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**The innate immune response to
Mycobacterium tuberculosis is
dependent on strain lineage and on host
population**

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**Thesis presented for the degree of
*Doctor of Philosophy***

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Abstract

The genome structure of *Mycobacterium tuberculosis* is strongly clonal, in the absence of horizontal gene transfer. Thus it is feasible that clonal lineages may exhibit particular phenotypic characteristics, which may, in turn, result in differences in virulence or influence their association with particular host populations. Indeed, the global distribution of *M. tuberculosis* strains is not uniform and certain strain lineages predominate in particular geographical areas. Further, there is evidence that some strain lineages are emerging, suggesting differences in virulence.

Firstly, we investigated the association between strain genotype of *M. tuberculosis* and *in vitro* correlates of virulence such as growth phenotype and cytokine induction in the monocyte-derived macrophage (MDM) model. We report that 'modern' clinical *M. tuberculosis* isolates from Cape Town (Lineage 2 and Lineage 4 strains) exhibit both lineage-specific patterns of growth *in vitro* (in broth and MDM) as well as cytokine responses in MDM.

Secondly, in order to study the interaction between host genetic background and the innate immune response to different strains of *M. tuberculosis* we conducted a cross sectional study comparing cytokine responses to *in vitro* infection of healthy donor MDM from individuals from different population groups with strains from different *M. tuberculosis* lineages. The inflammatory cytokines TNF, IL-12p40, IL-6, IL-1 β and GM-CSF were secreted at higher levels in response to infection with lineage 4 and lineage 3 strains as compared to lineage 2 and lineage 1 strains. Principal component analysis and linear modeling identified three inflammatory cytokines (IL-6, IL-12p40, and GM-

CSF) to be differentially secreted in all four-population groups. In addition, we noted evidence of possible strain lineage-host ethnicity interactions for several cytokines. Together, these studies suggests that genetic diversity of *M. tuberculosis* might influence the early innate immune response during tuberculosis infection, and that lineage-specific responses may be modulated by host genetic background.

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Declaration

I, Rajesh Sarkar, hereby declare that the work on which the thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work or any part thereof is being, has been, or is to be submitted for another degree in this or any other University.

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05 Feb 2013

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Abbreviations

ADC	Albumin-Dextrose-Catalase
AFB	acid-fast bacilli
ALOX5	5-lipoxygenase
ANOVA	analysis of variance
Ara-LAM	Non-mannose-capped-lipoarabinomannan
Atg12Atg5 complex	autophagy-related protein
ATPase	enzyme involved in catalysis of adenosine triphosphate decomposition
BAL	bronchoalveolar Lavage
BALB/c	albino laboratory breed of mice
<i>Bax</i>	anti-apoptotic gene
BCG	Bacillus Calmette –Guérin
<i>Bcl2</i>	anti-apoptotic gene
CAS	Central Asian Strain
CD14	cluster of differentiation 14
CFU	colony forming units
C _{max}	maximum number of colony forming units
CpG	regions of DNA where cytosine nucleotide occurs next to guanine
CR	complement receptor
CSF	cerebrospinal fluid
CTLA4	cytotoxic T lymphocyte antigen 4
<i>cyp12</i>	cytochrome 450 genes involved in bacterial electron transport chain
DC	dendritic cells
DCSIGN	dendritic cell specific adhesionmolecule-3 grabbing non-integrin
DNA	deoxyribonucleic acid

DosR	dormancy regulon
DR	direct Repeat region
DTH	delayed Type of hypersensitivity
ELISA	enzyme linked immune sorbitol assay
Fc receptor	fragment, crystallisable region
FCS	foetal calf serum
Fc γ RI	Fc gamma receptor 1
GM-CSF	Granulocyte Macrophage- Colony Stimulating Factor
GRO- α	growth-regulated oncogene α
GTPase	enzyme involved in hydrolysis of guanosine triphosphate
<i>Gyr A</i>	gene encoding the A subunit of DNA gyrase
HEK293	Human Embryonic Kidney 293 cells
HIV	human immunodeficiency virus
HLA-DR	MHC class II surface receptor encoded by human leukocyte antigen
HRP	horseradish peroxidase
IFN- γ	interferon-gamma
IL-1	interleukin -1
IL-10	interlukin-10
IL-12	interleukin-12
IL1- β	interlukin-1 β
IL-6	interleukin-6
IL-8	interleukin-8
IL1Ra	interleukin-1 receptor antagonist
iNOS	inducible nitric oxide synthase
IRF3	interferon regulatory factor 3
IS	insertion sequences
<i>Kat G</i>	gene encoding catalase peroxidase
kb	kilo bases

kDa	kilo Dalton
LAM	Latin American Mediterranean
LAM	Lipoarabinomannan
Log	logarithm
Lpr	lipoprotein
LPS	lipopolysaccharide
LSP	large sequence polymorphisms
LT	leukotrienes
LX	lipoxins
Man-LAM	mannosyl-lipoarabinomannan
MAP	multi analyte profiling
MCP1	Monocyte Chemoattractant Protein 1
MCSF	Macrophage Colony-Stimulating Factor
MDM	monocyte derived macrophages
MHC	major histocompatibility complex
MIC	minimal inhibitory concentration
MIP-1 α / CCL3	macrophage inflammatory protein-1 α
MIP-1 β / CCL4	macrophage inflammatory protein-1 β
MIRU-VNTR	mycobacterial interspersed repetitive units variable number
MLST	multi-locus sequence typing
MOI	multiplicity of infection
MPTR	major polymorphic tandem Repeats
mRNA	messenger ribonucleic acids
MTBC	<i>Mycobacterium tuberculosis</i> complex
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
MyD88	myeloid differentiation protein 88
NF κ B	nuclear factor transcription factors
NK cells	natural killer cell

NO	nitric oxide
NRAMP1	Natural Resistance Associated Macrophage Protein 1
nsSNP	Nonsynonymous single nucleotide polymorphism
NTF	noise transfer function region
OADC	Oleic acid-Albumin-Dextrose-Catalase
PAMP	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffer saline
PCA	principal component analysis
PCR	polymerase chain reaction
PGG	principal genetic groups
PGL	phenolic glycolipid
PILAM	phosphoinositide-LAM
<i>Pks</i>	polyketide synthase
PMN	polymorphonuclear leucocytes
PRR	pattern recognition receptors
<i>PvuII</i>	restriction endonuclease <i>Proteus vulgaris</i>
Rab	membrane protein of Ras superfamily
RAW264.7	mouse macrophage cell line
RD	regions of difference
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acids
RPMI	Roswell Park Memorial Institute
<i>Rv1354c</i>	conserved hypothetical protein of H37Rv
<i>Sig A</i>	<i>sigma factor A</i>
<i>SLC11A1</i>	gene member of solute carrier family 11 and encodes NRAMP1
SNP	single nucleotide polymorphism
SpolDB	spoligotyping database

sSNP	Synonymous single nucleotide polymorphism
TB	Tuberculosis
TbD1	tuberculosis specific deletion 1
TBM	tuberculosis meningitis
Th1	T-helper cell 1
THP1	Human acute monocytic leukemia cell line
TIRF	TIR Toll/IL1R (TIR) domain containing adaptor protein
TLR	toll like receptor
T_{\max}	time to reach C_{\max}
TNF	tumour necrosis factor

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Publications

Sarkar R, Lenders L, Wilkinson KA, Wilkinson RJ, Nicol MP (2012) Modern Lineages of *Mycobacterium tuberculosis* Exhibit Lineage-Specific Patterns of Growth and Cytokine Induction in Human Monocyte-Derived Macrophages. PLoS ONE 7(8): e43170. doi: 10.1371/journal.pone.0043170

Chapter 1

Host-pathogen interaction and the innate immune response to Tuberculosis

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1.1 Introduction

Despite available anti-tuberculosis drugs and widespread vaccination, there were an estimated 1.7 million deaths caused by tuberculosis globally in 2009 and most of the cases were reported from developing countries¹. In 2010, it was reported that the incidence of tuberculosis was 340 cases per 100,000 population per year in Africa (Figure 1.01)². South Africa has the 2nd highest global tuberculosis incidence with 981 tuberculosis cases per 100,000 people in 2010^{2,3}.

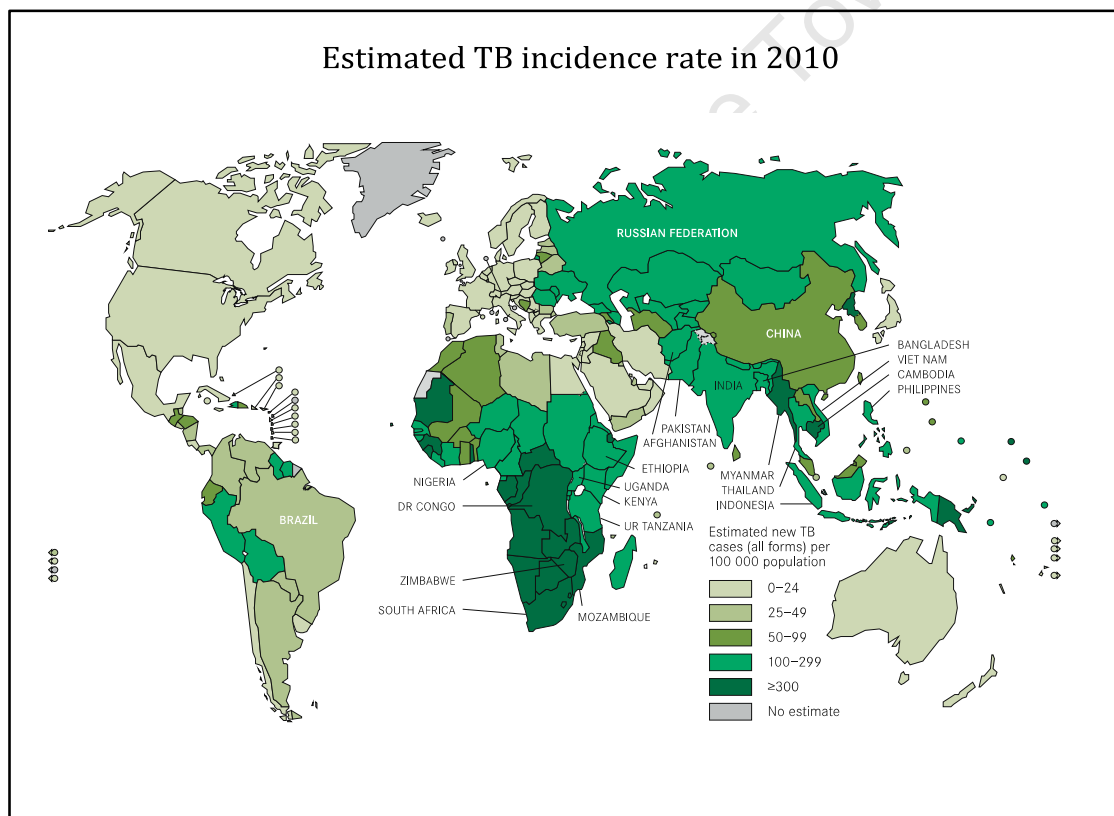


Figure 1.01 Worldwide estimation of TB incidence rates in 2010. Map showing total number of estimated new tuberculosis cases per 100,000 populations during 2010. The map illustrates the highest incidence of tuberculosis occurrence is in sub-Saharan-African countries (WHO, 2010).

Although, *Mycobacterium tuberculosis* is the major causative agent of tuberculosis, other mycobacteria forming part of the *M. tuberculosis* species complex including *M. bovis*, *M. africanum*, *M. microti* and *M. canetti* also cause the disease^{4, 5}. Evidence

suggests that *M. tuberculosis* is an ancient pathogen, which infected early hominids in East-Africa, as long as three million years ago⁶.

The majority of tuberculosis cases remain asymptomatic (i.e., latent tuberculosis), where the immune system controls the infection and no symptoms of active disease are observed⁷. In HIV-uninfected individuals one in ten of these latent infections develops active tuberculosis disease⁷, indicating that human protective immunity does not completely eradicate the bacillus from the body but is sufficient in preventing the development of tuberculosis disease in most individuals.

The success of *M. tuberculosis* in the human population is likely to be due, in part, to the successful evolution of *M. tuberculosis* to adapt to the human host; in particular, to the ability of the organism to evade or subvert the host immune response⁸⁻¹¹.

1.2 The innate immune response in tuberculosis

Both innate and adaptive immunity play important roles in *M. tuberculosis* infection. The innate immune response involves immediate phagocytosis of the bacilli upon infection together with the interaction of *M. tuberculosis* with various innate receptors and their activation. The adaptive immune response includes both cellular and humoral components. The interaction with T lymphocytes is thought to be of primary importance in the control of mycobacterial infection. Clonal expansion of T lymphocytes is important for the induction of an effective adaptive immune response by effector T cells. However, during *M. tuberculosis* infection, the onset of the T cell mediated acquired immune response is delayed. This delay permits bacteria to adapt to the infection site and modulate the T cell response¹². In contrast, the innate immune response begins immediately after inhalation of bacteria and remains until the

induction of adaptive immunity by T lymphocytes. Hence, it is clear that the innate immune system has a vital role in host defence and early control of infection.

M. tuberculosis uses the lung as the main port of entry and major site of replication. The macrophage is one of the principal innate immune components of the lung in human *M. tuberculosis* infection. The macrophage and its interaction with *M. tuberculosis* are central to the pathogenesis of human TB.

Upon inhalation of tubercle droplet nuclei, *M. tuberculosis* travels through respiratory tract and enters the lungs where it comes in contact with resident alveolar macrophages. Receptor mediated recognition of *M. tuberculosis* by alveolar macrophages initiates phagocytosis of infected mycobacteria. The ingested mycobacteria multiply inside the alveolar macrophages leading to rupture of the infected cells and the release of numerous bacilli. This aids early proinflammatory cytokine induction and recruitment of monocyte derived macrophages and other inflammatory cells from the blood stream into the lungs. Subsequently, extracellular mycobacteria multiply inside these cells and spill over from local infection site to neighbouring tissues and are carried to regional lymphnodes. After approximately 3 weeks post infection T cells immunity emerges which activates macrophages by production of inflammatory cytokines (TNF and IFN- γ) and thereby decreases bacterial multiplication. In time, the growth of mycobacteria is contained by 'granuloma' formation which triggers dormancy or latency of mycobacterial infection. A 'granuloma' consists of small numbers of infected phagocytes surrounded by blood derived monocytes, macrophages, epitheloid cells (differentiated macrophages) or foamy macrophages and CD4+, CD8+ lymphocytes along with fibroblasts and extracellular matrix protein. A characteristic feature of granulomas in tuberculosis infection is central solid necrotic tissue due to death of infected cells. Effective T cell

responses to *M. tuberculosis*-derived antigens may sterilize granulomas. Alternatively, the lesion may reactivate years after primary exposure due with liquefaction of necrotic tissue (Caseous necrosis) and destruction of lung tissue, resulting in entry of *M. tuberculosis* into the lung airspaces or blood vessels and result in further tuberculosis disease dissemination. The clinical consequences of *M. tuberculosis* infection (Figure1.02) involve several sequential events as outlined below:

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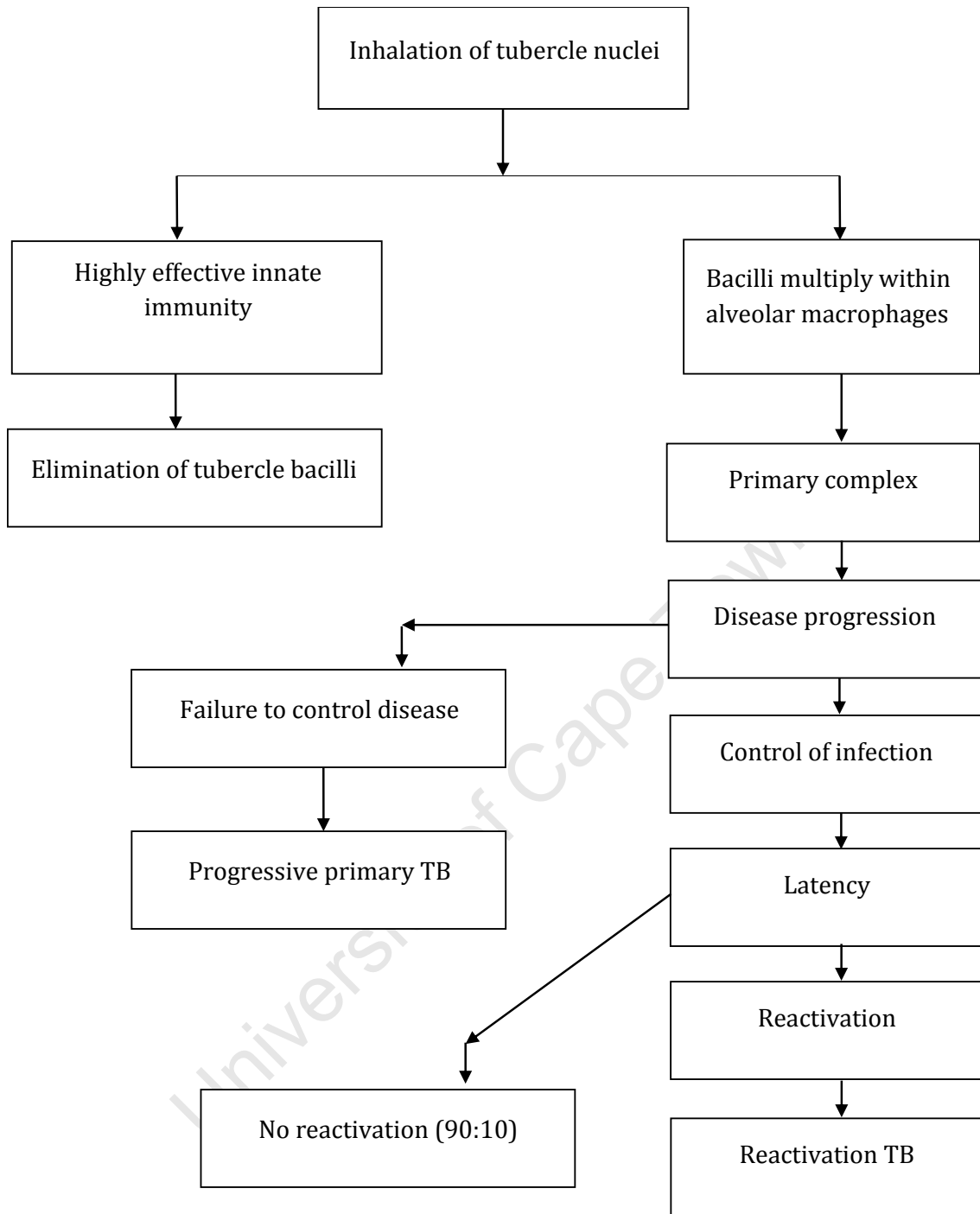


Figure 1.02 schematic diagrams illustrating the clinical consequences of *M. tuberculosis* infection.

The innate immune response is mediated by macrophages, dendritic cells (DCs), mast cells, neutrophils, eosinophils and Natural Killer cell (NK cells). The main role of these cells is to interact with the microbial pathogen and control the initial infection. This may involve non-specific recognition of pathogen, opsonisation, complement activation, phagocytosis and activation of pro-inflammatory signalling pathways and induction of apoptosis¹³.

1.2.1 The role of the macrophage

Macrophages are professional phagocytes, which are derived from monocytes and serve as an essential innate immune component in *M. tuberculosis* infection. The main roles of the macrophage are to destroy the pathogen by phagocytosis, to recruit other innate immune cells to infection sites and process and present antigen to T lymphocytes. Alveolar macrophages reside in lungs and are known as residential macrophages while monocytes travel through blood and are carried to the infection site to differentiate into macrophages during tuberculosis infection. Macrophages possess a range of receptors on the cell surface, which interact with *M. tuberculosis* to trigger early cytokine production such as IL-12 and TNF¹⁴. Macrophages can process the engulfed mycobacteria and present antigen to T cells, which in turn produce IFN- γ which enhances the anti-mycobacterial activity of macrophages. Macrophage - T cell interactions play a central role in bacterial containment during *M. tuberculosis* infection. IFN- γ activated macrophages show increased expression of major histocompatibility complex (MHC) class II molecules, which enhances antigen presentation. Hence, macrophages are essential in initiating the non-specific innate immune defence in tuberculosis infection and are also important to trigger the T cell

specific adaptive immune response. However, *M. tuberculosis* has evolved mechanisms to escape the bactericidal effects of activated macrophages¹⁵.

Generally, macrophage activation is studied *in vitro* by stimulation of mononuclear cells with microbial components or cytokines. Based on the activation pathway, human macrophages are differentiated into two different types: classically activated macrophages (M1) and alternatively activated macrophages (M2)^{16, 17}. Classically activated macrophages are activated by pro-inflammatory cytokines (or T helper type 1 (Th1) cytokines) such as TNF, IFN- γ , Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and bacterial cell wall component such as lipopolysaccharide (LPS). M1 macrophages have a bactericidal effect and produce various inflammatory cytokines (IL-1, TNF, IL-6, etc.) during infection. Prolonged activation of M1 macrophages may cause tissue damage. On the other hand, alternatively activated macrophages are activated by anti-inflammatory or Th2 cytokines (IL-4, M-CSF, IL-10). Moreover, M2 macrophages are subdivided into M2a (activated by IL-4 or IL-13), M2b (activated by immune complex or TLR agonists, e.g. LPS) and M2c (activated by IL-10 and glucocorticoid hormone). M2 macrophages are less bactericidal and modulate the immune response by producing regulatory cytokines such as IL-10¹⁸. Hence, macrophage activation is balanced by pro-inflammatory and anti-inflammatory phenomena.

