. *J Antimicrob Chemother*. **53(3):** 435–440.

Orme, I.M. and Cooper, A.M. 1999. Cytokine/chemokine cascades in immunity to tuberculosis. *Immunol Today*. **20:** 307-312.

Ormerod, L.P. and Horsfield, N. 1987. Short-course antituberculosis chemotherapy for pulmonary and pleural disease: 5 years' experience in clinical practice. *Br J Dis Chest.* **81(3):** 268–271.

Ormerod, L.P., McCarthy, O.R., Rudd, R.M. and Horsfield, N. 1991. Short course chemotherapy for pulmonary tuberculosis. *Respir Med.* **85(4):** 291–294.

Othieno, C., Hirsch, C.S., Hamilton, B.D., Wilkinson, K., Ellner, J.J. and Toossi, Z. 1999. Interaction of *Mycobacterium tuberculosis*-induced transforming growth factor β1 and interleukin-10. *Infect. Immun*. **67:** 5730–5735.

Ouyang, W., Kolls, J.K. and Zheng, Y. 2008. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity.* **28**: 454–467.

Ozinsky, A., Underhill, D.M., Fontenot, J.D., Hajjar, A.M., Smith, K.D., Wilson, C.B., Schroeder, L. and Aderem, A. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc. Natl. Acad. Sci. USA* **97:** 13766–13771.

Pablos-Mendez, A., Raviglione, M.C., Laszlo, A., Binkin, N., Rieder, H.L., Bustreo, F., Cohn, D.L., Lambregts-van Weezenbeek, C.S., Kim, S.J., Chaulet, P. and Nunn, P. 1998. Global surveillance for antituberculosis-drug resistance 1994–1997. World Health Organization– International Union against tuberculosis and lung disease working group on anti-tuberculosis drug resistance surveillance. *N Engl J Med.* **338**: 1641–1649.

Pan, Y., Lloyd, C., Zhou, H., Dolich, S., Deeds, J., Gonzalo, J.A., Vath, J., Gosselin, M., Ma, J., Dussault, B., Woolf, E., Alperin, G., Culpepper, J., Gutierrez-Ramos, J.C. and Gearing, D. 1997. Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. *Nature.* **387(6633):** 611-617.

Park, E.K., Jung, H.S., Yang, H.I., Yoo, M.C., Kim, C. and Kim, K.S. 2007. Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. *Inflamm Res.* **56(1):** 45–50.

Parrish, N.M., Dick J.D. and Bishai, W.R. 1998. Mechanisms of latency in *Mycobacterium tuberculosis*. *Trends Microbiol*. **6:** 107-112.

Pasula, R., Wright, J.R., Kachel, D.L. and Martin, W.J.2nd. 1999. Surfactant protein A suppresses reactive nitrogen intermediates by alveolar macrophages in response to *Mycobacterium tuberculosis*. *J Clin Investig*. **103(4):** 483-490.

Peters, W. and Ernst, J.D. 2003. Mechanisms of cell recruitment in the immune response to *Mycobacterium tuberculosis*. *Microbes and Infection.* **5:** 151–158.

Pieters, J. 2001. Evasion of host cell defense mechanisms by pathogenic bacteria. *Curr. Opin. Immunol.* **13(1):** 37–44.

Pethe, K., Swenson, D.L., Alonso, S., Anderson, J., Wang, C. and Russell, D.G. 2004. Isolation of *Mycobacterium tuberculosis* mutants defective in the arrest of phagosome maturation. *Proc Natl Acad Sci USA*. **101(37):** 13642-13647.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B. and Beutler, B. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutation in Tlr4 gene. *Science.* **282(5396):** 2085-2088.

Powrie, F. and Coffman, R.L. 1993. Inhibition of cell-mediated immunity by IL4 and IL10. *Res. Immunol*. **144:** 639–643.

Prieschl, E.E., Kulmburg, P.A. and Baumruker, T. 1995. The nomenclature of chemokines. *Int Arch Allergy Immunol.* **107:** 475-83.

Proudfoot, A.E.I., Handel, T.M., Johnson, Z., Lau, E.K., LiWang,P., Clark-Lewis, I., Borlat, F., Wells, T.N.C. and Kosco-Vilbois, M.H. 2003. Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc Natl Acad Sci USA.* **100(4):** 1885-1890.

Quenelle, D.C., Winchester, G.A., Staas, J.K., Barrow, E.L.W. and Barrow, W.W. 2001. Treatment of tuberculosis using a combination of sustained-release rifampin-loaded microsphere and oral dosing with isoniazid. *Antimicrobial Agents and Chemotherapy.* **45:** 1637-1644.