1.2.2 Recognition of *M. tuberculosis* by phagocytic cells

The strategy of the innate immune response is to recognize conserved structures present on bacterial pathogens. These structures are known as pathogen associated molecular patterns (PAMP)¹⁹, and the receptors of the innate immune system that recognizes them are known as pattern recognition receptors (PRR). The most familiar

examples of PAMPs are bacterial LPS, peptidoglycan, mannans, teichoic acids, bacterial DNA and RNA. *M. tuberculosis* has a thick cell wall containing 60% lipids and polysaccharides, including a rich mycolic acid layer²⁰. *M. tuberculosis* possesses a broad range of PAMPs (such as 19kDa and 24kDa lipoproteins, LprA, LprG, lipoarabinomannan, phosphoinositide lipoarabinomannan, triacyl lipopeptides, unmethylated CpG DNA), which are recognized by specific PRRs.

M. tuberculosis may interact with different types of receptors on the surface of macrophages either directly through recognition of PAMP by PRR, or indirectly by opsonins. Receptors include toll like receptor, the mannose receptor²¹, the Fc receptor²², complement receptor²³, CD14 receptor²⁴ and scavenger receptor²⁵.

The role of these receptors has been partially characterized in *in vitro* studies of tuberculosis. Mycobacteria encounter many different cell types and it is likely that the innate recognition is achieved through a range of ligand-receptor interactions. Multiple receptors may synergize in *M. tuberculosis* recognition, depending upon the distribution of a specific receptors on specific cell types²⁶. *M. tuberculosis* has adapted a unique mechanism to invade macrophages and its invasion strategy is vital in survival of *M. tuberculosis* in macrophages. The various cell surface receptors and mycobacterial ligands play an important role in innate immune response. The interaction between different mycobacterial ligands and macrophage cell surface receptors is therefore described in detail below.

1.2.3 Receptors and associated ligands

1.2.3.1 Toll like receptors (TLR)

Toll like receptors on macrophages and dendritic cells have been recognised as important receptors for mycobacterial recognition. The mammalian TLR family has 11 distinct toll-like receptors. TLR recognize intact *M. tuberculosis* or various

mycobacterial components including lipopolysaccharide, bacterial DNA and teichoic acids²⁷⁻³⁰. Tuberculosis recognition involves various TLRs including TLR2/1/6 (TLR2 is a heterodimer of TLR1 and TLR6), TLR9 and TLR4^{27, 31} (Figure 1.03) Interaction with these receptors leads to triggering of signalling pathways that promote phagocytosis and initiates pro-inflammatory pathways that act locally and systemically. Immune activation through TLR stimulation may occur with or without phagocytosis. Following interaction with bacterial PAMP, TLR signalling pathways are activated, which are based on two adaptor proteins- myeloid differentiation protein 88 (MyD88) and Toll/IL-1R (TIR) domain containing adaptor protein inducing interferon β (IFN β) [TRIF]. Firstly, the MyD88 dependent pathway is a cascade of different receptors which are engaged in the signalling of various TLRs (except TLR3 which only activates through TRIF, TLR4 activates through both MyD88 dependent pathway and TRIF), leading to the activation of the mitogen associated protein kinase (MAPK) pathways and nuclear translocation transcription factors (NF- κ B) which in turn switches on transcription of different genes involved in the production of various pro-inflammatory cytokines such as TNF- α , IL-12 and IL-1 β ³².

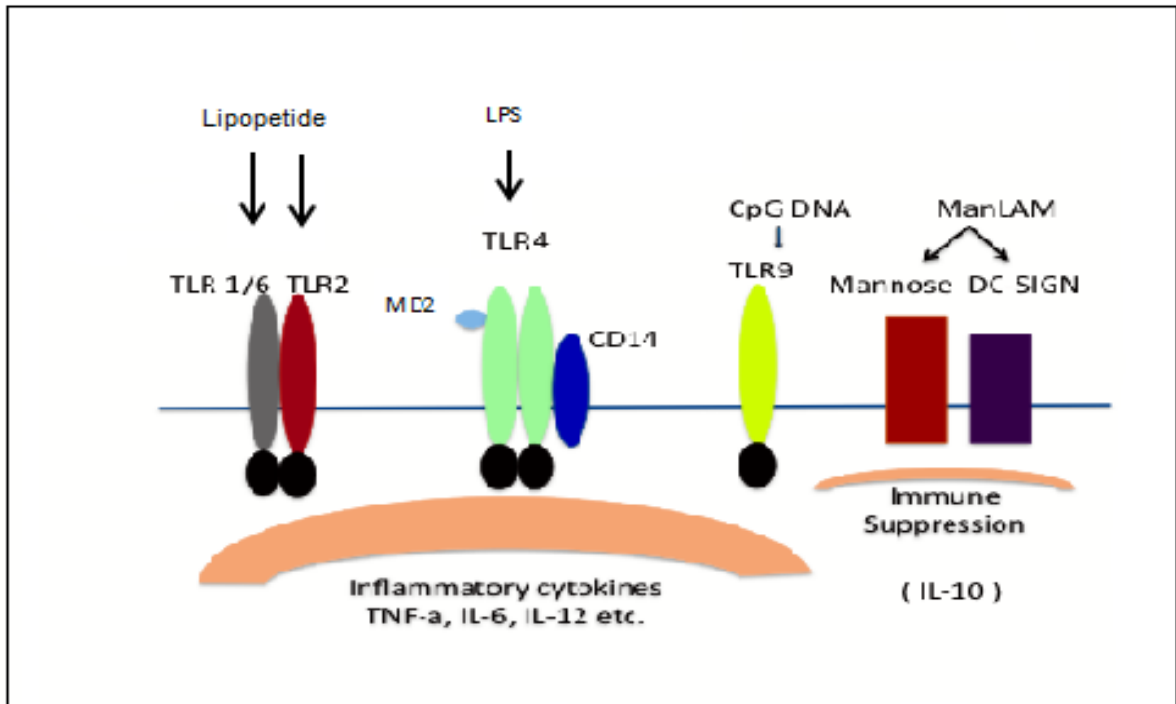


Figure 1.03 Diagram showing interaction of mycobacterial ligands with innate immune receptors on macrophages and induction of associated cytokines (Redrawn and modified from Jo, Eun-Kyeong, 2008).

Secondly, TIRF dependent pathways which are TLR4 mediated and involved in the activation of NF- κ B and interferon regulatory factor 3 (IRF-3) pathways are also involved in the transcription of pro-inflammatory type-I interferon³².

1.2.3.2 Experimental studies of TLR and associated ligands

M. tuberculosis possesses several lipoproteins (such as 19kDa lipoprotein, 27kDa lipoprotein, LprA, LprG) that interact with TLR and other receptors. Lipoprotein-mediated recognition of TLR modulates the function of innate and adaptive immunity in *M. tuberculosis* infection. The 19kDa lipoprotein plays an important role in immune recognition by TLRs during tuberculosis infection. The 19kDa lipoprotein induces IL-12p40 production by a human THP1 monocyte cell line³³. IL-12 is an important inducer of Th1 lymphocyte responses³⁴ that are essential to eliminate mycobacterial infection³⁵. IFN- γ produced by activated Th1 cells has an important role in anti-

mycobacterial activity in macrophages during tuberculosis infection. IFN- γ stimulated macrophages up-regulate MHC class II antigen processing and presentation to CD4 T cells and demonstrate increased bactericidal activity³⁶. However, in humans, IFN- γ stimulated macrophages cannot completely eradicate tuberculosis infection. The mechanism by which tuberculosis escapes the bactericidal effect of IFN- γ can be partly explained by the effect of lipoprotein-mediated stimulation on human macrophages. The 19kDa lipoprotein decreases IFN- γ regulated antigen processing and presentation by HLA-DR³⁷. Fc γ RI is considered as a marker of macrophage activation, which is regulated by IFN- γ . The expression of Fc γ RI was shown to be decreased following prolonged stimulation (>24h) of THP1 cells with 19 kDa lipoprotein and IFN- γ through a TLR-dependent pathway³⁷. Additionally, the 24kDa lipoprotein (LPr G) was found to be a TLR2 antagonist and inhibited MHC class II Ag processing following prolonged exposure (>16h) of THP1 cells and human primary macrophages³⁸.

Activation of TLR receptors has been implicated in the process of autophagy. Autophagy is an innate immune mechanism of host defence, which involves the formation of a membrane around the cytosol along with infected cell organelles and microbial pathogens, which is further supplied to lysosome for degradation. A recent study using human alveolar macrophages and a RAW264.7 cell line demonstrated enhanced mycobacterial co-localization with the autophagosome when stimulated with LPS (via the TLR4 mediated and MyD88 independent signalling pathway)³⁹. Various *in vitro* cell culture and murine studies demonstrating the role of TLRs in tuberculosis infection are summarised in Table 1.01

Table 1.01 *In vitro* studies of the role of TLR in the immune response to *M. tuberculosis*.

Receptors	<i>M. tuberculosis</i> / ligands	Effect of recognition/ infection	References
Toll like receptor TLR 2	19kDa lipoprotein (P19)	19 kDa lipoprotein stimulated THP1 cell line induces IL-12 which is TLR2 mediated.	40
	<i>M. tuberculosis</i>	TLR2 knockout mice showed decrease in production of pro-inflammatory cytokines such as IL-12p40 and TNF- α when compared with normal mice at 4 hours and 24 hours post infection.	41
	19kDa lipoprotein (P19)	19kDa lipoprotein suppressed NO production in presence of iNOS inhibitor (L-NIL, N-immunoethyl-L-lysine) in <i>M. tuberculosis</i> infected RAW264.7 murine cell line, which in turn reduced antimicrobial activity, suggesting NO is needed in TLR mediated antimicrobial response. Further, 19kDa lipoprotein-stimulated <i>M. tuberculosis</i> infected peritoneal macrophages from TLR 2 Knockout mice showed negligible level of reduction in NO level and intracellular growth of mycobacteria was not affected, suggesting that the antimicrobial activity of TLR 2 is NO dependent in murine macrophages. Alternatively, human monocytes showed reduction in intracellular growth mycobacteria was restricted when stimulated with 19kDa lipoprotein in presence of a TLR2-blocking antibody while iNOS inhibitor has no effect on NO production or reduction in bacterial number, suggesting that the antimicrobial activity of TLR is NO independent in human macrophages.	42

Receptors	<i>M. tuberculosis</i> / ligands	Effect of recognition/ infection	References
TLR 2	19kDa-glycolipoprotein (P19)	19kDa glycolipoprotein (P19) treated THP1 cells and MDM showed increase in apoptosis, using Annexin V binding to membrane phosphatidylserine and tunnel assays. The apoptosis was TLR2 mediated as blocking of TLR2 activity by a monoclonal antibody resulting in inhibition of apoptosis.	43
	19kDa glycolipoprotein (P19) & LprG	Regulates expression of MHC II molecules and inhibition of antigen processing and presentation in THP1 cell line and human primary macrophages. Blocking of TLR2 using a monoclonal antibody inhibited the lipoprotein-mediated inhibition of antigen processing and presentation.	37,38
TLR2/ TLR4	38kDa glycoprotein	38kDa glycoprotein-treated monocytes from pulmonary tuberculosis patient secreted more TNF- α and IL-6 than healthy donors and the cytokine secretion was TLR2 and TLR4 mediated. The pro-inflammatory cytokine secretion was enhanced by activation of mitogen activated protein kinase and extracellular signal regulated kinase 1/ 2.	44

Receptors	<i>M. tuberculosis</i> / ligands	Effect of recognition/ infection	References
TLR4	<i>M. tuberculosis</i> infection in TLR4 deficient mice and wild-type mice.	In a low dose model of bacillary infection, TLR4 deficient mice were more susceptible to <i>Mycobacterium tuberculosis</i> infection. TNF- α , IL-12p40 and Monocyte Chemotactic Protein-1 (MCP-1) production in BAL supernatant and lung homogenate was lower in TLR4 knockout mice than wild-type mice.	45
	<i>M. tuberculosis</i> infection in TLR4 defective mice (C3H/HeJ) and wild-type mice (C3H).	In a high dose aerosol infection, TLR4 knockout mice did not exhibit increased susceptibility to infection.	46
TLR9	CpG motifs in bacterial DNA <i>M. tuberculosis</i> in TLR2 and TLR9 double knockout mice.	Induce IL-12 by dendritic cells in <i>M. tuberculosis</i> infected mice. TLR2/9 double knockout mice showed greater susceptibility to <i>Mycobacterium tuberculosis</i> infection than a single TLR knockout mice (TLR2 or TLR9) and marked by defects in IL-12 and other pro-inflammatory cytokine production.	47

Together, these findings suggest that interaction of TLR and mycobacterial ligands induces pro-inflammatory responses during *ex-vivo* tuberculosis infection. Furthermore, TLR knockout mice have compromised immune responses and increased susceptibility to *M. tuberculosis* infection, suggesting an important role of TLR in tuberculosis disease susceptibility.

1.2.3.3 The role of TLR in human tuberculosis

Various human investigations have revealed the pivotal role of TLR-mediated resistance against *M. tuberculosis* infection. The Vitamin D3 pathway has been shown to be important in the control *M. tuberculosis* replication in macrophages⁴⁸. Vitamin D deficiency is considered to be a risk factor for *M. tuberculosis* infection⁴⁹,⁵⁰. Liu *et al.* showed that TLR2 mediated activation of macrophages up-regulates the Vitamin D receptor and Vitamin D- 1- hydroxylase genes, leading to the induction of the antimicrobial peptide, cathelicidin⁵¹. African American individuals who are more susceptible to *M. tuberculosis* infection have low levels of 25 hydroxyvitamin D (a precursor of Vitamin D, also known as calcidiol), resulting in the reduced production of cathelicidin⁵². Further, a non-synonymous single nucleotide polymorphism (SNP), TLR1 SNP I602S (T1805G), is reportedly associated with differential NF- κ B mediated signalling in HEK293 cells in response to stimulation with triacylated lipopeptides from *M. tuberculosis*²⁸. The T allele, which is highly prevalent (allelic frequency 0.99) in Vietnamese and African Americans (allelic frequency 0.750) compared to the European American population (allelic frequency 0.231), was associated with a higher level of inflammatory cytokine IL-6 induction in whole blood stimulated with lipopeptides, indicating polymorphism in TLR1 (I602S) might associate with a different spectrum of inflammatory responses that might be associated with the susceptibility to tuberculosis disease²⁸. Subsequently, a case control study from Vietnam has reported an association of tuberculosis with a polymorphism in Toll Interleukin Receptor Domain (TIRAP- a receptor protein which is involved in TLR signalling during *M. tuberculosis* infection). The study compared patients with pulmonary tuberculosis and tuberculosis meningitis; cord blood sample were used

as a control. The synonymous SNP C558T in TIRAP was associated with susceptibility to tuberculosis, while further genotyping frequency analysis found that the 558T haplotype was more frequent amongst the patients with tuberculosis meningitis than pulmonary tuberculosis. Additionally, the 558TT genotype was associated with a low level of IL-6 production in whole blood assays when compared with the 558CC genotype, suggesting TIRAP influenced the disease susceptibility by moderating inflammatory cytokine IL-6 production⁵³. A recent study has shown that a SNP rs352139 in an intron of TLR9 increases susceptibility to tuberculosis in the Vietnamese female population, suggesting TLR9 polymorphisms may have a role in susceptibility to tuberculosis in the Asian population⁵⁴.

1.2.3.4 Mannose receptor and associated ligands

The mannose receptor plays an important role in non-opsonised uptake of *M. tuberculosis* during phagocytosis and in host cell modulation. Mannosyl-lipoarabinomannan (Man-LAM), in the mycobacterial cell wall, serves as a ligand for mannose receptors.

Lipoarabinomannan (LAM) is a major cell wall component of *M. tuberculosis*⁵⁵. LAM is made up of a backbone of manosyl-phosphatidyl-inositol, which is attached to a D-mannan core and a D-arabino domain. The arabino domains may possess either a capping of mannosyl (Man-LAM) or phosphoinositide (PI-LAM) or remain uncapped (Ara-LAM). Man-LAM is present in the slow growing virulent Erdman strain of *M. tuberculosis* and *M. bovis* bacillus calmette –guérin (BCG) whilst the noncapped Ara-LAM is present in an avirulent strain of *M. tuberculosis* H37Ra^{56,57}.

PI-LAM has been found mostly in rapidly growing mycobacterial species such as *M. smegmatis*⁵⁸, also in *M. tuberculosis* H37Rv and *M. bovis* BCG⁵⁹.

LAM plays a key role in receptor-mediated phagocytosis of *M. tuberculosis*. Schlesinger *et al.* have shown that phagocytosis of two virulent strains (H37Rv and Erdman) by human monocyte-derived macrophages is mediated by the mannose receptor in addition to complement receptors (CR1, CR3 and CR4) whereas the avirulent strain H37Ra interacts only with complement receptors during phagocytosis²¹. Polystyrene microspheres coated with Man-LAM were shown to adhere to monocyte-derived macrophages (MDMs). This was confirmed by abrogating the effect of Man-LAM with exomanosidase treatment of Man-LAM or blocking Man-LAM with monoclonal antibody, both of which resulted in the inhibition of adherence of mycobacteria to MDM⁶⁰.

Additionally, structural variations in LAM are associated with differential cytokine induction in human and murine macrophages. Ara-LAM from H37Ra induced more TNF production by murine macrophages and human PBMC as compared to Man-LAM from the virulent Erdman strain of *M. tuberculosis*⁶¹.

Man-LAM antagonises mannose receptor-dependent IL-12 production⁶². In contrast, PI-LAM from non-pathogenic mycobacteria (eg. *M. smegmatis*) was shown to activate murine macrophages and a human monocytic cell line by a TLR2-dependent pathway resulting in the induction of pro-inflammatory cytokines TNF- α and IL-12, which are responsible for the induction of apoptosis⁶³⁻⁶⁵. Phagocytic uptake through mannose receptors in dendritic cells is associated with an anti-inflammatory response due to inhibition of IL-12 production, which in turn enhances survival of tubercle bacilli inside macrophages^{62, 66}. Thus, Man-LAM is a

virulence factor that suppresses pro-inflammatory cytokine production and enhances intracellular survival.

1.2.3.5 DC-SIGN receptor

The DC-SIGN receptor also plays an important role in mycobacterial recognition by the innate immune system. The DC-SIGN receptor is generally present on the surface of dendritic cells and is also expressed on the surface of macrophages and alveolar macrophages on exposure to microbial infection⁶⁷. DC-SIGN acts as a major phagocytic receptor for *M. tuberculosis* on human dendritic cells by binding to Man-LAM. It specifically recognizes those mycobacterial species which contain Man-LAM⁶⁸. The DC-SIGN receptor therefore recognizes *M. bovis* BCG and *M. tuberculosis* but it does not bind to mycobacterial species such as *M. smegmatis*. *M. tuberculosis* binds with DC-SIGN to induce the anti-inflammatory cytokine, IL-10, which results in the blocking of dendritic cell maturation⁶⁹.

1.2.3.6 Complement receptor

The complement system is a cascade of serum proteins which are present on cell surfaces and activated by antibodies (classical pathway) or microbes (alternative pathway)⁷⁰. The complement system is involved in opsonisation of invading bacteria, which in turn promotes phagocytosis and bacterial cell lysis. *M. tuberculosis* utilizes the complement system to invade macrophages. Macrophages express two-complement receptor proteins on their cell surface: CR1- a monovalent transmembrane protein and C3b/C4b- a heterodimer with a common β chain⁷¹. *M. tuberculosis* can be ingested by activation of complement receptors following opsonisation with C3b and iC3b, a derivative of C3, which permits the bacteria to be recognized by CR1 and C3b/C4b respectively²². Alternatively, *M.*

tuberculosis is capable of binding non-opsonically to CR3 in which case the capsular polysaccharide of *M. tuberculosis* binds to the β glucan-binding site on CR3⁷¹. CR3 is therefore involved in both opsonic and non-opsonic binding of *M. tuberculosis* but the function of this receptor in mycobacterial uptake is not clear from human and animal studies⁷².

1.2.3.7 Other receptors

Apart from the above major receptors, various other receptors may take part in phagocytosis and innate immune recognition. For instance, CD14 has been found to be associated with non-opsonic uptake of mycobacteria. The neutralization of CD14-receptor with monoclonal antibody inhibits uptake of H37Rv in human microglial cells⁷³. Subsequently, the blocking of CRs and mannose receptors together with monoclonal antibodies does not completely prevent mycobacterial uptake in macrophages and may play a role by scavenger receptors²⁵.

1.3 Cytokines as mediators of the innate immune response

Receptor-mediated recognition of *M. tuberculosis* results in cellular activation and production of cytokines⁷⁴. Triggering of the appropriate activating receptor by *M. tuberculosis* induces transcriptional activities resulting in an early inflammatory cytokine response³² (Figure 1.05). This in turn induces various chemokines and further promotes recruitment of macrophages, neutrophils, T cells and NK cells to the site of infection^{74, 75}, as part of non-specific host defence mechanisms. In turn, the innate immune response coordinates and directs the adaptive immune response⁷⁴.

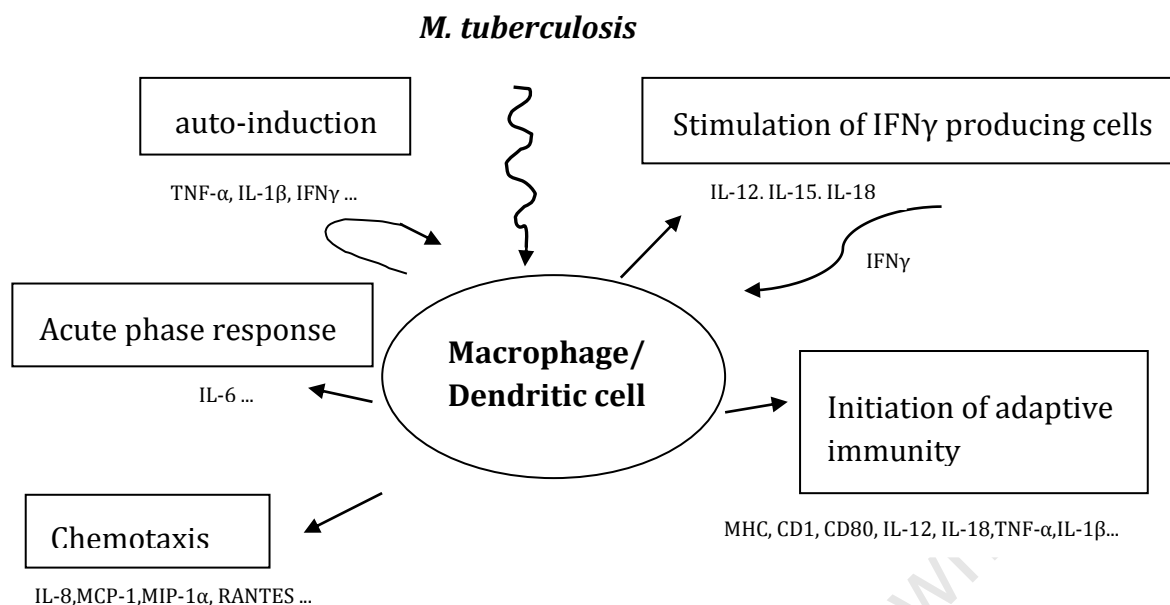


Figure 1.04 Diagram showing activation of macrophages and dendritic cells by *M. tuberculosis* and induction of various inflammatory cytokines (Adapted and modified from van Crevel *et al.*, 2002).

1.3.1 Tumor necrosis factor (TNF)

TNF is an essential pro-inflammatory cytokine, which is produced by monocytes, macrophages, dendritic cells, and T cells during tuberculosis infection. Experimental evidence suggests that TNF is an essential mediator in tubercular granuloma formation^{14, 76, 77}. It has a broad range of activity including macrophage activation and enhancement of chemokine production. In humans, neutralization of TNF using anti-TNF drugs in treatment of rheumatologic diseases increases the risk of reactivation of latent tuberculosis, clearly indicating that TNF plays a protective role in tuberculosis infection⁷⁸⁻⁸⁰.

1.3.2 Interleukin-12 (IL-12)

M. tuberculosis is a strong inducer of IL-12, which is mainly produced by macrophages and dendritic cells following phagocytosis of tubercle bacilli. IL-12 is essential to induce a protective Th1 response with production of IFN- γ ³⁴. In humans, mutations in the IL-12p40 and the IL-12 receptor genes result in enhanced susceptibility to mycobacterial infection^{81, 82}.

1.3.3 Interleukin-6 (IL-6)

IL-6 is produced by monocytes and macrophages following phagocytosis of *M. tuberculosis* and causes inflammation and an acute phase response^{83, 84}. However, IL-6 serves as a multifunctional cytokine with pro-inflammatory and anti-inflammatory properties. For instance, IL-6 has been found to inhibit IFN- γ secretion by human and mice macrophages, suggesting IL-6 is an antagonist of cellular immunity⁸⁵. On the other hand, IL-6 deficient mice have been shown to develop lethal tuberculosis infection, identifying a possible protective role of IL-6 in tuberculosis⁸⁶.

1.3.4 Interleukin- 1 β (IL-1 β)

IL-1 β , an important cytokine mediator in tuberculosis infection, is produced by monocytes, macrophages and dendritic cells^{87, 88}. IL-1 β is involved in multiple activities such as inflammation, cell proliferation, differentiation, and apoptosis. IL-1 β is induced primarily in precursor form and is activated by the caspase-1 enzyme upon bacterial stimulation. A soluble receptor, IL1Ra, acts as an antagonist of the inflammatory response induced by IL-1 β ⁸⁹. There is evidence of influence of

IL1Ra polymorphisms on *M. tuberculosis* infection⁹⁰. For instance, *in vitro* stimulation of PBMC with virulent H37Rv induced an increased level of IL1Ra mRNA and its protein in healthy homozygous IL1Ra A2⁺ subjects when compared to healthy IL1Ra A⁻ individuals. The ratio of IL1Ra/IL-1 β was higher in IL1Ra A2⁺ individuals⁹⁰. Additionally, a case control study amongst 89 Indian emigrants in the United Kingdom reported that IL1Ra A₂/ IL-1 β A1⁺, a pro-inflammatory haplotype (produces less IL1Ra and more IL1 β), was unevenly distributed amongst patients with tuberculosis pleurisy, resulting in reduced IL1 β antagonism, highly delayed type of hypersensitivity (DTH) and a strong inflammatory response in tuberculosis pleurisy⁹⁰. Furthermore, the IL1Ra A2⁺ homozygous genotype was associated with a reduced level of DTH in response to purified protein derivative (used for tuberculin skin testing) and was not associated with tuberculosis pleurisy⁹⁰.

1.3.5 Interleukin-10 (IL-10)

IL-10 is a regulatory cytokine, produced by alternatively activated macrophages, dendritic cells and regulatory T cells, which subverts innate immunity in bacterial infection^{18, 91}. Peripheral blood mononuclear cells (PBMCs) from HIV infected tuberculosis patients induced lower levels of IFN- γ as compared to HIV-uninfected tuberculosis patients and healthy donors. Blocking of IL-10 with a monoclonal antibody increased the production of IFN- γ and enhanced IL-12 production in HIV infected tuberculosis and HIV negative tuberculosis patients⁹². Additionally, the expression of the T cell stimulatory factor CTLA4 was reduced in PBMC from tuberculosis patients. Further, stimulation of PBMC from tuberculosis patients with anti-IL-10 antibody up-regulates IL-12 and CTLA4 expression. Hence, IL-10

down-regulates IL-12 secretion by down-regulating CTLA4 expression, resulting in the suppression of the Th1 response during *M. tuberculosis* infection⁹². Evidence suggests that IL-10 affects the Th1 response differently with different pathogens⁹³. For example, IL-10 knockout mice infected with different mycobacteria showed differences in bacterial clearance from various infected organs. *M. avium*-infected IL-10 knockout mice showed a sustained increase in IFN- γ producing T cell response, causing immediate clearance of *M. avium* from the lungs and liver. In contrast, *M. tuberculosis*-infected IL-10 knock-out mice showed a transient increase in the IFN- γ producing T cell response at 4 weeks post infection but this response diminished at 8 weeks and similar T cell responses were observed in both wild type and IL-10 knockout mice. There was reduction in bacterial number in lungs at four weeks post infection but bacteria were retained in the lungs and an equivalent amount of *M. tuberculosis* was recovered from lungs at 8 weeks in both wild type and IL-10 knockout mice. Additionally, IL-10 knockout mice showed a significant increase in IL-12 production at 48 hours post infection with *M. avium* but not with *M. tuberculosis*, suggesting endogenous IL-10 secretion by pathogenic mycobacteria plays a complex role in mycobacterial infection⁹⁴.

1.3.6 Chemokines

Several studies have reported the important role of chemokines in the innate and adaptive immune response to tuberculosis^{95, 96}. Chemokines are produced by monocytes, macrophages, and dendritic cells and also by CD4⁺ T cells. Chemokines influence cellular migration, recruitment, and activation of monocytes, macrophages, and leucocytes. Tuberculosis infection is followed by induction of TNF, which in turn regulates the production of various chemokines (CC) such as

CCL2 (macrophage chemotactic protein-1: MCP1), CCL3 (macrophage inflammatory protein 1- α : MIP1- α) and CCL4 (macrophage inflammatory protein 1- β : MIP1- β)^{97, 98}.

CCL2/MCP-1 plays a crucial role as a chemoattractant for monocytes and T lymphocytes⁹⁹. CCL2 mRNA and protein levels are elevated in PBMC-derived CD14+ cells in tuberculosis infection. Immunoblotting with anti-CCL2 antibody showed high levels of MCP-1 in tuberculous lymph nodes¹⁰⁰. A study in Brazilian tuberculosis patients has reported that the 17q11-17q21 regions of the human chromosome, which possess chemokine encoding genes for CCL2 and many other chemokines, are associated with susceptibility to tuberculosis¹⁰¹. Subsequently, Flores *et al.* reported that polymorphism in CCL2 promoter (MCP-1-2518) was associated with active pulmonary tuberculosis disease and increased plasma CCL2 levels in Mexican and Korean tuberculosis patients with G genotypes¹⁰². Tuberculosis was more common in patients with AG and GG than AA genotypes. Individuals with the CCL2 GG homozygous genotype showed a high level of CCL2 in plasma while the concentration of IL-12 was low¹⁰². Furthermore, monocytes from healthy carriers with the GG genotype produced more CCL2 than monocytes from individuals with the AA genotype, when stimulated with *M. tuberculosis* antigens. Neutralization of CCL2 activity with monoclonal antibody increased IL-12p40 in monocytes from the GG genotype while addition of exogenous CCL2 increased IL-12p40 production by monocytes from individuals with the AA genotype¹⁰². This suggests that individuals with CCL2 GG genotype may have enhanced MCP1 production, which in turn inhibits the production of IL-12p40 and increases the risk of active pulmonary tuberculosis¹⁰².

MIP1- α and MIP1- β are involved in the activation of macrophages¹⁰³ and activation and proliferation of T cells⁹⁹. Alveolar macrophages infected with H37Rv produce less MIP1- α and MIP1- β when compared to macrophages infected with H37Ra¹⁰⁴. Additionally, alveolar macrophages from HIV infected patients have shown to produce reduced levels of TNF and MIP1- β when compared with alveolar macrophages from healthy individuals¹⁰⁴. MIP1- β has been found to reduce the intracellular growth of *M. tuberculosis*¹⁰⁴.

Another important chemokine in *M. tuberculosis* infection is IL-8, which is primarily produced by monocytes and macrophages upon phagocytosis of tubercle bacilli¹⁰⁵ and serves as a chemoattractant to recruit neutrophils, lymphocytes, and monocytes to sites of infection^{106, 107}. IL-8 and other chemokine levels were found to be elevated in the bronchoalveolar lavage (BAL) fluid of patients with tuberculosis¹⁰⁸.

The major findings discussed in this part of the literature review clearly demonstrate the key role of the innate immune response in the outcome of *M. tuberculosis* infection. During course of evolution, *M. tuberculosis* has adapted various diverse mechanisms to evade host microbiocidal functions, leading to persistence and proliferation within favourable niches. Recognition of mycobacterial PAMP by different PRR phagocytic cells and their subsequent activation is important to switch on pro-inflammatory signalling pathways, which enable the effective innate immune response to mycobacteria. Virulent strains of *M. tuberculosis* modulate inflammatory response by recognition through alternative receptors (eg. Mannose, DC-SIGN), thereby subvert classical activation

of phagocytes. This ability of *M. tuberculosis* to modulate inflammatory cytokine responses emerges as one of its important evasion strategies.

Chapter 2

Strain diversity in *M. tuberculosis*

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2.1 Introduction

It was previously believed that genetic variation in *M. tuberculosis* was restricted and *M. tuberculosis* was considered to be a monomorphic species¹⁰⁹. More recently, studies have revealed that there is considerable variation in the genome of *M. tuberculosis*, and that evolution is based on deletion, duplication and single nucleotide polymorphism¹¹⁰⁻¹¹². Horizontal gene transfer is considered to be a very rare event. Since genetic transfer seldom, if ever, occurs with modern TB strains⁵, *M. tuberculosis* follows a clonal pattern of evolution^{113, 114}.

The range of clinical outcomes following infection with *M. tuberculosis* suggests that genetic diversity in *M. tuberculosis* strains may be an important determinant in the development of tuberculosis disease^{111, 115-123}. The term 'virulence' has many possible interpretations when applied to *M. tuberculosis*. It may be used to refer to lethality, to transmissibility, to a predilection for causing disease rather than latent infection, to a propensity for extra-pulmonary spread or the acquisition of multi-drug resistance¹²⁴⁻¹²⁸. Since virulence is complex, and difficult to assess in the human host, virulence of *M. tuberculosis* has been assessed in various *in vivo* animal¹²⁹⁻¹³¹ and *in vitro* tissue culture models¹³²⁻¹³⁴, which may be used to model growth or dissemination, disease pathology, fatality and transmissibility. Importantly, different genotypes of *M. tuberculosis* have been shown to induce distinct immune-pathological responses in various animal and human studies^{129, 130, 135-137}. Several outbreak strains have been shown to subvert the body's protective immune response with identification of specific virulence determinants that are involved in modulation of host protective immunity^{131, 138-140}.

The *M. tuberculosis* global phylogeny consists of six phylogeographic lineages¹¹¹. Evidence from epidemiological studies suggests that these lineages might be adapted to particular human populations^{111, 112, 117, 141}. For instance, Gagneux *et al.* observed, in a cosmopolitan setting in San Francisco, where diverse groups of humans and tuberculosis strain lineages co-exist, that prevalent strains were associated with their sympatric^a human population¹¹¹. When infection with allopatric^b strains occurred, it was mainly associated with risk factors such as HIV-TB co-infection, which perturb the normal host-pathogen relationship¹¹¹.

The question therefore arises whether differences in virulence may be ascribed broadly to *M. tuberculosis* strain lineages, or whether such differences exist only at the level of the individual strain. This review will describe the consequences of genetic diversity between *M. tuberculosis* strain lineages and the associated virulence characteristics that have been assessed previously in various studies in human tissue culture and animal models.

2.2 Molecular typing to detect genetic diversity in *M. tuberculosis*

In past years, the identification of diversity within the *M. tuberculosis* complex (MTBC^c) was restricted to morphological characteristics, growth phenotype and biochemical characteristics¹⁴². These techniques were highly laborious, time consuming and poorly discriminatory.

Molecular biological techniques such as whole genome sequencing, restriction fragment length polymorphism (RFLP) analysis, spoligotyping, Mycobacterial

^a Occupying the same geographical location

^b Occupying different geographical locations

^c MTBC : *Mycobacterium tuberculosis* complex, including *M. tuberculosis*, *M. africanum*, *M. bovis* and *M. microti*

Interspersed Repetitive Units-Variable Number Tandem Repeat (MIRU-VNTR) and deletion analysis have provided the opportunity to analyse genetic variation in different *M. tuberculosis* isolates and contribute to the construction of a global phylogenetic classification of *M. tuberculosis*. Moreover, these genotyping techniques have proven useful in tuberculosis control efforts by identifying clonal outbreaks, distinguishing between reactivation and reinfection and in mapping transmission within communities¹⁴³⁻¹⁴⁶.

2.2.1 IS6110 restriction fragment length polymorphism

Insertion sequences (IS) are generally small DNA sequences (less than 2.5 kb) distributed through the bacterial genome as mobile genetic elements. The most commonly found IS element in MTBC is IS6110, a 1355 bp insertional sequence of the IS3 family, and is exclusively present within the MTBC¹⁴⁷. IS elements have two particular characteristics: they code for a protein (usually denoted as transposase) which catalyses the enzymatic reaction to promote movement of the IS element throughout the bacterial genome as well as one regulatory protein which regulates transposition activity. Transposition of IS elements can cause gene disruption and activation or change of the adjacent gene expression due to the presence of included regulatory elements. IS elements are important from an evolutionary perspective. IS elements can act as genome parasites to harm the host bacteria¹⁴⁸ but also play an important role in bacterial adaptive evolution¹⁴⁹.

In 1993, Van Embden *et al.* developed a restriction fragment length polymorphism (RFLP) analysis incorporating restriction digestion by the enzyme *PvuII* followed by Southern blotting and hybridization with a probe for IS6110¹⁵⁰. This method is effective for generating clear and discriminatory RFLP band patterns^{150, 151}. Several

studies in the past have reported that RFLP has important advantages in terms of stability, reproducibility and high discriminatory value^{152, 153}. MTBC members possess between 0 and 25 copies of IS6110^{154, 155}. RFLP has low discriminatory power for *M. tuberculosis* strains that contain five or fewer copies of IS6110¹⁵⁶. Further disadvantages of RFLP are that it is a slow, time consuming, labour intensive and technically demanding technique which requires large amounts of DNA from mycobacterial isolates.

2.2.2 Spoligotyping

The *M. tuberculosis* genome shows diverse repetitive sequences: transposable elements¹⁴⁷, trinucleotide repeats¹⁵⁷, mycobacterial interspersed repetitive units variable number tandem repeats^{114,158} and the direct repeat (DR) region¹⁵⁹. The DR region in *M. tuberculosis* comprises multiple 36bp direct repeat sequences which are interspersed with 35 to 41bp unique spacer DNA sequences. The number of repeats in a DR sequence varies from 10 to 50 in various strains within the *M. tuberculosis* complex. The DR region is one of the most extensively studied loci for studying strain diversity amongst MTBC.

Spoligotyping is a combination of polymerase chain reaction (PCR) and hybridization techniques which identify the presence or absence of 43 interspersed spacer sequences in the DR regions of different strains of *M. tuberculosis*. The first step in this method is to amplify the DR regions using primers directed at the DR sequences which also amplify the adjacent spacer region present between two DR loci. The presence or absence of a spacer sequence in a test strain is determined by hybridization to a membrane coated with 43 synthetic oligonucleotides representing the complete set of spacer sequences.

Hybridization is detected by chemiluminescence. Spoligotyping results are highly reproducible; data can easily be allocated numerical values and computerized and are available for interlaboratory comparison. An international spoligotyping database (SpolDB4) has been developed which includes at least 39,295 entries from 122 countries¹⁶⁰. The groups of isolates are assigned into different clades or families based on the similarity in spoligotyping pattern. Spoligotyping is typically performed from cultured isolates but can also be performed directly from some clinical samples without prior culturing, as well as from paraffin-embedded material and Ziehl-Neelson stained slides. Spoligotyping can also be useful to differentiate various species of the MTBC depending on the species-specific variation of spacer sequences. The major limitations of spoligotyping are a relatively low discriminatory power, and the possibility of convergent evolution giving rise to identical or similar spoligotype patterns in strains which are only distantly related¹⁶¹.

2.2.3 Mycobacterial interspersed repetitive unit- variable number tandem repeat (MIRU-VNTR) genotyping

The genome of *M. tuberculosis* contains diverse repetitive sequences including several short nucleotide sequences which are structured as tandem repeats and often exhibit a variation in repeat number (known as variable number tandem repeat [VNTR]). Earlier, systematic analysis of MTBC strains described 11 loci comprising five major polymorphic tandem repeats (MPTR, A to E) and six exact tandem repeats (ETR, A to F, 53-79bp)¹⁵⁸. Supply *et al.* recognized the presence of human minisatellite-like structures in the genome of MTBC. The minisatellite structures consist of 40-100bp repetitive sequences called Mycobacterial Interspersed Repetitive Units (MIRU) that are scattered in 41 loci throughout the

chromosome of *M. tuberculosis* H37Rv¹¹⁴. Initially, 12 MIRU within the 41 loci were reported to be highly polymorphic and suitable for strain genotyping¹¹⁴. The MIRU-VNTR genotyping method is based on the detection of the number of tandem repeats at several loci in the *M. tuberculosis* genome. One commonly used MIRU-VNTR method for typing *M. tuberculosis* clinical isolates is based on the detection of 12 of the 41 MIRU loci. MIRU-VNTR involves PCR amplification of each of these 12 loci to determine the size of the amplicon and hence the number of repeats at each locus. This is denoted by a 12 digit format corresponding to the number of repeats at each locus. It has been reported that 12-locus MIRU is less discriminatory compared to IS6110-RFLP typing with high IS6110 copy number strains¹⁶². More recently, additional loci have been included in the MIRU-VNTR typing method, e.g., 24-locus MIRU-VNTR¹⁶³.

2.2.4 Large Sequence Polymorphisms

Fleischmann *et al.* performed comparative analysis on the genome sequences of H37Rv and CDC1551 and identified single nucleotide polymorphisms (SNPs) and deletions in the coding sequences, the latter of which are known as large sequence polymorphisms (LSPs) or regions of difference (RD)¹⁶⁴. LSPs have been identified throughout the *M. tuberculosis* genome and are irreversible and aggregative in nature. Various studies have used deletion mapping to distinguish between strains within the MTBC^{111, 165, 166}. LSPs were used by Gagneux *et al.* to construct a global phylogenetic classification of MTBC as indicated in Figure 2.01¹¹¹.

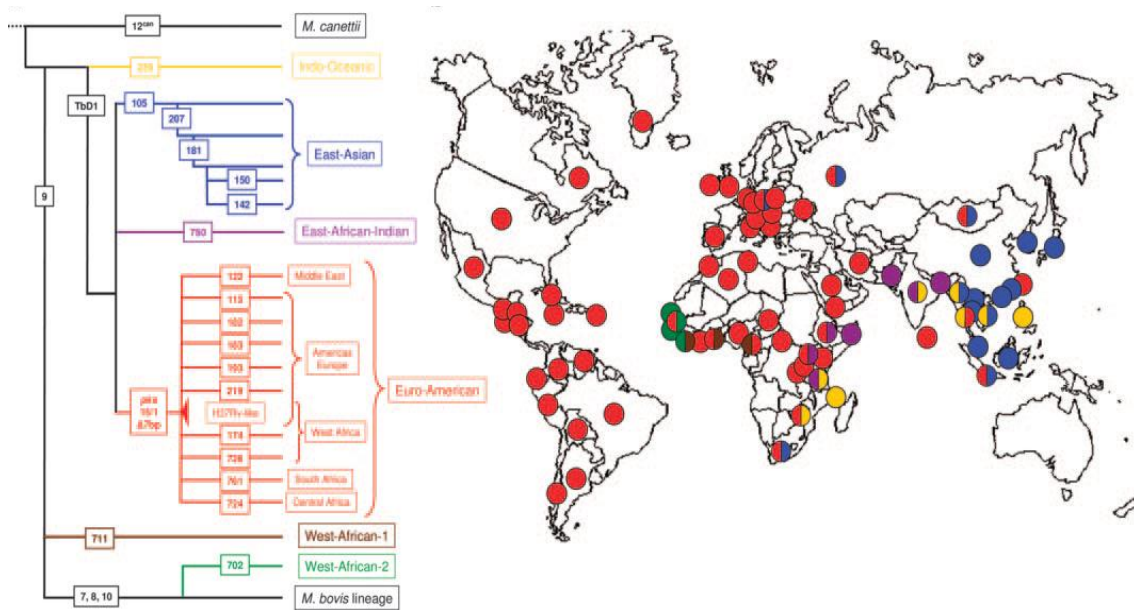


Figure 2.01 Global phylogeny of *M. tuberculosis* based on large sequence polymorphisms. (Taken directly from Gagneux *et al.*, 2006).