Quesniaux, V., Fremond, C., Jacobs, M., Parida, S., Nicolle, D., Yeremeev, V., Bihl, F., Erard, F., Botha, T., Drennan, M., Soler, M.N., Le Bert, M., Schnyder, B. and Ryffel, B. 2004. Toll-like receptor pathways in the immune responses to mycobacteria. *Microbes Infect*. **6(10):** 946-959.

Raja, A. 2004. Immunology of tuberculosis. *Indian J Med Res*. **120:** 213-232.

Ramasesh, N., Krahenbuhl, J.L. and Hastings, R.C. 1989. In vitro effects of antimicrobial agents on *Mycobacterium leprae* in mouse peritoneal macrophages. *Antimicrob Agents Chemother*. **33:** 657-62.

Raviglione, M.C. 2003. The TB epidemic from 1992 to 2002. *Tuberculosis* (Edinb). **83:** 4-14.

Reddy, V.M., O'Sullivan, J.F. and Gangadharam, P.R. 1999. Antimycobacterial activities of riminophenazines. *J Antimicrob Chemother*. **43(5):** 615-623.

Reed, S.G. and Dalemans, W. 2003. Prospects for a better vaccine against tuberculosis. *Tuberculosis*. **83:** 213–219.

Riley, R.L., Mills, C.C., Nyka, W., Weinstock, N., Storey, P.B., Sultan, L.U., Riley, M.C., and Wells W.F. 1959. Aerial dissemination of pulmonary tuberculosis. *Am. J. Hyg*. **70:** 185-196.

Riley, R.L. and O'Grady, F. 1961. Airborne contagion. Macmillan Publishing Co., Inc., New York.

Roach, D.R., Briscoe, H., Baumgart, K., Rathjen, D.A. and Britton, W.J. 1999. Tumor necrosis factor (TNF) and a TNF-mimetic peptide modulate the granulomatous response to *Mycobacterium bovis* BCG infection in vivo. *Infect. Immun*. **67:** 5473–5476.

Rockett, K.A., Brookes, R., Udalova, I., Vidal, V., Hill, A.V. and Kwiatkowski, D. 1998. 1,25 Dihydroxyvitamin D3 induces nitric oxide synthase and suppresses growth of *Mycobacterium tuberculosis* in a human macrophage-like cell line. *Infect Immun* **66:** 5314-5321.

Rojas, M., Olivier, M., Gros, P., Barrera, L.F. and Garcia, L.F. 1999. TNF-a and IL-10 modulate the induction of apoptosis by virulent *Mycobacterium tuberculosis* in murine macrophages. *J Immunol*. **162:** 6122-31.

Rollins, B.J. 1997. Chemokines. *Blood*. **90:** 909–928

Rook, G.A.W. 2007. Th2 cytokines in susceptibility to tuberculosis. *Curr Mol Med*. **7:** 327–37.

Rot, A. and von Andrian, U.H. 2004. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. *Annu Rev Immunol*. **22:** 891-928.

Russell, D.G. 2007. Who puts the tubercle in tuberculosis? *Nat Rev Microbiol*. **5:** 39.

Ryffel, B., Fremond, C., Jacobs, M., Parida, S., Botha, T., Schnyder, B. and Quesniaux, V. 2005. Innate immunity to mycobacterial infection in mice: critical role for toll-like receptors, *Tuberculosis* (Edinb). **85:** 395-405.

Sadek, M.I., Sada, E., Toossi, Z., Schwander, S.K. and Rich, E.A. 1998. Chemokines induced by infection of mononuclear phagocytes with mycobacteria and present in lung alveoli during active pulmonary tuberculosis. *Am J Respir Cell Mol Biol.* **19:** 513-521.

Salgame, P. 2005. Host innate and Th1 responses and the bacterial factors that control *Mycobacterium tuberculosis* infection. *Curr Opin Immunol*. **17:** 374.

Sarracent, J. and Finlay, C.M. 1982. The action of clofazimme on the level of lysozomal enzymes of cultured macrophages. *Clin Exp Immunol.* **48:** 261-72.

Sasindran, S.J. and Torrelles, J.B. 2011. *Mycobacterium tuberculosis* infection and inflammation: what is beneficial for the host and for the bacterium? *Frontiers in Microbiology | Cellular and Infection Microbiology.* **2(2):** 1-16.

Saunders, B.M. and Cooper, A.M. 2000. Restraining mycobacteria: role of granulomas in mycobacterial infections. *Immunol Cell Biol*. **78:** 334-341.

Schlesinger, L.S. 1993. Macrophage phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. *J. Immunol.* **150:** 2920.