2.2.5 Single nucleotide polymorphisms

Since, in general, the genome structure of MTBC is highly conserved, polymorphism at the nucleotide level is an important source of genetic variation in mycobacteria as well as a useful tool for detecting strain diversity in MTBC. Non-synonymous SNPs (nsSNPs) alter the single amino acid sequence and may confer a specific phenotypic characteristic such as drug resistance¹¹⁰. Synonymous SNPs (sSNPs) are useful to study bacterial evolution and genetic drift amongst *M. tuberculosis* strains. Various studies have used SNPs as a basis for phylogenetic analysis^{109, 112, 167} including an important recent global phylogeny by Hershberg *et al.* illustrated in Figure 2.02¹¹².

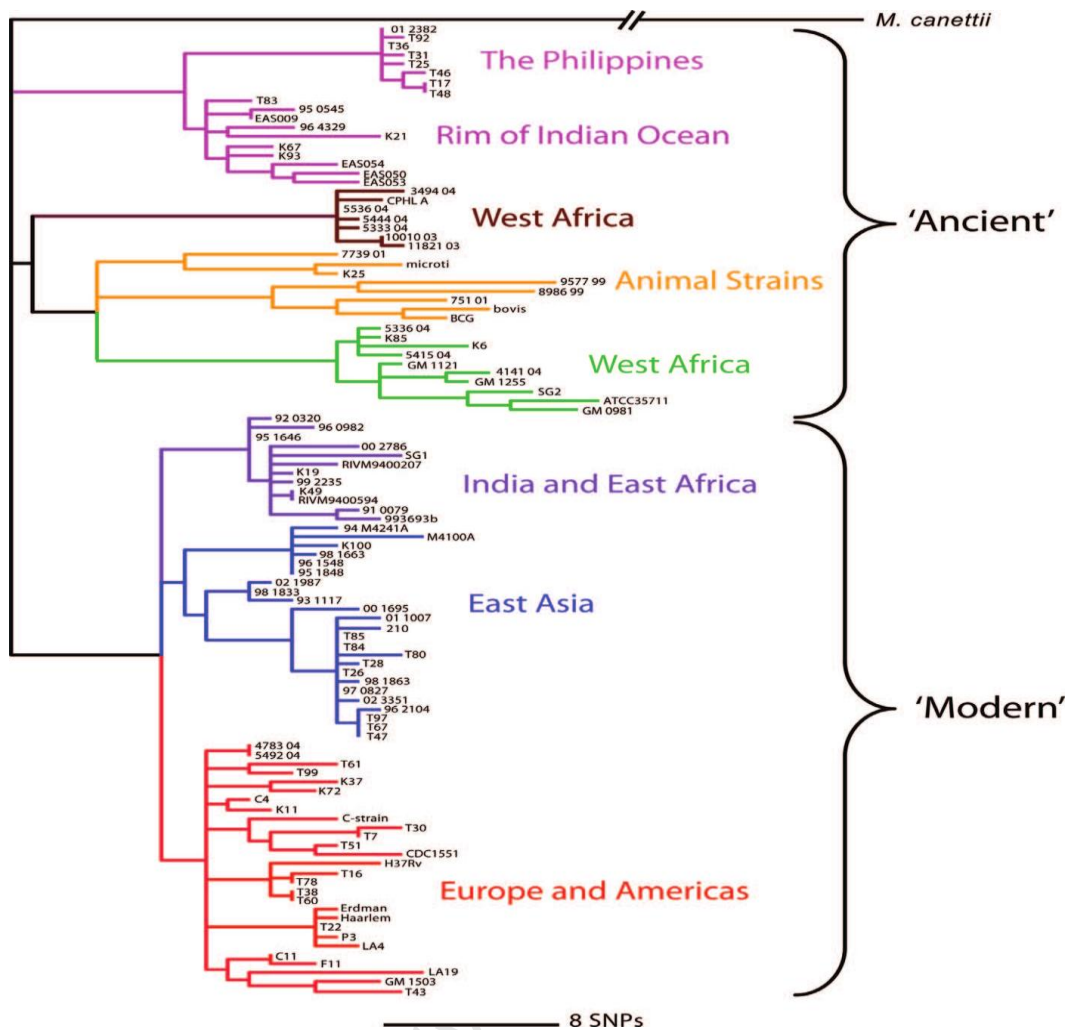


Figure 2.02 Global phylogeny of *M. tuberculosis* based on multi-locus sequencing of 89 genes from 108 strains. (Taken directly from Hershberg *et al.*, 2008).

2.3 The Global Phylogeny of *M. tuberculosis*

Early studies using single nucleotide polymorphism analysis, based on the sequence analysis of 26 structural genes of MTBC, revealed two non-synonymous single nucleotide polymorphisms (nsSNP) at *katG* codon 463 (the gene encoding catalase peroxidase) and *gyrA* codon 95 (the gene encoding the A subunit of DNA gyrase). Based on these polymorphisms, *M. tuberculosis* was classified into three principal genetic groups (PGG) as described in Figure 2.03¹⁰⁹.

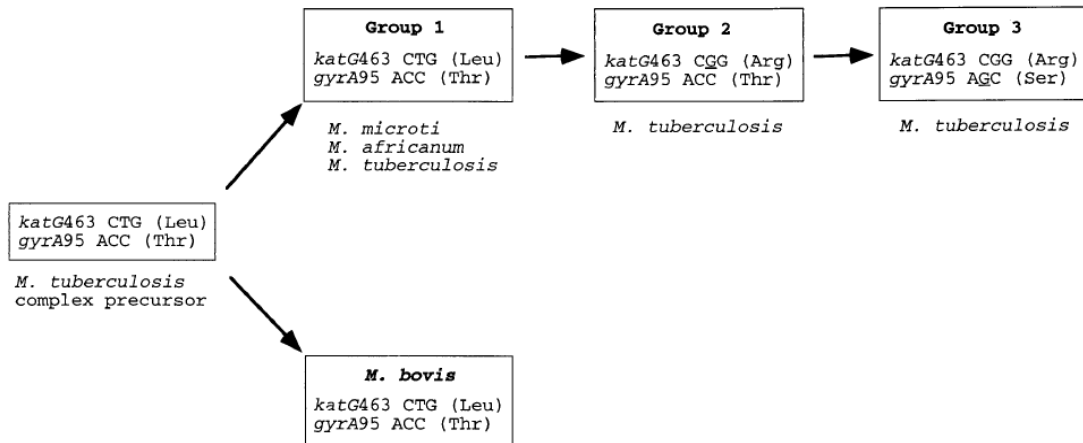


Figure 2.03 The classification of MTBC into principal genetic groups based on two non synonymous Single Nucleotide Polymorphisms. (Taken directly from Sreevatsan *et al.*, 1997).

Later studies used LSP analysis to construct phylogenetic classification schemes. Brosch *et al.* proposed a new MTBC phylogenetic classification based on analysis of 20 variable genetic markers in 100 strains of MTBC. The majority of these polymorphisms resulted from irreversible genetic events in ancient progenitor mycobacterial strains. The authors divided *M. tuberculosis* into “Modern” and “Ancestral” strains based on the *M. tuberculosis* specific deletion 1 (TbD1). The genetic deletion RD9 defines an evolutionary lineage represented by *Mycobacterium africanum*, *Mycobacterium microti* and *Mycobacterium bovis* that diverged from the progenitor of present *M. tuberculosis* strains before TbD1 occurred. Since RD9 is not present in *Mycobacterium canetti* strains, these strains are considered to be direct descendants of tubercle bacilli that existed before the *M. africanum* and *M. bovis* lineage separated from the *M. tuberculosis* lineage (Figure 2.04)¹⁶⁶.

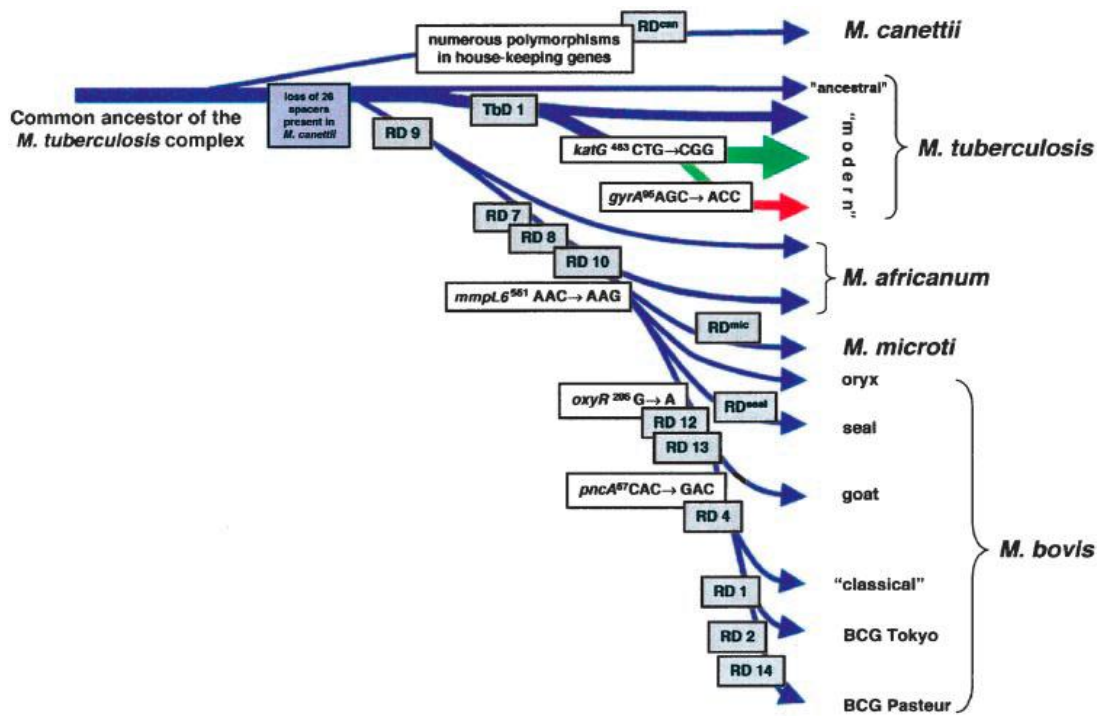


Figure 2.04 The evolution of *M. tuberculosis* complex as determined by region of deletion region of deletion analysis. (Taken directly from Brosch et al., 2002).

Baker *et al.* utilized multi-locus sequence typing (MLST) of seven housekeeping genes, which are associated with antimicrobial drug resistance in 225 clinical isolates from hospitals in England and Wales. The study identified 36 sSNPs that were geographically associated with the patients' country of origin and classified *M. tuberculosis* strains into four distinct lineages. However, the numbers of genes selected in this study were limited and so unable to resolve a more detailed phylogeny of *M. tuberculosis*¹⁶⁷.

Subsequently, Brudey *et al.* constructed a large spoligotype database (SpolDB4) including a broad global collection of *M. tuberculosis* spoligotype patterns and classified them according to spoligotyping families¹⁶⁰. This phylogeny broadly coincided with previous, SNP-based phylogenetic classification schemes.

Gagneux *et al.* classified *M. tuberculosis* into six major lineages, based on LSP analysis of 875 MTBC strains from 80 countries (Figure 2.01). Certain lineages were predominant in specific geographic areas and named according to the geographical distribution: the East Asian lineage (commonly known as the W-Beijing strain family), East-African-Indian lineage, Euro-American lineage, Indo-Oceanic lineage, and West African 1 and West African 2 lineages¹¹¹.

Although LSP based approaches of defining tuberculosis phylogeny are broadly effective in classifying *M. tuberculosis* strains, the genetic distances between the lineages are not clear and mycobacterial evolution pattern might not be completely imitated by such classification¹¹⁰.

In an important recent study, Hershberg *et al.* performed multi-locus sequence analysis of 89 complete genes in 108 strains, which includes 1.5% of the total genome from each strain. The study identified a total of 488 SNPs and established a phylogenetic correlation between genetic variability in *M. tuberculosis* strains and the geographical distance travelled by these strains from the ancestral progenitor¹¹². The phylogenetic tree produced by this study was concurrent with the LSP-based phylogenetic analysis (Figure 2.02)¹¹¹. Further, the authors identified three ancient strain lineages in the African zone including the Philippines and the Rim of the Indian Ocean, suggesting an origin for the MTBC in Africa and subsequent spread throughout the world following human migration patterns¹¹². This hypothesis also supported the anthropological evidence that modern humans spread out from Africa¹⁶⁸. Additionally, this phylogenetic analysis divided modern *M. tuberculosis* strains into three modern lineages (Euro-American, East African Indian and East Asian). Furthermore, these modern strains were thought to have originated in Western Europe, Northern India, and East Asia

respectively and spread worldwide due to more recent migration and proliferation of the human population. The route of the modern strain transfer was hypothesized to have been via water transportation due to the contemporary use of ships during this period¹¹². The presence of the 'red strains' (Euro-American) in Asia, Africa and in the Middle East indicated the spread of these strains due to European colonization. Further, the spread of the 'blue strains' (East-Asian/Beijing) in South Africa may have been related to human migration from South-East Asia to South Africa due to slavery in the gold mines during Dutch colonization. Additionally, the presence of the 'purple strains' (East-African-Indian strains) in East-Africa was supported by the history of migration of human populations to East-Africa from the Indian subcontinent. This study also highlighted a molecular evolutionary approach to detect fitness in *M. tuberculosis* complex. It was found that purifying selection against potentially deleterious mutations was highly reduced in MTBC compared with other bacteria resulting into a high ratio of non-synonymous to synonymous SNPs (DN/DS). The authors suggested that increase in non-synonymous mutation might associate with random increase in the genetic drift, which in turn was influenced by the human demography, migration of human populations and patient-to-patient transmission¹¹².

Comas *et al.* performed whole genome DNA sequencing of 22 strains representative of the six global phylogenetic lineages. Comparative sequence analysis of these strains provided 9037 unique SNPs from single or multiple strains¹⁶⁹. The classification system derived from this analysis was highly congruent with the LSP and SNP based classification system¹⁶⁹. Based on this genome-based classification, the authors suggest a change to the nomenclature of

MTBC lineages: “Lineage 1 (also known as Indo-Oceanic lineage)”, “Lineage 2 (also known as East Asian; includes “Beijing”)", “Lineage 3 (also known as CAS/Delhi)”, “Lineage 4 (also known as Euro-American)”, “Lineage 5 (also known as West African 1)” and “Lineage 6 (also known West African 2)”¹⁶⁹. This nomenclature will be used from this point in this thesis, since it avoids confusion caused previously by the use of conflicting names for different lineages.

A comparison in spoligotyping, LSP and genome-based classification is given in Table 2.1. Note the confusion generated by the different use of the terms “East African Indian” lineage and EAI spoligotype family, which refer to completely different strain lineages.

Table 2.01 Global phylogenetic classification of *M. tuberculosis* strain lineages based on large sequence polymorphisms (LSP) and spoligotyping.

MTBC species	Spoligotyping based families (Brudey <i>et al.</i> , 2006)	LSP based lineages (Gagneux <i>et al.</i> , 2006)	Genome-based phylogeny (Comas <i>et al.</i> , 2010)
<i>M. tuberculosis</i>	EAI	Indo Oceanic	Lineage 1
<i>M. tuberculosis</i>	Beijing	East –Asian	Lineage 2
<i>M. tuberculosis</i>	CAS	East-African Indian	Lineage 3
<i>M. tuberculosis</i>	H, LAM, X, T, S others	Euro-American	Lineage 4
<i>M. africanum</i>	AFRI2	West African-I	Lineage 5
<i>M. africanum</i>	AFRI1	West African-II	Lineage 6

EAI- East African Indian, CAS- Central Asian, H- Haarlem, LAM-Latin American Mediterranean, AFRI- *M. africanum*.

2.4 Description of modern strain lineages of *M. tuberculosis*

2.4.1 Lineage 2 (East – Asian, includes Beijing strains)

Strains from the Beijing genotype (lineage 2) possess a characteristic spoligotyping pattern, which involves deletion of spacer 1 to 34, and the presence of spacer 35 to

43 in the direct repeat region^{121, 170}. Beijing genotype strains (lineage 2) predominate in eastern Asia¹⁷⁰, however these strains are ubiquitous and have been found to be transmitted globally^{171, 172}. Evidence suggests these strains are emerging in many parts of the world. A study from major paediatric referral hospitals in Cape Town, South Africa identified a 20% increase in the proportion of W-Beijing strains between 2000 to 2003 amongst isolates of *M. tuberculosis* from children¹²¹. Furthermore, the study also found that these strains were absent in histological samples over the period of 1930-1965, rare in 1966-1995 (2.8% samples) and rapidly emerging during 1996-2005 (20% samples)¹²¹.

Based on the presence of IS6110 in the NTF-1 region of the chromosome, Beijing strains have been divided into two sub-lineages. Firstly, modern or 'typical' Beijing strains possess an IS6110 insertion in the NTF region and are distributed worldwide. In addition, so-called 'W' strains possess a mutation in the *mut* gene, which encodes a DNA repair enzyme^{121, 171, 173-176}. These 'W-Beijing' strains are responsible for worldwide outbreaks, are major causes of tuberculosis in China, Russia and South Africa and are associated with multidrug resistant tuberculosis infection¹⁷⁰⁻¹⁷². Secondly, ancient or 'atypical' Beijing strains (these are still included amongst modern strain lineages of *M. tuberculosis*) do not possess an insertion in the NTF region and are less widely distributed, but are found frequently in Japan and Korea^{173, 177-179}.

In a recent study from Japan, modern Beijing strains were shown to transmit more frequently amongst the younger generation than ancient Beijing strains¹⁷⁶. The reason behind the variation in worldwide distribution of the two subgroups of

Beijing lineage is not clear. The modern Beijing lineage might be better adapted to spread in modern human societies^{119, 171, 172}. However, despite the global spread of modern Beijing strains, an ancient Beijing strain caused a serious outbreak amongst the homeless population in the United States¹⁸⁰. Furthermore, ancient Beijing strains with low-fitness cost rifampicin resistance mutations have been identified as a cause of tuberculosis amongst HIV-infected patients in the Eastern Cape province of South Africa¹⁸¹.

Other classification schemes have been proposed for W-Beijing strains, including a scheme based on LSP analysis which divides the Beijing lineage (Lineage 2) into five subgroups¹⁶⁵.

2.4.2 Lineage 3 (CAS/ Delhi)

Lineage 3 (CAS) strains are largely distributed in the Indian subcontinent, the Middle East, and Central Asia and comprise approximately 30% of circulating strains in these regions¹⁶⁰. This lineage is also common in East Africa, which has experienced substantial Indian migration^{120, 160} and amongst the Indian population in the United Kingdom¹⁸². The Lineage 3 is designated as the Central Asian strain (CAS) family in the spoligotype database and has a particular spoligotype signature that involves deletion in spacers 4 to 27 and 23 to 24. This lineage is related to lineage 2 strains (Beijing) and shows a close relationship with Beijing strains on spoligotyping and MIRU-VNTR¹⁸³.

2.4.3 Lineage 4 (Euro-American)

Lineage 4 (Euro-American strains) predominates in Europe, America, the Middle East and Africa¹¹¹. This is a diverse lineage, which covers the principle genetic

group 2 and 3 as described by Srevatsan *et al.*¹⁰⁹ and consists of a wide range of spoligotype families including Latin American Mediterranean (LAM), Haarlem, X and T families of strains¹⁶⁰. Based on spoligotype data, Brudey *et al.* proposed various subgroups within the Latin American Mediterranean family - LAM1 to LAM12¹⁶⁰. Haarlem strains are distributed worldwide, including in Europe¹⁶⁰ and the Caribbean¹⁸⁴. Low IS6110 copy number-containing 'X' family strains are common in the United Kingdom and in North America¹⁸⁵. In Africa, LAM, Haarlem, and T family strains are common¹⁸⁶. LAM3 strains are common in South Africa^{187, 188}.

2.5 Virulence of *M. tuberculosis*

Virulence can be defined as the ability of a pathogen to cause severe disease in the human host¹⁸⁹. It is not possible to define virulence in *M. tuberculosis* simply in terms of specific virulence determinants as it does not have clearly defined virulence factors such as exotoxin production by *Clostridium tetani*¹⁹⁰ or *Staphylococcus aureus*¹⁹¹. Host and bacterial factors both play an important role in the outcome of infection with *M. tuberculosis*. For example the level of serum vitamin D is considered to be a risk factor in *M. tuberculosis* infection; individuals with low level vitamin D levels have shown increased risk of developing detectable tuberculosis infection⁵⁰. Age¹⁹² and host immune status play critical roles. As an example of the interplay of host and strain factors, the relatively less virulent *M. africanum* strains cause tuberculosis disease in HIV-infected patients in West Africa, but less commonly in HIV-uninfected individuals^{193, 194}. Differences in the outcome of infection by different strains may be mediated by strain-specific immune responses. For example, the outbreak strain, CDC1551 (a lineage 4 strain), induced a high number of tuberculin skin test conversions amongst contacts in the

Kentucky region in United States but few cases of active tuberculosis disease, suggesting high immunogenicity, but low virulence¹⁴⁰.

Early comparative studies of virulence were limited by the lack of genotyping information, which made it difficult to assign particular virulence characteristics to specific strains or strain families. Recently, there has been substantial work to assess the relation between *M. tuberculosis* genotype and virulence in human cell cultures and animal infection models. However, findings are not always consistent, due to variation in the specifics of the model used, the selection of strains and, probably operator-dependent issues.