Scott Algood, H.M., Chan, J. and Flynn, J.L. 2003. Chemokines and tuberculosis. *Cytokine & Growth Factor Reviews.* **14:** 467–477.

Serbina, N.V., Jia, T., Hohl, T.M. and Pamer, E.G. 2008. Monocyte-Mediated defense against microbial pathogens. *Annu Rev Immunol*. **26:** 421–452.

Shah, N.S., Wright, A., Bai, G.H., Barrera, L., Boulahbal, F., Martin-Casabona, N., Drobniewski, F., Gilpin, C., Havelkova, M., Lepe, R., Lumb, R., Metchock, B., Portaels, F., Rodrigues, M.F., Rüsch-Gerdes, S., Van Deun, A., Vincent, V., Laserson, K., Wells, C. and Cegielski, J.P. 2007. Worldwide emergence of extensively drug-resistant tuberculosis. *Emerg Infect Dis* **13(3):** 380-387.

Shen, G.H., Wu, B.D., Hu, S.T., Lin, C.F., Wu, K.M. and Chen, J.H. 2010. High efficacy of clofazimine and its synergistic effect with amikacin against rapidly growing mycobacteria. *Int J Antimicrob Agents.* **35(4):** 400-404.

Shibasaki, T., Katayama, N., Ohishi, K., Fujieda, A., Monma, F., Nishi, K., Masuya, M. and Shiku, H. 2007. IL-3 cannot replace GM-CSF in inducing human monocytes to differentiate into Langerhans cells. *Internat J Oncol*. **30(3):** 549-555.

Shintani, T. and Klionsky, D.J. 2004. Autophagy in health and disease: a double-edged sword. *Science.* **306:** 990-995.

Sibille, Y. and Reynolds, H.Y. 1990. Macrophages and polymorphonuclear neutrophils in lung defense and injury. *Am.Rev. Respir. Dis.* **141:** 471-501.

Spigelman, M.K. 2007. New tuberculosis therapeutics: a growing pipeline. *J Infect Dis.* **196(Suppl 1):** S28-34.

Steel, H.C., Matlola, N.M. and Anderson, R. 1999. Inhibition of potassium transport and growth of mycobacteria exposed to clofazimine and B669 is associated with a calcium-independent increase in microbial phospholipase A2 activity. *J Antimicrob Chemother.* **44:** 209–216.

Stenger, S. and Modlin, R.L. 1999. T cell mediated immunity to *Mycobacterium tuberculosis*. *Curr Opin Microbiol*. **2:** 89-93.

Sturgill-Koszycki, S., Schlesinger, P.H., Chakraborty, P., Haddix, P.L., Collins, H.L., Fok, A.K., Allen, R.D., Gluck, S.L., Heuser, J. and Russell, D.G. 1994. Lack of acidification of Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. *Science*. **263(5147)**: 678-681.

Su, J., Gong, H., Lai, J., Main, A. and Lu, S. 2009. The potassium transporter Trk and external potassium modulate Salmonella enteric protein secretion and virulence. *Infect Immun*. **77(2):** 667-675.

Sugawara, I., Yamada, H., Mizuno, S. and Iwakura, Y. 2000. IL-4 is required for defense against mycobacterial infection. *Microbiol. Immunol*. **44:** 971–979.

Sundaramurthy, V. and Pieters, J. 2007. Interactions of pathogenic mycobacteria with host macrophages. *Microbes Infect* **9:** 1671-1679.

Suzuki, H., Katayama, N., Ikuta, Y., Mukai, K., Fujieda, A., Mitani, H., Araki, H., Miyashita, H., Hoshino, N., Nishikawa, H., Nishii, K., Minami, N. and Shiku, H. 2004. Activities of granulocyte-macrophage colony-stimulating factor and Interleukin-3 on monocytes. *Am J Hematol*. **75(4):** 179-189.

Swenson, C.R., Popescu, M.C. and. Ginsberg R.S. 1988. Preparation and use of liposomes in the treatment of microbial infections. *Crit. Rev. Microbiol*. **15:** S1–S31.

Takeda, K. and Akira, S. 2005. Toll-like receptors in innate immunity. *Int Immunol.* **17:** 1-4.

Thelen, M. 2000. Dancing to the tune of chemokines. *Nature Immunol.* **2:** 129–134.

The World Health Report 1999. Making a difference. Geneva: World Health Organization; 1999. p. 116.

Thoma-Uszynski, S., Kiertscher, M.S., Ochoa, M.T., Bouis, D.A., Norgard, M.V., Miyake, K., Godowski, P.J., Roth, M.D. and Modlin, R.L. 2000. Activation of Toll-like receptor 2 on human dendritic cells triggers induction of IL-12, but not IL-10. *J. Immunol.* **165:** 3804.