2.5.1 The human macrophage model of *M. tuberculosis* virulence

Tissue culture models are less time consuming and resource intensive than animal models. However, a tissue culture model can only partially represent the very early stages of tuberculosis infection while animal models may also be useful to understand later stages of infection. *M. tuberculosis* invades alveolar macrophages, which are the primary site of intracellular replication and persistence. Early control of replication by macrophages is likely to play an important role in determining the outcome of infection. Since *M. tuberculosis* is an intracellular pathogen which has adopted various strategies to adapt to the host's cellular environment, it is useful to consider the host-pathogen relationship at the cellular level as a virulence characteristic. *M. tuberculosis* utilizes different strategies to survive and replicate in the intracellular environment. Following ingestion by macrophages, *M. tuberculosis* is channelled to the phagosome¹⁹⁵. Following phagocytosis, phagosomes fuse with lysosomes, which results in degradation of microbes by endolytic acid hydrolases. *M. tuberculosis* reduces the

antimycobacterial activity of macrophages by arresting fusion of phagosomes with lysosomes¹⁹⁶. *M. tuberculosis* also modulates H⁺ ion concentration in the phagolysosome by inhibiting fusion of infected vacuoles with the proton pump-ATPase, responsible for creating an acidic environment within the phagolysosome, resulting in reduced acidity inside the phagolysosome and functional failure of lysosomal acid hydrolases¹⁹⁷. Furthermore, the phagosomal pathway interacts with the endocytic pathway involving the Ras family GTPase. Experimental evidence in mycobacterial infection shows the presence of the early endosome marker Rab-5 instead of the late endosome marker Rab-7 on mycobacterial phagosomes, indicating phagosomal arrest during *M. tuberculosis* infection¹⁹⁸. Additionally, the tryptophan aspartate containing coat protein (TACO), which is normally associated with cholesterol in the plasma membrane¹⁹⁹, is recruited during early phagosome formation and is essential for phagosomal maturation. However, mycobacteria are able to retain this protein in phagosomes, thus inhibiting its supply to lysosomes, resulting in inhibition of phagosomal maturation during *M. tuberculosis* infection²⁰⁰. Since the ability of *M. tuberculosis* to survive and replicate in the host macrophage is key to the development of disease, the ability of strains of *M. tuberculosis* to survive and grow in the macrophage or monocyte model may be a useful surrogate marker of virulence. Various human monocyte cell lines such as THP1 (the acute monocytic leukaemia cell line) have also been used in *ex vivo* tuberculosis models to measure intracellular growth and cytokine induction^{134, 201}. While monocytic cell lines are similar in many respects to primary human macrophages, they are produced from transformed cells from a single individual and may differ in important characteristics from primary cells,

e.g., THP1 cells possess Fc and C3b receptors on the cell surface but lack surface and cytoplasmic immunoglobulins²⁰².

All models of infection have limitations in their ability to replicate human disease, however they may offer useful insights that can be validated in studies of humans with tuberculosis.

2.5.2. Intracellular growth within monocytes/macrophages as a virulence characteristic

Lineage 2 strains (Beijing) have been the most extensively studied clinical strains. *In vitro* studies demonstrated that individual strains from lineage 2 grew rapidly in human cell culture models using monocyte derived macrophages (MDM) or monocytes or human macrophage cell lines^{132, 203}. Zhang *et al.* utilized a MDM model to study the correlation between the extent of the spread of *M. tuberculosis* strains in a Los Angeles community setting and the ability of the strains to grow in human macrophages¹³². In this study, a lineage 2 strain 210 responsible for 25% of tuberculosis cases in Los Angeles, grew more rapidly in human MDM than strains which caused disease in single patients who came in contact with many persons in a homeless shelter¹³². Li *et al.* found significant differences in the growth of different *M. tuberculosis* strains in human monocytes and found that four lineage 2 strains grew more rapidly than Erdman, CDC1551 and several other non-Beijing strains²⁰³. Subsequently, Theus *et al.* also demonstrated that the growth of lineage 2 strain 210 was significantly faster than that of unique strains in activated THP1 monocytic cells¹³⁴. Theus *et al.* further demonstrated that strains that clustered using RFLP analysis grew more rapidly than unique RFLP pattern strains. This was attributed, in part, to the rapid induction of interleukin-10 (IL-10) by clustered

strains, which was hypothesized to decrease the production of tumour necrosis factor (TNF) during early stages of infection²⁰⁴.

Taken together, the above experimental evidences suggest that lineage 2 strains (Beijing) replicate more rapidly in human macrophages than other strain lineages, indicating a possible virulence-associated characteristic for this lineage. However, there are contradictory studies, which suggest that rapid growth in macrophages is not a common characteristic of all lineage 2 strains. In a further study, Theus *et al.* performed intracellular growth assays in a THP1 cell line using various lineage 2 strains to assess whether rapid growth was characteristic for a broad range of lineage 2 strains. The authors found that three lineage 2 strains grew more slowly than the others whilst strain 210 grew faster, suggesting variation in the intracellular growth rate amongst lineage 2 strains²⁰⁵.

Intracellular growth of *M. tuberculosis* in macrophages is partly controlled by cellular apoptosis since programmed cell death kills bacteria and enhances containment of the bacterial infection. A recent study from Korea has reported that K strain, a frequently found virulent lineage 2 strain in Korea, causes less cellular apoptosis and induces more necrosis in human monocytic THP1 cell lines than H37Rv. K strain causes more up regulation of the anti-apoptic *Bcl-2* family gene than H37Rv and slight up regulation of anti-apoptic *Bax* gene similarly as H37Rv in the THP1 cell line²⁰⁶.

Intracellular growth of strains from other lineages has also been examined, but data are more limited. A recent study from Pakistan demonstrated that a lineage 3 strain (CAS1) with deletion in RD149 grew slowly in THP1 cells when compared with a lineage 3 strain without the corresponding deletion or H37Rv and lineage 3 strains having deletion in both RD149 & RD152²⁰¹. Strains from lineage 4 (Euro-

American) have been frequently studied, probably as this lineage predominates in Europe and the United States. Li *et al.* demonstrated that the outbreak strain CDC1551 (lineage 4- X family strain), which was responsible for a high degree of skin test conversion amongst contacts in the South-Eastern USA (and reportedly grew faster than the Erdman strain but slower than H37Rv in mice), grew more slowly than the Erdman strain in human monocytes²⁰³. Haarlem strains (lineage 4, Euro-American), which have been found to grow faster in activated murine macrophages than H37Rv²⁰⁷ and cause extensive outbreaks of multidrug resistant tuberculosis in Argentina¹⁸³, were shown to be characterized by six LSPs which disrupted physiologically important genes such as *Rv1354c* and *cyp121*. Interestingly, both of these genes are considered a drug target for *M. tuberculosis*. *Rv1354* encodes cyclic- di-GMP, a messenger molecule involved with the bacterial inner membrane²⁰⁸. The cytochrome 450 *cyp121* gene in *M. tuberculosis* H37Rv was found to be involved in bacterial electron transport system, producing an enzyme that serves as an active site for binding to anti-mycobacterial (azole) drugs²⁰⁹. In an another report, strain-5 (lineage 4), a prevalent mycobacterial isolate in Madrid, where it was associated with HIV-infection and prison outbreaks, showed more rapid growth in THP1 cells during the first 24 hours of infection than H37Rv and other circulating orphan strains from this region²¹⁰.

2.5.3 Animal models of *M. tuberculosis* virulence

Virulence can be assessed more directly in animal models of disease. Although animal models have limitations²¹¹, various animal studies have used mice, guinea pigs, rabbits, and primates to identify virulence characteristics and compare virulence between strains of *M. tuberculosis*. Virulence has been most commonly assessed in the mouse model by measuring bacterial burden, histopathological evidence of inflammation and tissue destruction and the induction of delayed type hypersensitivity (DTH)^{129, 212}. Further, rabbit models have been extensively used in tuberculosis research because they show disease similar to humans, characterized by typical granuloma formation with caseation, liquefaction, and cavitation^{213, 214}. Recently, non-human primates have been used to study tuberculosis, with close replication of a broad range of tuberculosis disease manifestations from latency to fulminant tuberculosis²¹⁵.

2.5.4 Growth of *M. tuberculosis* strains in animal models as a virulence characteristic

The virulence of *M. tuberculosis* strains has been assessed in various animal infection models by measuring the extent of bacillary replication in different primary target organs (such as lung and spleen), the extent of end organ damage (measured using histopathological score, organ weight) and lethality. Lopez *et al.* demonstrated that Beijing strains (lineage 2) produce extensive pneumonia and early death in mice as compared to *M. canetti* and Haarlem (lineage 4) strains of tuberculosis¹³⁵. In an attempt to compare the virulence of multiple different strains of *M. tuberculosis*, Dormans *et al.* determined the virulence of 19 strains in BALB/c mice with respect to survival, lung pathology, bacterial load and delayed-type

hypersensitivity. The study reported that Beijing genotype strains 2 & 3 (lineage 2) were hypervirulent, as were Somali (lineage 3) and Haarlem (lineage 4) strains as manifested by early death and increased bacillary burden when compared with H37Rv, *M. canneti* and Beijing strain 1 (lineage 2). This suggests that lineage 2 strains may differ in virulence¹²⁹. These data suggest that lineage 2 strains differ with respect to virulence and that virulence correlates with the transmissibility of the lineage 2 strains¹³⁰. Further, Agruiler *et al.* selected lineage 2 strains from different sub lineages (according to a classification scheme by Hanekom *et al.*, 2007¹⁷⁵) based on transmission criteria in series of tuberculosis cases in patients. The transmissibility of lineage 2 strains was categorized in mice by co-housing the mice with or without infection with different strains from lineage 2 subgroups¹³⁰. The author reported that mice infected with highly transmissible lineage 2 strains died earlier (within 5 weeks post-infection) with a higher histopathological score than did mice infected with non-transmissible lineage 2 strains (died within 4 months post-infection). The mice infected with highly virulent Beijing strains produced low level of IFN- γ , ephemeral TNF and high levels of IL-4 (in the later stages of infection) than those infected with low virulence Beijing strains.

2.5.5 Induction of innate immune responses by *M. tuberculosis* strains as a virulence characteristic

There is evidence of variation in the induction of immune responses by different *M. tuberculosis* strains^{132, 136, 138, 204, 206, 216-219}. Individual strains differ in their ability to induce cytokine production in various *in vitro* and *in vivo* models of infection. However, findings are not consistent across different reported studies. Possible

explanations for this variation could be the use of slightly different experimental models and the lack of standardization in strain selection.

Lineage 2 strains have been most widely studied. The propensity of lineage 2 strains to induce low levels of pro-inflammatory cytokines (TNF, IL-6, and IL12p40) relative to comparator strains is consistently reported in various *in vitro* studies. For instance, a study from Korea found that a lineage 2 strain, K strain, induces lower levels of protective cytokines such as TNF α , IL-6, and IL12p40 than H37Rv in a human monocytic THP1 cell line²⁰⁶. This finding was further supported by recent studies by Tanveer *et al.* who demonstrated that lineage 2 strains, along with lineage 3 strains (CAS), induce low levels of pro-inflammatory cytokines such as TNF and IFN- γ in THP1 cells and in whole blood assays²¹⁹. Subsequently, Wang *et al.* studied various sublineages of the lineage 2 strains and demonstrated consistently low cytokine induction in *in vitro* models²¹⁶. The authors describe low levels of IL-6, TNF, IL-10 and GRO- α production by a range of lineage 2 strains from different sublineages in human macrophages and dendritic cells as compared to H37Rv²¹⁶. A recently reported human clinical study from Madagascar found that PBMCs infected with modern group-1 strains [including CAS (lineage 3) and Beijing strains (lineage 2), according to a classification scheme by Arnold *et al.*²²⁰] induce lower levels of antigen-specific IFN- γ than the modern group-2 [Harlem, LAM and X strains (lineage 4)] and ancient strains (lineage 1, Indo-Oceanic lineage), suggesting that these strains might have evolved mechanisms to subvert the Th1 response that may provide a selective advantage to these lineages²¹⁸. Similarly, Portevin *et al.* found that modern strain lineages (Lineage 2,3 & 4) induce low levels of inflammatory cytokines, such as TNF, IL-6, IL-12p40, IFN- γ , as compared with ancient lineages (lineage 1, 5& 6) in GM-CSF derived macrophages

and monocyte derived dendritic cells²¹⁷. Of interest, before the development of genotyping techniques, very early studies reported that southern Indian strains were less virulent than strains from the United Kingdom in guinea pig models²²¹. The predominant strain lineage in Southern India is Lineage 1 (Indo-Oceanic, an ancient strain lineage) whilst lineage 4 strains (Euro-American, a modern strain lineage) predominate in the United Kingdom¹¹¹.

The specific mechanism by which containment of intracellular growth occurs in *M. tuberculosis* infection is unknown. In murine studies, containment depends, in part, on reactive oxygen intermediates and nitric oxide, whereas in humans the role of these molecules remains unclear. BALB/c mice produce high levels of Th1 cell cytokines and inducible nitric oxide synthase (iNOS) in the early stages of infection, which facilitate containment^{222, 223}. Lopez *et al.* showed that BALB/c mice demonstrated transient increases in TNF and iNOS mRNA and a low level of IFN- γ expression following infection with Beijing strains (lineage 2), whilst infection with *M. canetti* resulted in high and sustained TNF- α and iNOS mRNA expression¹³⁵.

Manca *et al.* studied levels of cytokine induction in B6D2F mice after infection with outbreak strains of *M. tuberculosis*²²⁴. The lineage 4-outbreak strain (Euro-American), CDC1551, induced high levels of TNF- α , IFN- γ , IL-12, IL-10, and IL-6 mRNA in the lungs of mice¹³³. Conversely, the lineage 2 outbreak strain (Beijing), HN878, (which caused early death of mice) induced lower levels of TNF- α , IL-6, IL-12 and IFN- γ mRNA in lungs, while IL-10, IL-4 and IL-5 mRNA levels were significantly increased, suggesting a shift from Th1 to Th2 cytokine induction. The hypervirulent characteristic of HN878 was associated with an increase in production of innate type I interferon (IFN α/β). The intranasal administration of

purified IFN α/β resulted in an increase in the bacillary load in lungs 28 days post-infection and was associated with a reduction in IL-12 mRNA in lungs and decreased survival²²⁵.

Subsequently, Manca *et al.* further demonstrated that HN878 as well as lipid extracts from this strain induced low levels of IL-12 (a Th1 cytokine) and increased Th2 cytokines such as IL-4 and IL-13. In contrast, the lineage 4 strain CDC1551 induced more Th1 cytokines such as IL-12 and TNF¹³⁶. Therefore, the outcome of tuberculosis infection may relate, in part, to the balance between Th1/Th2 cytokines²²⁶. The immune suppressive phenotype of HN878 is associated with the presence of biologically active phenolic glycolipid (PGL-tb) in the cell wall of this strain¹³⁶. PGL-tb has been identified in many lineage 2 strains (Beijing subgroups 3, 4, 5, according to a subgrouping of the Beijing lineage as described by Gagneux *et al.* 2006¹¹¹) having an intact *pks1-15* gene (polyketide synthase). This enzyme is involved in synthesis of PGL-tb¹³⁹. PGL-tb is associated with suppression of pro-inflammatory Th1 cytokines such as TNF, IL-6 and IL-12 and has been associated with virulence in the mouse model^{136, 139, 224}. However, PGL-tb is also present in other *M. tuberculosis* strains such as *M. canetti*²²⁷. Most other strain lineages have a 7bp deletion in *pks1-15*, which causes a frameshift mutation and makes them incapable of synthesizing PGL-tb²²⁷. In order to determine the impact of PGL-tb on bacterial virulence and the immune response, Sinsimer *et al.* constructed PGL-producing recombinant H37Rv by transforming the *pks1-15* gene into H37Rv. The transformed H37Rv propagated at the same rate as the wild-type strain in human monocytes, suggesting that PGL-tb does not affect *in vitro* growth in monocytes. Further, based on bacterial colony count on lungs, spleen, and survival, recombinant H37Rv (PGL-tb positive) was found to be less virulent in mice and

rabbits than wild type H37Rv²²⁸. However, several cytokines (IL-1 β , TNF, IL-10 and IL-17) levels were lower in the PGL-transformed H37Rv mutant than the wild type H37Rv strain in human monocytes²²⁸. However, PGL-tb is not the sole mediator of the immune-suppressive phenotype of Beijing strains (lineage 2). Low levels of pro-inflammatory cytokines have been shown to be induced by both PGL-tb positive (W4) and PGL-tb negative (N4) Beijing strains²²⁸, suggesting that PGL may work together with other lipid or protein factors which could also be virulent determinants in lineage 2 strains²²⁸. As PGL-tb is absent in most *M. tuberculosis* clinical strains, this lipid molecule is clearly not a fundamental virulence determinant, but it may impart a 'hypervirulent' characteristic to strains with certain genetic backgrounds²²⁸.

It has been suggested that alternative molecular mechanisms are likely to be involved in the success of lineage 2 strains. As such an alternative, Reed *et al.* demonstrated that the lineage 2 strains constitutively overexpressed genes of the DosR regulon (including *dosR*, *Rv3130c*, *hspX*, *fdxA* and *narX*) in normal *in vitro* growth condition, while other lineages only showed induction of the regulon following exposure to hypoxia or nitric oxide¹³⁹. Domenech *et al.* further demonstrated that the modern group of Beijing strains possess a duplicate copy of the *dosR* gene, which might be partly responsible for overexpression of DosR²²⁹. Lineage 2 strains produce large numbers of triacylglycerides in *in vitro* aerobic cultures, which interestingly, coincides with the up-regulation of the gene *Rv3130c*¹³⁹. This gene is responsible for production of the enzyme triacylglyceride synthase and is a member of the *M. tuberculosis* DosR regulon. Thus, lineage 2 strains are capable of synthesizing fatty acids such as triacylglycerides, which are useful as an energy sources during hypoxia, resulting in a unique survival strategy

during infection¹³⁹. Hence, activation of the DosR regulon is thought to confer an adaptive advantage to *M. tuberculosis* to facilitate survival within hypoxic conditions in granulomas¹³⁹.

Much attention has focused on lineage 2 strains, however outbreak strains from other families have also been associated with immune subversion. The lineage 3 (CAS) strain, CH, caused a tuberculosis outbreak amongst school children in Leicester, in the United Kingdom. CH was reported as a cause of 254 cases of latent tuberculosis infection and 77 cases of active pulmonary tuberculosis within a year¹⁴⁰. CH-infected MDMs induced less protective IL12p40 and more anti-inflammatory IL-10 than H37Rv. CH also induced up-regulation of IL-10 mRNA expression in MDMs¹³⁸. This immune phenotype was ascribed, in part, to a deletion in *Rv1519*. The *Rv1519* deletion is common to lineage 3 strains, and so the immune-subversive phenotype may represent a lineage-specific (rather than strain-specific) characteristic, although this has not yet been demonstrated.

This evidence may suggest that the success of certain strain lineages of *M. tuberculosis* may be dependent upon their ability to subvert the normal host protective immune response.

Chapter 3

The *in vitro* growth phenotype of *M. tuberculosis* strains from modern lineages

University of Cape Town

3.1 Introduction

Variability in the outcome of infection with *M. tuberculosis*, such as latent²³⁰ and active tuberculosis²³¹, treatment failure²³² and emergence of drug resistance²³³ has highlighted the potential role of strain variation in influencing these outcomes. Assessment of virulence or fitness of different strains of *M. tuberculosis* is complicated by the lack of suitable animal models and the complexity and prolonged nature of human disease.

The simplest measure of bacterial fitness is rate of growth, as determined by enumeration of bacterial growth by counting of acid-fast bacilli (AFB), by enumerating colony-forming units (CFU) or by estimating the turbidity of liquid bacterial culture²³⁴. These measures may give differing results as progression of bacterial growth may lead to the accumulation of dead bacilli, which may add to total count (AFB) but not to the actual viable count (CFU) of bacteria. Furthermore, evidence suggests that bacilli in human tissues with latent infection may lose their acid fast characteristics²³⁵. Hence, viable bacterial count by CFU estimation is considered as more reliable method for enumerating mycobacterial growth kinetics in various models of host pathogen interaction. In the experiments below, we compared growth of different strains in axenic media by CFU estimation.

Cell culture models have been widely used to assess various aspect of host pathogen interaction in tuberculosis including immunological responses and intracellular growth^{132, 134, 203, 236, 237}. In this study, we used primary monocyte derived macrophages (MDM) from peripheral blood mononuclear cells (PBMC) from human subjects as an *in vitro* model. The monocyte-derived macrophage (MDM) model may mimic the early stages of human tuberculosis infection.

Tuberculosis in the Western Cape Province of South Africa is predominately caused by strains from two globally successful lineages - lineage 4 and lineage 2¹⁸⁸. Lineage 2 strains have been found to be transmitted globally with a high tendency to cause outbreaks and multidrug-resistant tuberculosis infection, suggesting a better adaptation of this phylogenetic lineage to causing disease in the current epidemiological context^{172, 238}. Strains from this particular genotype have been shown to replicate more rapidly in the human cell culture model^{132, 203, 204}. Lineage 4 (Euro- American lineage, including LAM, X and Harlem subfamilies of strains) is a highly successful lineage in Europe, America, and parts of Africa and the Middle East^{111, 188, 239, 240}. The lineage 4 strain CDC1551 grew at a similar rate to H37Rv in human monocytes^{133, 241}. Further, lineage 3 (CAS, uncommonly seen in the Western Cape) strains predominate on the Indian sub-continent²⁴²⁻²⁴⁴ and have been shown to exhibit slower growth than H37Rv in axenic liquid media but similar growth in a cell culture model^{138, 201, 237}.