Thoma-Uszynski, S., Stenger, S., Takeuchi, O., Ochoa, M.T., Engele, M., Sieling, P.A., Barnes, P.F., Rollinghoff, M., Bolcskei, P.L., Wagner, M., Akira, S., Norgard, M.V., Belisle, J.T., Godowski, P.J., Bloom, B.R. and Modlin, R.L. 2001. Induction of direct antimicrobial activity through mammalian Toll-like receptors. *Science.* **291:** 1544-46.

Tillotson, G.S. Tuberculosis- new aspects of chemotherapy. 1996. *J. Med Microbiol.* **44:** 16-20.

Toossi, Z., Gogate, P., Shiratsuchi, H., Young, T. and Ellner, J.J. 1995a. Enhanced production of TGF-beta by blood monocytes from patients with active tuberculosis and presence of TGF-beta in tuberculous granulomatous lung lesions. *J Immunol.* **154:** 465-473.

Toossi, Z., Young, T.G., Averill, L.E., Hamilton, B.D., Shiratsuchi, H. and Ellner, J.J. 1995b. Induction of TGF-b by purified protein deriverative of *Mycobacterium tuberculosis. Infect. Immun.* **63:** 224.

Tyagi, J.S. and Sharma, D. 2004. Signal transduction systems of mycobacteria with special reference to *M. tuberculosis*. *Curr Sci*. **86**: 93-102.

Ulrichs, T. and Kaufmann, S.H. 2006. New insights into the function of granulomas in human tuberculosis. *J Pathol.* **208:** 261.

Underhill, D.M., Ozinsky, A., Hajjar, A.M., Stevens, A., Wilson, C.B., Bassetti, M. and Aderem, A. 1999a. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature.* **401:** 811.

Underhill, D.M., Ozinsky, A., Smith, K.D. and Aderem, A. 1999b. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. *Proc. Natl. Acad. Sci. USA.* **96:** 14459.

Van Crevel, R., Ottenhoff, T.H.M. and van der Meer, J.W.M. 2002. Innate immunity to *Mycobacterium tuberculosis*. *Clin Microbiol Rev*. **15:** 294-309.

Van Furth, R. and Cohn, Z.A. 1968. The origin and kinetics of mononuclear phagocytes. *J Exp Med* **128:** 415–435.

Van oud Alblas, A.B. and van Furth, R. 1979. Origin, Kinetics, and characteristics of pulmonary macrophages in the normal steady state. *J Exp Med.* **149:** 1504–1518.

Van Rensburg, C.E., Gatner, E.M., Imkamp, F.M. and Anderson, R. 1982. Effects of clofazimine alone or combined with dapsone on neutrophil and lymphocyte functions in normal individuals and patients with lepromatous leprosy. *Antimicrob Agents Chemother.* **21(5):** 693-697.

Van Rensburg, C.E, Jooné, G.K. ,O'Sullivan, J.F. and Anderson, R. 1992. Antimicrobial activities of Clofazimine and B669 are mediated by lysophospholipids. *Antimicrobial Agents and Chemotherapy*. **36(12):** 2729-2735.

Van Rensburg, C.E., Van Staden, A.M. and Anderson, R. 1993. The riminophenazine agents clofazimine and B669 inhibit the proliferation of cancer cell lines in vitro by phospholipase A2 mediated oxidative and non oxidative mechanisms. *Cancer Res.* **53:** 318-323.

Venkatesan, K. 1989. Clinical pharmacokinetic consideration in the treatment of patients with leprosy. *Clin Pharmacokinet* **16:** 365–386.

Verhoef, J. 1991. Host-pathogen relationships in respiratory tract infections. *Clin. Ther*. **13:** 172-180.

Verstack, B., Hertzog, P. and Mansell, A. 2007. Toll-like receptor signaling and the clinical benefits that lie within. *Inflamm. Res*. **56:** 1–10.

Vischer, W.A. 1969. The experimental properties of G 30 320 (B 663)—a new anti-leprotic agent. *Lepr Rev*. **40:** 107-110.

Wadee, A.A., Anderson, A. and Rabson, A.R. 1988. Clofazimine reverses the inhibitory effect of *Mycobacterium tuberculosis* derived factors on phagocyte intracellular killing mechanisms. *Journal of Antimicrobial Chemotherapy.* **21:** 65-74.

Wadee, A.A., Cohen, J.D. and Rabson, A.R. 1987. Gamma interferon reverses inhibition of leukocyte bactericidal activity by a 25-kilodalton fraction from *Mycobacterium tuberculosis*. *Infection Immunity* **55:** 2777-2782.