Various mouse studies have reported that recombinant IFN- γ pre-treated murine macrophages are able to induce anti-mycobacterial activity by a nitric oxide (NO)-mediated pathway. The catalytic oxidation of arginine to citrulline is mediated by iNOS (inducible nitric oxide synthase) that generates NO as a by-product. NO has potent bactericidal effect²⁴⁵. In contrast, the role of NO mediated containment of mycobacterial growth remains unclear in humans with low levels of measurable NO following *M. tuberculosis* infection^{245, 246}. We therefore compared the effect of IFN- γ pre-treatment of MDM on the intracellular growth of different strains of *M. tuberculosis*.

3.2 Aims of the study

- To compare the rate of growth of modern strains of *M. tuberculosis* in axenic media in order to identify lineage-specific patterns of growth
- To compare the rate of growth of modern strains of *M. tuberculosis* in primary human monocyte-derived macrophages in order to identify lineage-specific patterns of growth
- To determine the growth modulatory effect of IFN- γ on different *M. tuberculosis* strains in primary human monocyte derived macrophages

3.3 Methods

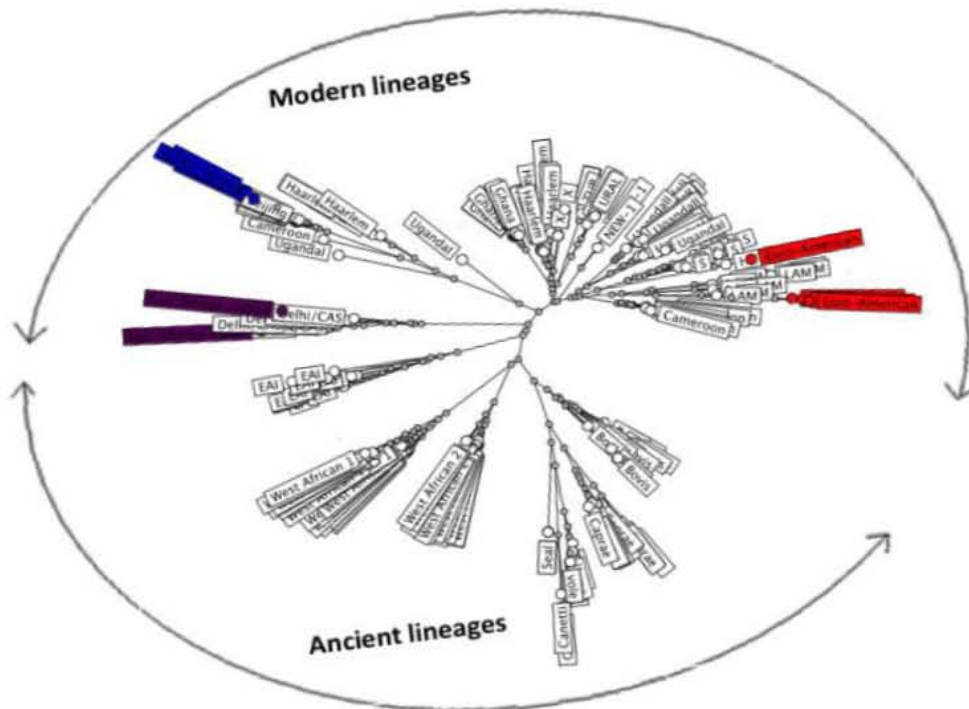
3.3.1 Selection of *M. tuberculosis* strains

We selected strains of *M. tuberculosis* from a previously described collection of paediatric isolates from the broader Cape Town region of South Africa¹⁸⁸. Strains from children with tuberculosis are likely to be representative of circulating *M. tuberculosis* strains in the region, as childhood tuberculosis classically follows exposure to an infectious adult source case. It should be noted that these strains could have been conditioned differentially in their respective hosts when compared to strains derived from adults. Isolates were initially cultivated using BACTEC MGIT960 automated liquid culture and grown further in 7H9 Middlebrook Medium prior to genotyping and *in vitro* cell culture assays. Overall, these strains were passaged approximately five times during the study period.

We performed spoligotyping, 15-locus multiple interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) analysis as well as region of deletion (RD) typing (lineage 2 strains only) in order to identify the major modern

strain lineages and strain clusters (Table 3.01). For these studies, all strains selected were from 'modern' lineages of *M. tuberculosis*, as representative strains from 'ancient' lineages were not available (Figure 3.01). We selected strains from each of the most common lineages in this strain collection, including three lineage 2 strains (Beijing, including two RD150/RD181 and one RD181 deleted strain) and three lineage 4 strains (Euro-American, belonging to the LAM3/F11 subfamily). In addition, we selected two lineage 3 (CAS) strains (the previously described CH strain, as well as a second CAS strain from the Cape Town paediatric collection).

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Lineage 4 (Euro-American)

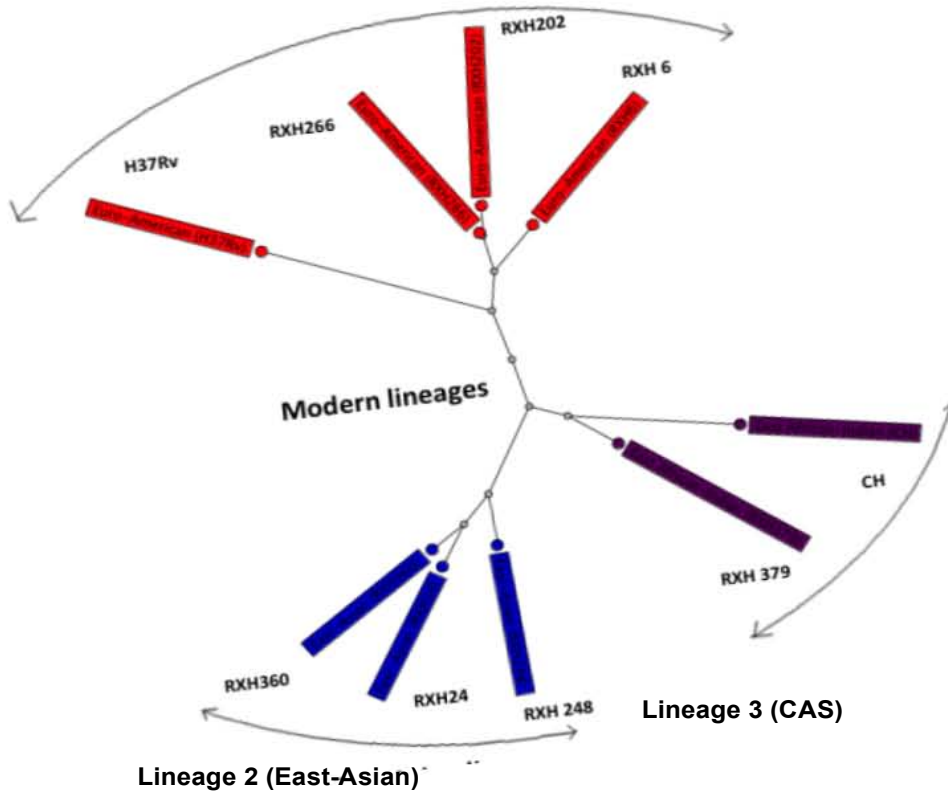


Figure 3.01 Phylogenetic relationships of selected *M. tuberculosis* strains in radial trees based on 15 loci MIRU-VNTR and spoligotyping.

Table 3.01 Genotypes of selected *M. tuberculosis* strains used in phenotypic studies.

Strain	MIRU-VNTR	Spoligotype	Lineage
H37Rv	243132253233552	777777477760771	Lineage 4, Laboratory strain
RXH248	442335464485372	00000000003771	Lineage 2, Beijing, RD150/RD181 deleted
RXH24	442345553575482	00000000003771	Lineage 2, Beijing, RD181 deleted
RXH360	442325554475472	00000000003771	Lineage 2, Beijing, RD150/RD181 deleted
CH	522355424275384	703777740003731	Lineage 3, CAS2
RXH379	4423664?? 285373	702777740003771	Lineage 3, CAS1
RXH266	442344442253172	774177407760771	Lineage 4, LAM3
RXH202	442344442253172	776177607760740	Lineage 4, LAM3
RXH6	442365542253173	774377007760771	Lineage 4, LAM3

3.3.2 Preparation of mycobacterial culture

3.3.2.1 Culturing of *M. tuberculosis* strains

To ensure a synchronous growth phase amongst all strains, *M. tuberculosis* strains were grown at 37°C in a shaking incubator (120 rpm) to mid log phase (0.6 to 0.9 OD) in Middlebrook 7H9 broth (Difco, USA) containing 0.2% glycerol, 0.05% Tween 80, and 10% albumin-dextrose-catalase (ADC) growth enrichment (BD, USA). Stock vials of *M. tuberculosis* culture from different strains were supplemented with 15% glycerol and preserved in multiple aliquots at -80°C for growth assays. All experimental procedures with viable tuberculosis strains were carried out in a biosafety cabinet in a biosafety level III laboratory facility.

3.3.2.2 Standard stock culture preparation

The total standard colony forming unit (CFU) concentration of the stock vial was calculated by serial dilution and plating in multiple replicate on Middlebrook 7H11 agar (Difco, USA) containing 0.5% glycerol and 10% Oleic acid-albumin-dextrose-catalase (OADC) growth enrichment (BD, USA). A new vial was thawed before each experimental procedure. The colony forming unit (CFU) content of an aliquot of each stock was re-confirmed during each experiment by plating on Middlebrook 7H11 agar.

3.3.3 Axenic growth assay

3.3.3.1 *M. tuberculosis* growth in liquid 7H9 broth

For axenic growth assays, frozen stock of known CFU concentration were freshly thawed and multiple replicates of 10^5 CFU/ml of bacilli of *M. tuberculosis* were inoculated into 10 ml pre-warmed 7H9 Middlebrook broth supplemented with 0.2% of glycerol, 0.05% Tween 80, and 10% ADC growth enrichment (BD, USA) and cultured in a shaking incubator (120rpm) at 37°C for 360 h.

3.3.3.2 Quantification of *M. tuberculosis* growth in axenic culture

Increase in bacterial number in liquid broth was evaluated at different time intervals. Aliquots were withdrawn at intervals and diluted up to six fold in Phosphate Buffered Saline (PBS). Bacterial clumping was minimized by thorough repetitive pipetting of diluted samples. Serial dilutions were plated in triplicate onto Middlebrook 7H11 agar plates containing 10% OADC growth enrichment and incubated at 37°C. Colonies on plates were enumerated after 3 weeks of incubation and results are expressed as \log_{10} CFU per millilitre.

3.3.4 Intracellular growth assay

3.3.4.1 Ethical considerations

The study was conducted with ethical approval from the Faculty of Health Sciences Research Ethics Committee, University of Cape Town (REC REF 261/2008). The healthy donors buffy coats were collected from South African Blood Transfusion Services.

3.3.4.2 Monocyte isolation, culture, and maturation to monocyte derived macrophages (MDM)

MDM were prepared from healthy donor buffy coats supplied by South African Blood Transfusion Services. Peripheral blood mononuclear cells (PBMC) were separated by centrifugation over Ficol-paque Plus (Pharmacia, Uppsala, Sweden). Cells were washed in RPMI, pooled, and counted. Cells were incubated in a large tissue culture flask (175 cm²) at 300×10^6 PBMC per 25 ml RPMI per flask for 2 h at 37°C in 5% CO₂. This step allows monocytes to adhere to the bottom of the flask. Non-adherent cells were removed by three washes with 10 ml of pre-warmed RPMI medium. Finally, 10 ml of ice-cold phosphate buffered saline (PBS) was added and the flask incubated at 40°C for 20 minutes. Using a long handled scraper, monocytes were dislodged from the bottom of the flask and pooled in a 50 ml falcon tube for counting. Cells were plated in R10 medium (RPMI containing 10% foetal calf serum (FCS)) at a concentration of 2×10^5 cells/well in a 96 well tissue culture plate and cultured at 37°C, in 5% CO₂, for 6 days in R10 medium.

3.3.4.3 MDM infection with *M. tuberculosis* strains and intracellular growth assay

For the intracellular growth assay, frozen *M. tuberculosis* stock were freshly thawed and reconstituted at room temperature at the time of infection. Before infection, each stock vial was plated for CFU enumeration to re-confirm the CFU content of the inoculum. Adherent MDM were co-cultured in duplicate with bacilli at a 1:1 ratio in R10 medium (RPMI+ non-heat inactivated 10% FCS). After 4 h of incubation, extracellular bacteria were removed gently by washing four times with pre-warmed PBS and replenished with fresh R10 medium. We confirmed removal of extracellular bacilli from the infected MDM by plating the washing buffer from the last wash onto 7H11 agar plates.

3.3.4.4 CFU analysis from infected MDM lysates

After 4, 24, 48 or 96 h of infection, MDM from each of the duplicate wells were subjected to complete lysis by mixing with 100 µl of 0.1% SDS and incubated at room temperature for 12min. Lysates were mixed thoroughly ten times, serially diluted (10^{-1} to 10^{-6}), and plated, in triplicate, on 7H11 agar plates. Plates were incubated for 3 weeks at 37°C and CFU enumerated using an inverted microscope.

3.3.4.5 Intracellular growth assay in IFN-γ stimulated MDM

Monocytes were isolated from buffy coats and matured into macrophages as described above. 24h prior to infection mature macrophages were pre-stimulated with R10 medium containing 100 U/ ml of recombinant human IFN-γ (BD Pharmingen). Each experiment was set up with an IFN-γ unstimulated control. Infection of macrophages was then performed as above. At the 4h time point, extracellular bacteria were removed by washing with pre-warmed PBS and each IFN-γ stimulated well was replenished with R10 medium containing 100 U/ ml of

IFN- γ . Unstimulated control wells were replenished with fresh pre-warmed R10 medium. After 4, 24, 48 and 96 h, infected MDM were lysed for CFU enumeration as described above.

3.3.4.6 MTT assay

The cytotoxicity of IFN- γ treatment of monocyte derived macrophages was determined by MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. Cytotoxicity is measured by reduction of the yellow tetrazolium salt of MTT to purple formazan by mitochondrial enzymes of living cells. 2×10^5 monocytes were plated onto 96-well flat bottom plate and cultured to form MDM using R10 medium for 6 days at 37^o C and 5% CO₂. Monocyte derived macrophages (MDM) were pre-treated with 100 U/ml of IFN- γ at 24h prior to MTT assay. The remaining IFN- γ treated wells were washed off after 24 h of incubation and the well replenished with fresh R10 media containing 100 U/ ml of IFN- γ . A control well, not treated with IFN- γ , was set up for each test well. At 24h, 48h and 96h 10 μ l of MTT (5mg/ml) was added into each well, followed by incubation at 37^oC for 2 h. Purple formazan was solubilized in 0.04N HCl in isopropanol, incubated on a shaker for 30 min and absorbance read at 570nm.

3.3.5 Statistical analysis

Graphpad Prism 5.00 was used for statistical analysis of intracellular growth in monocyte-derived macrophages. The intracellular growth index in MDM infection was compared between different groups using the unpaired t test for each time point. One-way ANOVA was used to compare the growth in MDM to measure the effect of IFN- γ . Dunnett's multiple comparison test was used for post-test

comparison. Axenic growth curve analysis was performed with one-way ANOVA and Bonferroni's post hoc test.

3.4 Results

3.4.1 *M. tuberculosis* growth in broth

M. tuberculosis strains belonging to different *M. tuberculosis* lineages were grown in liquid 7H9 Middlebrook broth to compare the growth rate of different lineages (two independent cultures, each performed in triplicate) (Figure 3.02 & 3.03 A, B). We used C_{\max} and T_{\max} to compare growth of various strains. C_{\max} is defined as the peak point on the bacterial growth curve (maximum number of colony forming units) whilst T_{\max} is the time required to reach C_{\max} . H37Rv grew to a higher C_{\max} than clinical strains (Table 3.02). Lineage 4 strains showed a higher C_{\max} than clinical strains from other lineages. (Figure 3.03 C) Lineage 2 strains showed an intermediate level of growth whilst lineage 3 strains had the lowest C_{\max} [C_{\max} , H37Rv vs. lineage 4 /lineage 2 / lineage 3 all $p < 0.0001$; lineage 4 vs. lineage 2 $p = 0.0024$; lineage 4 vs. lineage 3 $p = 0.0005$]. T_{\max} for H37Rv was 288 h. Lineage 3 strains showed significantly shorter T_{\max} than H37Rv ($p < 0.05$) (Figure 3.03D). The replicates of dilutions gave very reproducible results; suggesting that minimal clumping occurred during these assays.

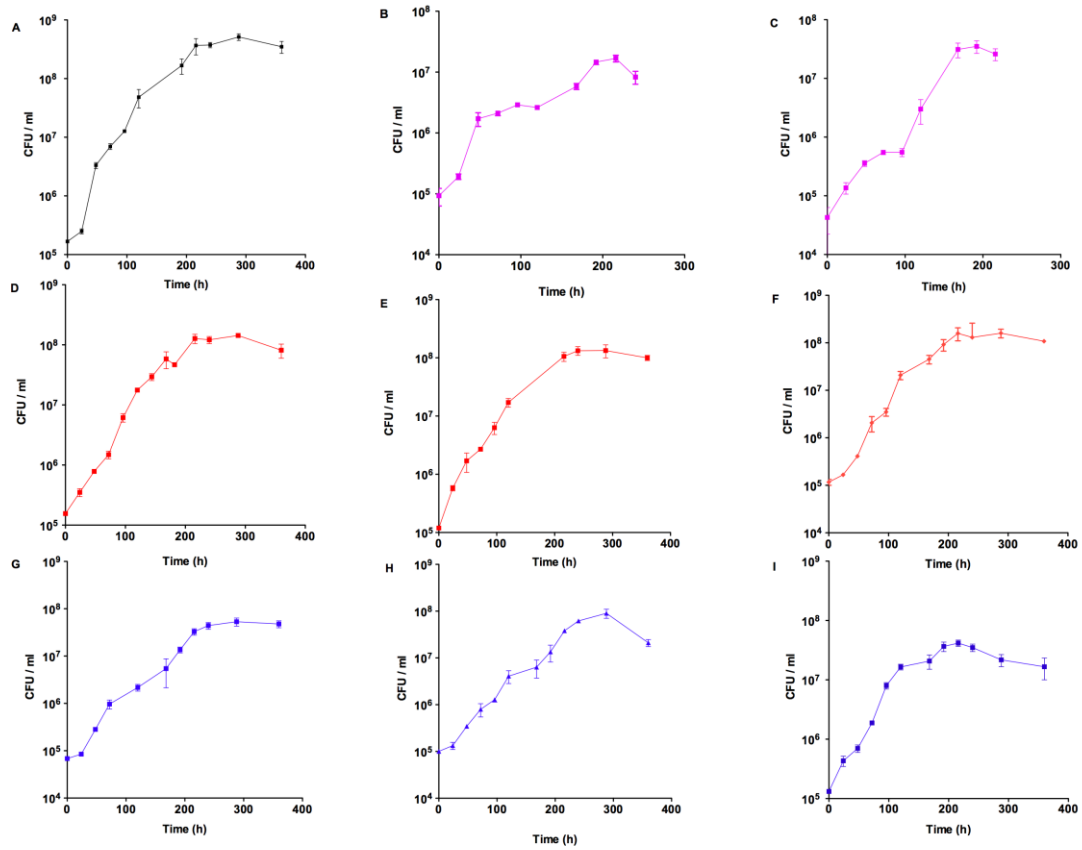


Figure 3.02 Growth of *M. tuberculosis* strains in liquid broth. (A, B, C) H37Rv; Lineage 3 strains (purple): CH and RXH379; (D, E, F) Lineage 4 strains (Red): RXH202; RXH266; RXH6; (G, H, I) Lineage 2 strains (Blue): RXH248; RXH360; RXH24. Each data point represents the mean and range (n=2) values.

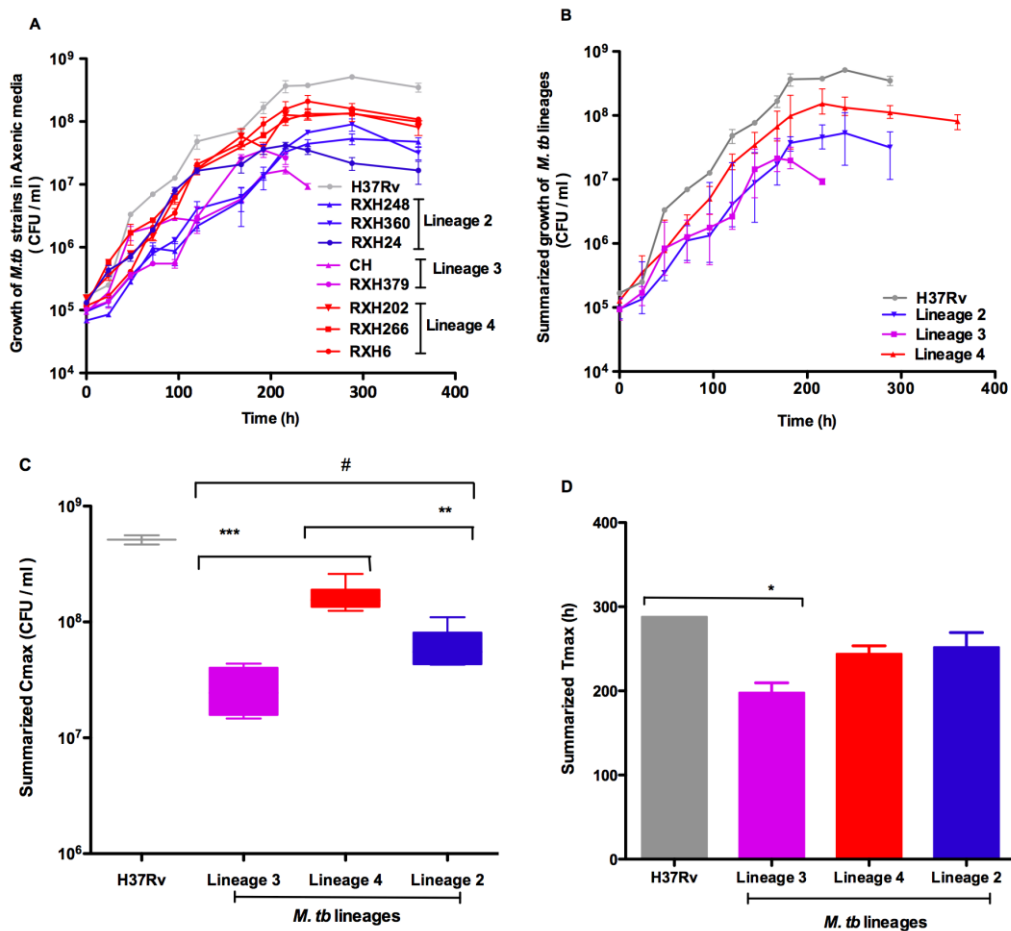


Figure 3.03 Growth of *M. tuberculosis* strains in liquid 7H9 broth. (A) Growth of individual *M. tuberculosis* strains from different lineages in liquid 7H9 broth. Data represent the mean and range (n=2) values. **(B)** Summarized (mean) growth of *M. tuberculosis* strain lineages. **(C)** Comparison of C_{max} (maximum point on the growth curve) for different strain lineages. Lineage 4 strains showed higher C_{max} than lineage 2** (p=0.01) and lineage 3*** strains (p=0.001). H37Rv showed significantly higher C_{max} than all clinical strains# (p=0.001) **(D)** Comparison of T_{max} (time to reach to C_{max}) for different strain lineages. H37Rv reached T_{max} significantly faster than lineage 3 strains* (p=0.05).