Wadee, A.A., Kuschke, R.H., Dooms, T.G. and Anderson, R. 1995. The pro-oxidative riminophenazine B669 neutralizes the inhibitory effects of *Mycobacterium tuberculosis* on phagocyte antimicrobial activity. *International Journal of Immunopharmacology.* **17:** 849-856.

Waksman, S.A. 1944. Suppressive effect of streptomycin on the growth of tubercle bacilli in laboratory animals. *Am J Public Health.* **34:** 358.

Watt, B. 1997. In vitro sensitivities and treatment of less common mycobacteria. *J Antimicrob Chemother.* **39:** 567-574.

Watts, H.G. and Lifeso, R.M. 1996. Current Concepts Review Tuberculosis of Bones and Joints. *The Journal of Bone and Joint Surgery, Incorporated.* **78 (2):** 288-298.

Wells, W.F. 1955. Airborne contagion and air hygiene. Harvard University Press, Cambridge, Mass.

Wheeler, D.S., Chase, M.A., Senft, A.P., Poynter, S.E., Wong, H.R. and Page, K. 2009. Extracellular Hsp72, an endogenous DAMP, is released by virally infected airway epithelial cells and activates neutrophils via Toll-like receptor (TLR)-4. *Respir Res*. **10:** 31.

Wiid, I., Hoal-Van Helden, E., Hon, D., Lombard, C. and Van Helden, P. 1999. Potentiation of isoniazid activity against *Mycobacterium tuberculosis* by melatonin. *Antimicrobial Agents and Chemotherapy.* **43:** 975-977.

Wilson, M., Seymour, R. and Henderson, B. 1998. Bacterial perturbation of cytokine networks. *Infect Immun*. **66:** 2401-2409.

Winder, F.G. 1982. Mode of action of the antimycobacterial agents and associated aspects of the molecular biology of the mycobacteria. In: Ratledge C, Stanford J, eds. The Biology of the Mycobacteria. **Volume 1**, Physiology, Identification and Classification. London: Academic Press, pp 353-438.

Working Group on New TB Drugs. Riminophenazines. http://www.newtbdrugs.org/project.php (1 July 2011, date last accessed).

World Health Organization. Global Tuberculosis Control - A short update to the 2009 report. Geneva, WHO, 2009.

World Health Organization. *Treatment of tuberculosis. Guidelines for National Programmes*, 3rd ed. Geneva: World Health Organization, 2003 (*WHO/CDS/TB 2003.313*).

World Health Organization. WHO report 2007. Global tuberculosis control: surveillance, planning, financing. WHO/HTM/TB/2007.376. Geneva, Switzerland: WHO, 2007.

Wu, M-H., Zhang, P. and Huang, X. 2010. Toll-like receptors in innate immunity and infectious diseases. *Front. Med. China.* **4(4):** 385–393.

Yew, W.W. and Leung, C.C. 2008. Update in tuberculosis 2007. *Am J Respir Crit Care Med* **177:** 479–485.

Yano, T., Kassovska-Bratinova, S., Teh, J.S., Winkler, J., Sullivan, K., Isaacs, A., Schechter N.M. and Rubin, H. 2011. Reduction of clofazimine by mycobacterial type 2 NADH: quinone oxidoreductase: a pathway for the generation of bactericidal levels of reactive oxygen species. *J Biol Chem.* **286:** 10276–10287.

Zhang, D., Zhang, G., Hayden, M.S., Greenblatt, M.B., Bussey, C., Flavell, R.A. and Ghosh, S. 2004. A toll-like receptor that prevents infection by uropathogenic bacteria. *Science.* **303(5663):** 1522–1526.

Zhang, X. and Mosser, D.M. 2008. Macrophage activation by endogenous danger signals. *J. Pathol.* **214:** 161–178.

Zhang, Y., Broser, M., Cohen, H., Bodkin, M., Law, K., Reibman, J. and Rom, W.N. 1995. Enhanced interleukin-8 release and gene expression in macrophages after exposure to *Mycobacterium tuberculosis* and its components. *J Clin Invest.* **95(2):** 586-592.

Zhang, Y. and Mitchison, D. 2003. The curious characteristics of pyrazinamide: a review. *Int J Tuberc Lung Dis.* **7:** 6-21.

Zwilling, B.S., Kuhn, D.E., Wikoff, L., Brown, D. and Lafuse, W. 1999. Role of iron in *Nramp1*-mediated inhibition of mycobacterial growth. *Infect. Immun*. **67:** 1386–1392.