Table 3.02 Strain wise comparison of *M. tuberculosis* growth in axenic media based on C_{max}[#] and T_{max}^{##} values.

Strain	Lineage	C _{max} (CFU / ml)	T _{max} (h)
H37Rv	Laboratory strain	5.14 ± 0.68 x 10 ⁸	288
RXH24	Lineage 2	4.50 ± 0.02 x 10 ⁸	204
RXH248	Lineage 2	5.33 ± 1.4 x 10 ⁷	288
RXH360	Lineage 2	9.07 ± 2.7 x 10 ⁷	264
CH	Lineage 3	1.69 ± 0.34 x 10 ⁷	216
RXH379	Lineage 3	3.68 ± 0.97 x 10 ⁷	180
RXH202	Lineage 4	1.44 ± 0.082 x 10 ⁸	240
RXH266	Lineage 4	1.46 ± 0.29 x 10 ⁸	252
RXH6	Lineage 4	2.10 ± 0.70 x 10 ⁸	240

C_{max}: maximum number of colony forming units, ## T_{max}: Time required to reach C_{max}. Data represent mean ± SD obtained from two separate growth experiments.

3.4.2 *M. tuberculosis* growth in monocyte derived macrophage

We used the MDM infection model to evaluate differences in intracellular growth in MDM from different donors (n= 7). We determined initial strain uptake (after 4 h) by expressing the number of CFU at the 4 h time point from MDM lysates as a percentage of the initial inoculum (Figure 3.04). H37Rv showed lower strain uptake by MDM than the clinical strains tested (p <0.001). There were no significant differences in strain uptake between clinical strains.

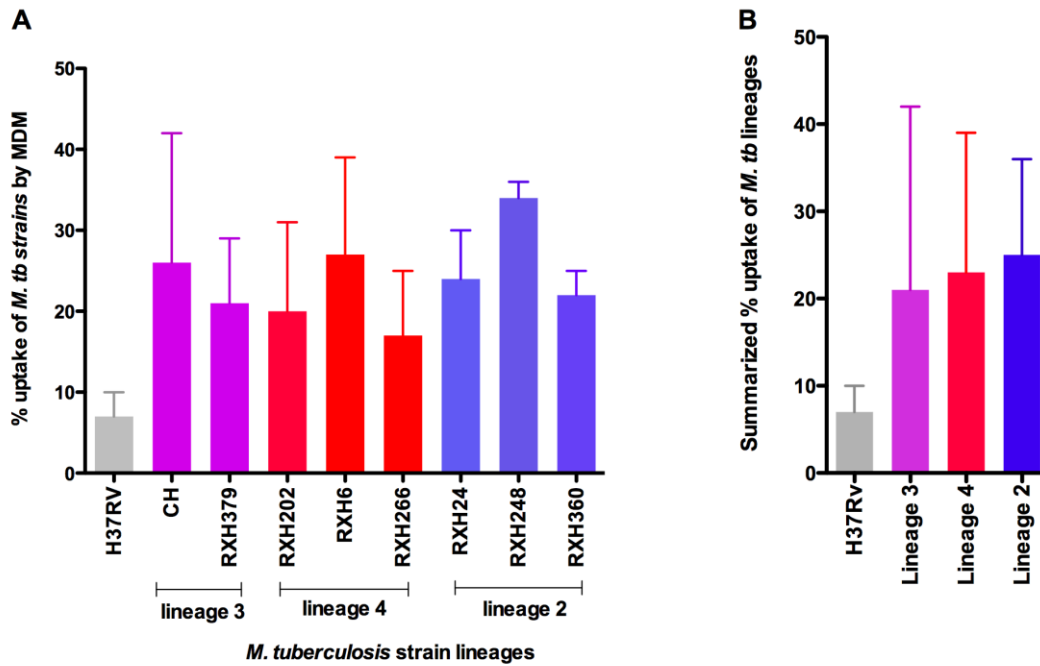


Figure 3.04 Strain uptake by monocyte derived macrophages (A) Uptake of *M. tuberculosis* strains by MDM (4h CFU calculated as a percentage of initial inoculum). **(B)** Summarized uptake of *M. tuberculosis* strain lineages by MDM. H37Rv showed significantly lower uptake by the than clinical strains[#] ($p < 0.001$). Data represent the median \pm range ($n = 7$).

To compare intracellular growth amongst different strain lineages, we determined a growth index, calculated from the \log_{10} of number of CFU at each time point divided by the \log_{10} of number of CFU at the 4h time point Figure 3.05. Growth of all clinical strains was slow for the first 24 h. Lineage 2 and lineage 4 strains grew faster over the first 48 h than lineage 3 strains (Lineage 3 vs. Lineage 4: $p < 0.01$, Lineage 3 vs. Lineage 2: $p < 0.001$). Lineage 2 strains grew significantly faster than lineage 3 strains ($p < 0.01$) over 96 h. The mean growth index of lineage 4, lineage 2, lineage 3 and H37Rv was 1.19 ± 0.22 , 1.25 ± 0.17 , 1.11 ± 0.10 and 1.69 ± 0.13 respectively over 96 h. (Figure 3.05 A&B). Despite low initial uptake, H37Rv multiplied more rapidly in MDM than clinical strains [H37Rv vs. lineage 2 and lineage 4: $p < 0.001$ at 24h and 48 h, H37Rv vs. lineage 3: $p < 0.001$ at all-time points]. As an alternative approach to compare the growth rate amongst these

strains, we also determined the doubling time of these strains. Doubling time was calculated based on the mean number of CFU at 4 h and 96 h (Table 3.03). The mean doubling time of lineage 2 strains was 23.34 ± 3.0 h, lineage 4 strains 25.10 ± 4.67 h, lineage 3 strains 26.14 ± 2.45 h and H37Rv 17.12 ± 1.61 h (mean \pm SD) over the 96 h time period.

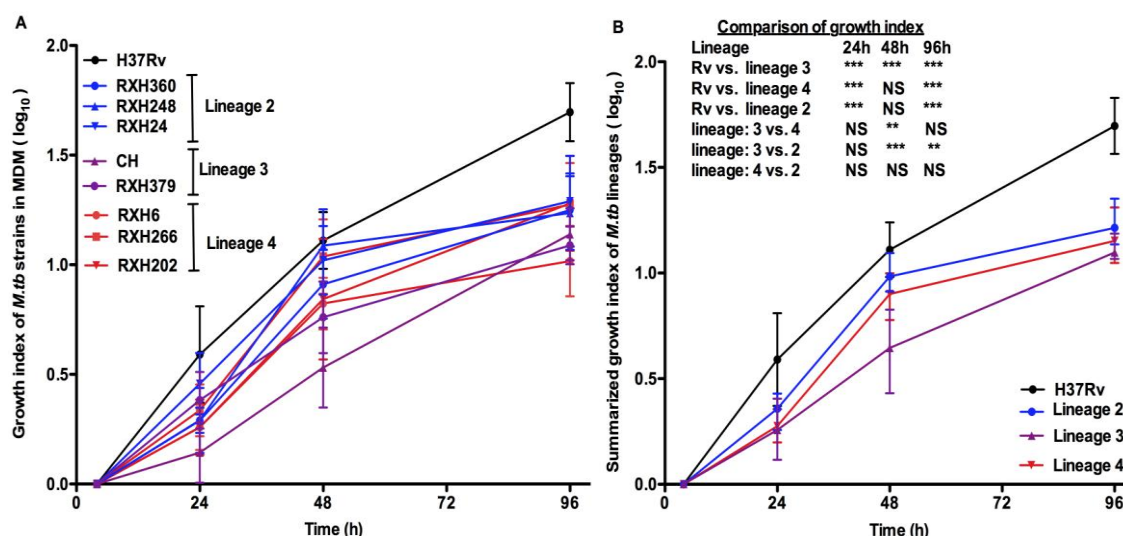


Figure 3.05 Growth index (calculated by the CFU at each time point divided by the CFU at initial time point) reflecting the intracellular growth of *M. tuberculosis* strains in MDM (A) Growth index of *M. tuberculosis* strains. (B) Summarized growth index of *M. tuberculosis* strain lineages. The data represents the mean \pm SD (n=7). Statistical significance is denoted as *p<0.05, **p<0.01, ***p<0.001.

Table 3.03 Doubling time⁺ for *M. tuberculosis* strains from different lineages in human monocyte derived macrophages

Strain	Lineage	24hrs	48hrs	96hrs
H37Rv	Laboratory strain	13.71 \pm 2.17	13.16 \pm 1.44	17.12 \pm 1.61
RXH24	Lineage 2	17.23 \pm 2.59	14.41 \pm 1.59	22.85 \pm 2.11
RXH360	Lineage 2	38.99 \pm 13.2	16.31 \pm 1.82	23.47 \pm 2.11
RXH248	Lineage 2	25.78 \pm 2.71	13.52 \pm 1.53	23.72 \pm 2.10
CH	Lineage 3	44.97 \pm 8.9	29.79 \pm 3.75	25.60 \pm 2.09
RXH379	Lineage 3	20.93 \pm 3.05	19.72 \pm 2.18	26.69 \pm 2.08
RXH202	Lineage 4	35.14 \pm 7.45	18.92 \pm 2.8	23.14 \pm 2.26
RXH266	Lineage 4	24.48 \pm 4.08	14.21 \pm 1.57	23.03 \pm 2.05
RXH6	Lineage 4	36.37 \pm 9.91	17.66 \pm 1.81	29.03 \pm 2.59

*Doubling times $G = t/3.3 \log (b/B)$ (t= time, B= initial colonies, b= final colonies). Results represent mean \pm S.E from n=7 donors.

3.4.3 Effect of pre-stimulation with IFN- γ on growth of *M. tuberculosis* in

MDM

We evaluated the antimyobacterial effect of IFN- γ when used to pre-stimulate human monocyte derived macrophages prior to infection with selected strains. MDM were isolated from three healthy donor buffy coats supplied by South African Blood Transfusion Services. These donors were anonymous and they were not from the subset of the earlier 7 donors as mentioned in the section 3.4.2. Monocytes were isolated as described above and matured to macrophages for 6 days. MDM (n=3 donors) were pre-stimulated with 100 U/ml of recombinant human IFN- γ for 24h prior to infection. As shown in Figure 3.06 (A-C) there was a small but consistent decrease in intracellular growth of *M. tuberculosis* strains in IFN- γ treated vs. untreated MDM. However, there was no clear difference between strains in this regard. Reduction in growth could potentially be attributed due to reduction in cell viability in MDM treated with IFN- γ (43). We tested cell viability by MTT assay. The Figure 3.06 showed that cells in control wells demonstrated an increase in OD at the 96h time period MDMs are generally non-proliferative but can undergo further differentiation with prolonged culture into differentiated macrophages (eg. M1 and M2) with associated change in size and morphology²⁴⁷ which may explain this finding.

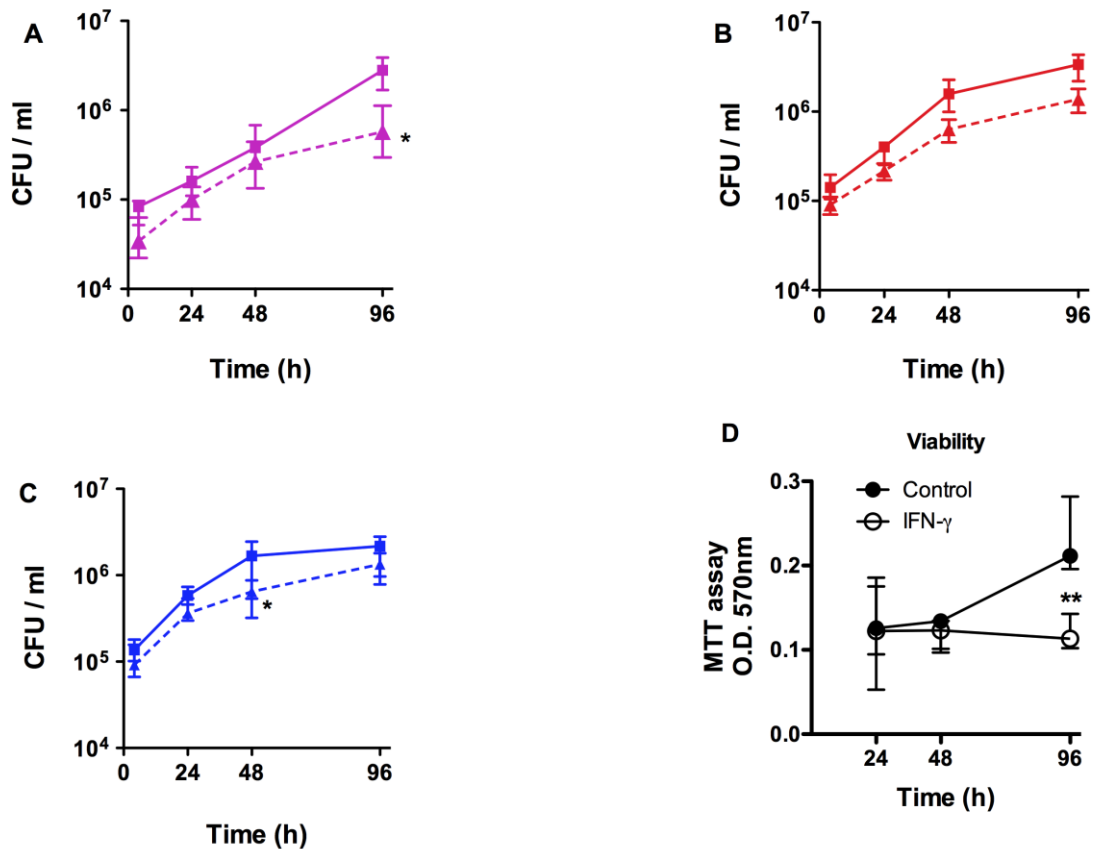


Figure 3.06 The effect of recombinant IFN- γ on the growth of different strains of *M. tuberculosis* in human MDM [Without IFN- γ (solid line) and with IFN- γ (dotted line)]. Six day old monocyte derived macrophages were pre-treated with 100 U/ ml of IFN- γ 24 h before infection. MDM were infected with different strains of *M. tuberculosis* with MOI 1 [2×10^5 MDM (10^6 cells/ ml) infected with 2×10^5 CFU (10^6 CFU /ml) of bacilli]. **(A)** H37Rv; **(B)** RXH202 (LAM3); **(C)** RXH24 (Beijing). The data represent median and interquartile range (n=3). **(D)** Viability of MDM measured by MTT assay: MDM were treated with 100 U/ ml of IFN- γ . The data represent the O.D. at 570nm (median and range) for two individual donors in triplicate. Level of statistical significance is denoted as * (p<0.05), ** (p<0.01), *** (p<0.001).

3.5 Discussion

We compared lineage-specific *in vitro* growth phenotypes (mycobacterial growth in liquid broth and intracellular growth in MDM) of the two prevalent strain lineages (lineage 2 and 4) from the Western Cape region of South Africa with those of the laboratory strain H37Rv as well as strains from lineage 3.

In this study, the laboratory-reference strain H37Rv grew most rapidly in liquid culture, suggesting that H37Rv is highly adapted to laboratory media. However it is relevant to note that strains of H37Rv used in different laboratories may differ both phenotypically and genotypically²⁴⁸. Interestingly, lineage-specific differences in growth patterns in axenic media were observed, with lineage 4 strains reaching a higher C_{max} than the other two lineages and lineage 3 strains peaking early at a lower C_{max} . Lineage 2 strains had an intermediate phenotype. Our results are in lined with previous findings, which showed slow growth of Lineage 3 strains in 7H9 broth^{138, 201, 237}. An outbreak lineage 3 strain, CH, grew slowly in axenic media⁴¹. Tanveer *et al.* found that a CAS1 (lineage 3) strain grew more slowly in liquid broth than a Beijing strain and H37Rv²⁰. Kanji *et al.* further demonstrated that a CAS1 strain with the RD149 deletion grew more slowly than H37Rv and a CAS1 strain with the double deletion RD149 & RD152⁴⁰.

Several previous studies have assessed intracellular growth of *M. tuberculosis* in human macrophages as a marker of virulence^{132, 249, 250}. Different strains may vary in their capacity to invade macrophages. We noted lower initial uptake of H37Rv when compared with clinical strains. There is evidence of variation in phagocytosis of different strains²⁵¹. For example Schlesinger *et al.* found that phagocytosis of two virulent strains (H37Rv and Erdman) by human monocyte derived macrophages is mediated by the mannose receptor in addition to complement receptor (CR1, CR3 and CR4) whereas the avirulent strain H37Ra uses only complement receptor for phagocytosis²⁵². Hence, higher initial bacterial uptake by clinical strains (compared with H37Rv) may reflect differential expression of pathogen-associated molecular patterns (PAMP), which are recognised by innate immune receptors; however this was not specifically addressed by these studies.

We determined the growth index, rather than absolute growth, which expresses the growth of all strains irrespective of their variable uptake. We observed that lineage 2 and lineage 4 strains grew more rapidly in MDM than lineage 3 strains between 24 and 48h but that growth slowed over the next 48 h. H37Rv grew more rapidly than all clinical strains over the first 24 h as well as over 96h. Rapid entry into the phagosome through receptor mediated uptake may trigger faster downstream events such as endosomal sorting inside the endosomal machinery, which in turn may accelerate the early biogenesis of phagosome and disease progression²⁵³. We noted differences in mycobacterial doubling time, which was variable over the time period [Doubling time was calculated by the formula $G = t/3.3 \log (\text{initial colonies} / \text{final colonies})$, t= time]. Torreles *et al.* also reported that doubling time of *M. tuberculosis* strains in MDM differs over time, which supports our findings²⁵¹. However, there were no significant differences in doubling time between clinical strains over the full 96 h period, suggesting that there do not appear to be major differences between modern lineages in this regard.

A growing body of literature suggests that individual strains from lineage 2 (Beijing) grow rapidly in *in vitro* human cell culture models^{132, 203, 204}. For example, Li *et al.* found several Beijing strains grew faster in human monocytes than non-Beijing strains¹⁸. However, there are differences between lineage 2 strains in this regard²⁰⁵. Epidemiological evidence suggests that lineage 2 has recently emerged in the Cape Town region of South Africa¹²¹. Our findings show similar growth rates for the two successful strain lineages (lineages 2 and 4) in the region, which grew more rapidly over 48 h than lineage 3. Other recent reports have shown that lineage 3 strains are slow growers in the THP1 monocytic cell line when compared to H37Rv^{201, 237}, which supports our findings.

The successful intracellular survival of *M. tuberculosis* within macrophages has been attributed to various virulence determinants such as up regulation of principal sigma factor A (*Sig A*), which was found to enhance the growth of a lineage 2 strain²⁵⁴. Further, phenolic glycolipid (PGL) was shown to modulate the pro-inflammatory cytokine response, causing enhanced survival of a lineage 2 strain in mice and monocytes¹³⁷. Accumulation of triacylglycerol within the cytoplasm of lineage 2 strains may provide an additional energy source during infection, perhaps affording a survival advantage during intracellular infection¹³⁹. In contrast, induction of a vigorous pro-inflammatory cytokine response by a lineage 4 strain was reported to restrict its survival in mice and in monocytes¹³³. A genetic polymorphism in *Rv 1519* was associated with suppressed IL-12 induction and induction of increased IL-10 in a lineage 3 outbreak strain, which, although it demonstrated impaired growth in axenic media, was not attenuated in the MDM model⁴¹. Man-LAM, found in virulent strains of *M. tuberculosis*, antagonises mannose receptor-dependent IL-12 production⁶² and induces an anti-inflammatory response, which enhances survival of tubercle bacilli inside macrophages^{62, 66}.

The early control of bacterial growth in tuberculosis infection relies on the activation of phagocytic cells, which is regulated by cytokine secretion. Various pro-inflammatory cytokines (such as TNF, IFN- γ , GM-CSF and M-CSF) are considered as an important mediators of macrophage activation^{17,255}. Mutations in the IFN- γ receptor are associated with disseminated *M. tuberculosis* infection²⁵⁶. Exogenous administration of IFN- γ has improved clinical outcome of drug resistant pulmonary tuberculosis patients²⁵⁷. IFN- γ stimulation has been shown to up regulate MHC class II expression; augment cytokine secretion and production of

nitric oxide (NO) in mice. The anti-mycobacterial property of NO in macrophages has been well documented in mice^{222, 258}, however, the role of iNOS in human *M. tuberculosis* infection remains controversial²⁵⁹⁻²⁶². There is evidence of expression of NOS2 in macrophages from tuberculosis bronchoalveolar lavage specimens^{263, 264}. Furthermore, there is evidence of NO production in human monocytes, macrophages, and a human monocytic cell line when infected with *M. tuberculosis*^{265, 266}. However, previous findings have demonstrated that a NO-independent TLR2 pathway mediates antimicrobial activity in human macrophages⁴². In contrast, a recent study by Lee *et al.* demonstrated that combined treatment with Vitamin D (1,25 D3) and IFN- γ augmented NO synthesis and iNOS expression in monocyte-derived macrophages, which was mediated by NO-dependent TLR2 pathways²⁶⁷. The early literature has conflicting reports on the effect of exogenous IFN- γ on the ability of human macrophages to *kill M. tuberculosis*, with some of these studies describing increased killing²⁶⁸ and others enhanced growth of *M. tuberculosis*²⁶⁹. The anti-microbial activity of IFN- γ is likely to be dependent on its concentration and timing of administration. Stimulation with higher concentrations of IFN- γ could be harmful during MDM differentiation. We noted a slight, but consistent decrease in *M. tuberculosis* growth in MDM, pre-stimulated with IFN- γ . There was no clear association between the effect of IFN- γ and the genotype of the infecting strain. We did not measure the NO level during infection. Since only two clinical strains and one laboratory strain were tested these findings should not be extrapolated more broadly.

MDMs are generally non-proliferative but it could undergo further differentiation into different macrophages (eg. M1 and M2) with changes in its size and morphology²⁴⁷. We have plated 2×10^5 cells in replicates in each well and observed

under microscope as generally advised prior to MTT assay. The data in control wells were in replicates and reproducible, suggest an increase in OD during MTT assay due to increase in formazan formation. Cells in the control wells were supplemented with RPMI and foetal calf serum (FCS) and cells were healthy and happily growing cells. In presence of nutrient medium cells in the control well might be further differentiated and changed in its morphology and size. Healthy cells in control wells could produce high amount of mitochondrial enzyme dehydrogenase, which might produce more formazan in 96 h time period. IFN- γ treated cells were activated and highly sensitised. Evidence suggests that MDM activation with pro-inflammatory (including IFN- γ) and anti-inflammatory molecule showed reduction in cell viability. Therefore, the decrease in OD observed in IFN- γ treated wells could be due to reduction in cell viability. Furthermore, IFN- γ is cytotoxic in nature and prolong activation might cause damage in cells. Previous studies showed that immunomodulator could sensitize the MDM such an extent that monolayer get destroyed causing death of the cells²⁶⁹⁻²⁷¹. Therefore, it is not clear to what extent the decrease in cell viability contributed to the effect of IFN- γ on mycobacterial growth.

There are a number of limitations of this *in vitro* model. Short term growth in macrophages only partially reflects the more lengthy and complex pathogenesis in humans, we were unable to distinguish bacterial uptake from simple adherence to macrophages and PBMC-derived macrophages differ phenotypically from alveolar macrophages²⁷². Rapid growth in macrophages may not necessarily represent a virulence characteristic²⁰⁶. Furthermore, due to the limited number of MDM available, we were only able to compare a few strains from each lineage. All of these strains (except CH) were isolated from South African patients, and only

represent specific sub-lineages. They do not therefore represent the global diversity of 'modern' TB strains.

In summary, we have demonstrated that the laboratory reference strain grew faster than clinical strains and that lineage-specific difference in growth patterns in axenic media were observed, with lineage 4 strains reaching a higher C_{max} than the other two lineages and lineage 3 strains peaking early at a lower C_{max} . Lineage 2 strains had an intermediate phenotype. We also observed initial uptake of H37Rv by MDM was lower as compared to clinical strains but that it grew faster in MDM than the clinical strains. Moreover, selected lineage 2 and lineage 4 strains grew faster in MDM than the lineage 3 strains. Pre-stimulation of IFN- γ resulted in an overall decrease in bacterial growth but no clear difference was observed between lineages.

Chapter 4

Induction of cytokine responses by *M. tuberculosis* strains from modern lineages

University of Cape Town

4.1 Introduction

Cytokines, potent mediators of body's immune response, are produced by different immune cells (such as macrophages, dendritic cells and T cells) following infection with *M. tuberculosis*^{74, 75, 89}. Cytokines regulate the immune response and also have effector functions. They play a key role in control of tuberculosis infection and serve as a marker of severity of tuberculosis infection^{75, 108, 204, 273, 274}. Several investigators have suggested that suppression of protective cytokine responses by *M. tuberculosis* is a key pathological mechanism in disease²⁷⁵⁻²⁷⁸.

Human cytokine levels can be measured in tuberculosis infection using *in vivo*²⁷⁹ (in lungs or in circulation) or *ex vivo* model systems (in isolated cells)^{133, 216, 280, 281}. *In vitro* studies have measured human cytokines following infection with *M. tuberculosis* using whole blood assays^{219, 282, 283}, primary cell isolates (mononuclear cells separated from peripheral blood mononuclear cells [PBMC])^{136, 284} or using commercially available monocytic cell lines such as THP-1^{201, 204}. Key cytokines involved in the control of tuberculosis infection are produced by mononuclear phagocytes (including tumor necrosis factor (TNF), interleukin-12 (IL-12), interleukin-1 (IL-1) interleukin-10 (IL-10) and interleukin-6 (IL-6))^{14, 285}.

The Th1 cytokine response is important in control of tuberculosis infection²⁸⁶ and IL-12 plays a crucial role in inducing a protective IFN- γ (Th1 response) response, which is associated with activation of macrophages to kill intracellular *M. tuberculosis*^{34, 287}. The key role of TNF in protective immunity in tuberculosis infection, and in maintaining granuloma formation, is demonstrated by studies showing reactivation of latent tuberculosis infection following use of therapeutic monoclonal antibodies directed against TNF²⁸⁸. TNF is involved in the

inflammatory response, macrophage activation, chemokine secretion and recruitment of monocytes, neutrophils, and lymphocytes to the infection site to form the granuloma^{14, 76, 77}.

In animal models, high levels of pro-inflammatory cytokines have been associated with increased survival of mice when infected with different *M. tuberculosis* strains^{130, 135}. For example, CDC1551 (a lineage 4 strain) has been shown to induce vigorous cytokine response in mice resulting in prolonged survival of mice when compared to lineage 2 (Beijing) strains such as HN878 and HN60, which fail to induce strong pro-inflammatory responses¹⁴. In *in vitro* macrophage models of human infection, reduced levels of pro-inflammatory cytokine secretion have been associated with higher intracellular growth rates^{138, 204, 250}.

Several *in vitro* studies have described diverse cytokine responses induced by various genotypes of *M. tuberculosis*^{136, 137, 201, 204, 216, 219, 280, 289}. For instance the outbreak strain CDC1551 (lineage 4 strain, X family strain) induced rapid and high levels of pro-inflammatory cytokines such as TNF, IL-6 and IL-12¹³³. Another outbreak strain, CH (lineage 3 strain, CAS strain), was found to produce less protective IL-12p40 and more anti-inflammatory IL-10 than the reference strain H37Rv²⁸⁴.

In this part of the study, we used strains representative of two predominant lineages from Western Cape region of South Africa lineage 4 and lineage 2, as well as comparator strains from the less prevalent lineage lineage 3. We elected to use human primary monocyte-derived macrophages (MDM) as a model to evaluate early cytokine response in *M. tuberculosis* infection. We were only able to assess two pro-inflammatory cytokines (TNF, IL-12p40) as large numbers of strains were

compared, and the amount of supernatant obtained from the intracellular MDM infection was limited in the 96-well plate format used. These cytokines were selected because of their important role in early tuberculosis infection^{14, 219, 280, 284}.

4.2 Aims of the study

- To determine the time course of TNF and IL12p40 responses human MDM infected with different modern strains of *M. tuberculosis*.
- To determine patterns of TNF and IL-12p40 production by MDM infected with different modern strains of *M. tuberculosis*.

4.3 Methods

4.3.1 Subjects

The experiment was conducted with human primary monocyte derived macrophages (MDM). Monocytes were isolated from healthy donors buffy coats supplied by South African Blood Transfusion Services. The samples were provided without donor identifiers; therefore demographic information was not available. The 6 donors selected to measure IL-12p40 were subset of 8 donors selected to measure TNF response. The study was conducted with ethical approval from the Research Ethics Committee, Faculty of Health Sciences, University of Cape Town (REC REF 261/2008).

4.3.2 *M. tuberculosis* strains

The time course experiment was performed with three lineage 2 strains [(RXH 24, RXH 65, RXH 169) and three lineage 3 strains (CH, CH1519 and CH1996) and H37Rv]. Subsequently we used the different panel of strains as described in Chapter 3 (which includes lineage 4 strains) to perform various growth assays and to investigate cytokine induction at the 48h time point.

4.3.3 Monocyte derived macrophage infection model

Monocytes were isolated from healthy donors, matured to macrophages, and infected with *M. tuberculosis* strains from different modern lineages. Culture supernatants were harvested following post infection at specific time points and stored at -80°C until ELISA was performed. TNF and IL-12p40 levels were measured in filtered sterilized culture supernatant using ELISA (BD Biosciences Pharmingen, San Diego, CA, USA).

We conducted a time course experiment to measure TNF and IL-12p40 at three different time points after infection (24h, 48h and 72h) in which 10^6 cells (n=5) were infected with 10^6 CFU of bacteria in 24 well flat bottom plates. These experiments were conducted with larger numbers of cells than later experiments, where we had refined the technique to utilize smaller cell numbers and allow comparison of more strains. For the comparison of larger numbers of strains, we selected the 48h time point for measurement of TNF and IL-12p40 levels. For these experiments, due to limited numbers of MDM, we used a 96-well format, in which 2×10^5 cells were infected with 2×10^5 CFU of bacteria in 96 well flat bottom plates. The six donors selected in this section to measure IL-12p40 were a subset of the 8 donors selected to measure TNF responses (where sufficient cells were obtained).

4.3.4 Cytokine measurement by ELISA

Sandwich ELISAs were performed to determine cytokine levels in cell supernatants. Microtiter plates were coated with 100 μ l purified capture antibody of IL-12p40 or TNF (BD Pharmingen) diluted in phosphate buffered saline (PBS, pH 9.5) and incubated overnight at 4°C. Plates were aspirated and washed 3 times with 300 μ l/well wash buffer (PBS with .05% Tween 20). Plates were blocked for 1h at room temperature and aspirated and washed three times. Serially diluted 100 μ l purified recombinant IL-12p40 or TNF standards (BD Pharmingen) were used as standard curves. 100 μ l cell supernatants were then added diluted in ELISA assay diluent and incubated for 3h at room temperature. Plates were washed 5 times and 100 μ l of secondary detection antibodies conjugated with Streptavidin-horse radish peroxidase (Save-HRP) for IL-12p40 or TNF (BD Pharmingen) added to each well for 1h at room temperature. ELISA wash buffer was used to wash plates 7 times. Subsequently, plates were incubated with 100 μ l

substrate (tetra-methyl-benzidine), for 10 minutes in room temperature in the dark and the reaction was stopped using stop solution (1M H₃PO₄ or 2 N NH₂SO₄). Absorption was measured at 450 nm using a Bio-Rad spectrophotometer.

4.3.5 Statistical analysis

Graphpad Prism 5.00 was used for statistical analysis of cytokines. The normality of the data was assessed by the D'Agostino & Pearson omnibus normality test. Since cytokine data were not normally distributed, cytokines were compared using the unpaired, non-parametric Mann Whitney test.

4.4 Results

Firstly, in order to determine the optimal time point for TNF sampling, we measured TNF levels at 24 h, 48 h and 72 h post infection of MDM. Secondly, in order to find out whether there was any pattern of cytokine induction characteristic of different *M. tuberculosis* lineages, we investigated TNF and IL-12p40 production at the 48 h time point.

4.4.1 Production of TNF by MDM infected with *M. tuberculosis* strains at different time points

To determine the optimal time point for sampling post infection, supernatant was collected at various time points (24h, 48h and 72h) and cytokine response (TNF and IL-12p40) measured by ELISA (Figure 4.01). MDM infected with lineage 3 strains and the laboratory strain H37Rv induced higher amount of TNF at all the time points compared with lineage 2 (Beijing) strains (H37Rv vs. lineage 2 p=0.001, lineage 3 vs. lineage 2 p= 0.001). IL-12p40 secretion was significantly higher in MDM infected with H37Rv than those infected with lineage 2 strains (p=

0.04) at the 48h time point. Since there was no significant difference in TNF response at different time points and IL-12p40 level was higher in MDM infected with H37Rv at the 48h time point we selected the 48h time point for further experiments.

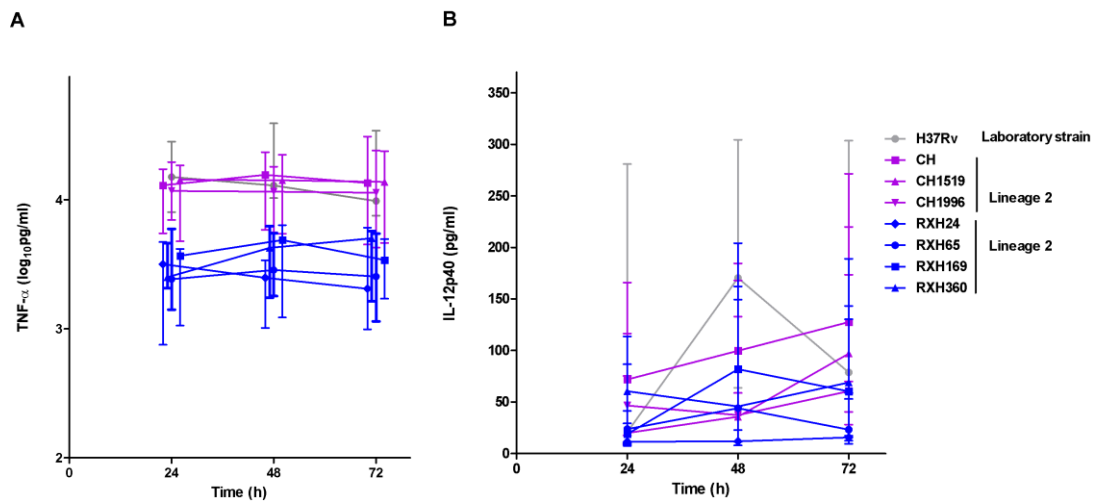


Figure 4.01 Production of TNF and IL-12p40 by MDM infected with *M. tuberculosis* strain lineages at different time points. Data from 5 donors; 10⁶ cells were infected with 10⁶ CFU of bacteria per well. TNF p values: H37Rv vs. lineage2 =0.001, lineage 3 vs. lineage 2 =0.001. Induction of IL-12p40 was significantly higher in MDM infected with H37Rv than the lineage 2 strains (p=0.04) at 48 h time point Data represent in the median and range.

4.4.2 Production of TNF and IL-12p40 by MDM infected with *M. tuberculosis* strains at 48 h

ELISA was performed on supernatant collected at 48 h post infection to measure the release of TNF and IL-12p40 from monocyte-derived macrophages (MDM) following infection with *M. tuberculosis* strains (Figure 4.02). MDM infected with Lineage 2 strains and Lineage 3 strains induced lower levels of IL-12p40 when compared with both H37Rv (H37Rv= 385 ±40 pg /ml, lineage 2= 91 ± 18 pg /ml,

lineage 3 = 123.8 ± 10 pg /ml, median and range, p<0.001) and lineage 4 strains (lineage 4 = 205 ± 15 pg /ml, p<0.001, median and range) (Figure 4.02 A).

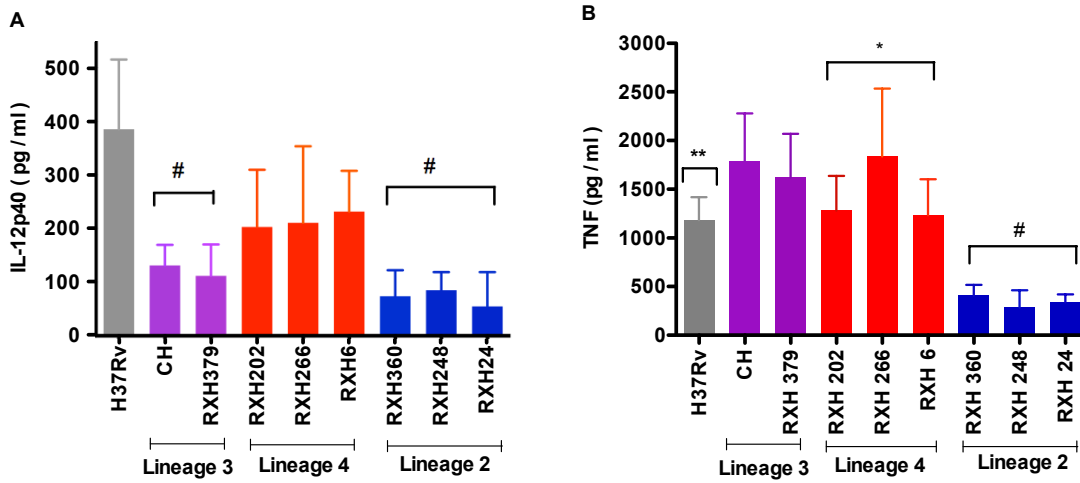


Figure 4.02 Cytokines detected in culture-supernatant (at 48h) from MDM infected by different strains of *M. tuberculosis* (A) IL-12p40 (n=6 donors). H37Rv and lineage 4 strains induced significantly higher level of IL-12p40 as compared lineage 3# and lineage 2# lineages (p<0.001 for both comparisons) (B) TNF (n= 8 donors). Lineage 3 strains induced significantly higher levels of TNF as compared to lineage 2# (p=0.001), lineage 4 * (p<0.05) and H37Rv (p=0.01). H37Rv and lineage 4 strains induced higher level of TNF than lineage 2 strains (lineage 4 vs. lineage 2 p = 0.001, H37Rv vs. lineage 2 p= 0.01). Data represent in the median and range. 2X10⁵ cells were infected with 2X10⁵ CFU of bacteria per well. Data represent in the median and range.**

Further, the release of TNF by MDM infected with lineage 2 strains was significantly reduced when compared with both, H37Rv (H37Rv= 1253 ± 84 pg /ml, lineage 2= 362 ± 28 pg /ml, p<0.01, median and range) and other lineages (lineage 3= 1818 ± 123 pg /ml, p<0.001; lineage 4 = 1207 ± 112 pg /ml, p<0.001, median and range) [Figure 4.02 B]. In contrast to IL-12p40, higher levels of TNF were induced by MDM infected with lineage 3 strains when compared to H37Rv (p <0.01) and lineage 4 strains (p<0.05). Induction of both TNF and IL-12p40 was higher in MDM infected with lineage 4 strains and H37Rv, but not significantly

different between these lineages (TNF: $p=0.556$; IL-12p40: $p=0.0574$, medians are significantly different if $p<0.05$).

4.5 Discussion

We have demonstrated that the pro-inflammatory cytokine (TNF and IL-12p40) profiles induced by representatives of the three major modern strain lineages of *M. tuberculosis* [lineage 4, lineage 2 and lineage 3] are distinct.

The collective evidence from several *in vitro* studies reinforces the view that *M. tuberculosis* strains induce variable innate immune responses^{201, 204, 216, 284}. However, the cytokine response may differ with the experimental model due to bacterial strains, stimulation conditions, time course and the cells used in the experiment. Several studies have reported that heterogeneity exists in the cytokine response induced by various genotypes of *M. tuberculosis*^{216, 284}. Recently, modern lineages (lineage 2, lineage 4, lineage 3) were shown to induce lower levels of pro-inflammatory cytokines in GM-CSF derived macrophages and monocyte derived dendritic cells when compared with ancient lineages [lineage 1(Indo-Oceanic), lineage 5 (West-African-1) and lineage 6 (West-African-2)]²⁸⁰. Moreover, in a recently reported study from Madagascar PBMC infected with strains from modern lineages (Beijing and CAS strains) produced low levels of antigen-specific protective IFN- γ response when compared with strains from ancient lineages (Indo Oceanic lineage), suggesting that modern strains might have evolved mechanisms to suppress production of IFN- γ which may provide a selective advantage to such modern strains²⁸⁹.

The results from the present study demonstrate that strains from modern lineages differ in their immune response. MDM infected with lineage 4 strains induced

similar levels of pro-inflammatory cytokines to the reference strain H37Rv (also a lineage 4 strain). This finding is compatible with previous findings that lineage 4 strains (including Harlem and LAM strains) induced similar patterns of TNF, IL-6, IL-10, and GRO- α as H37Rv in human macrophages²¹⁶. The outbreak strain, CDC1551, which is a representative of the lineage 4 (belonging to the X family), also induced high levels of pro-inflammatory cytokines in monocyte culture^{133, 136}.

Furthermore, in this study the selected Beijing strains induced low levels of pro-inflammatory cytokines (TNF and IL-12p40). This finding is in line with previous findings of low level cytokine induction (TNF, IL-6, IL-10 and GRO- α) by lineage 2 strains, irrespective of subfamily, in human macrophages²¹⁶. Low pro-inflammatory cytokine induction by individual lineage 2 strains has been reported in various human cell culture models including human monocyte-derived macrophages and THP1 cell line^{137, 204, 206, 216, 219, 290}. In contrast, a recent study demonstrated that 'ancient' lineage 2 strains from Brazil (characterized by the absence of *IS6110* within the [NTF]) induced similar levels of TNF and IL-10 to H37Rv whilst 'modern' multidrug-resistant Beijing strains isolated in Russia, induced low TNF and high IL-10 in the THP1 macrophage cell line²⁹⁰. Tanveer *et al* found lineage 2 strains to produce a weaker IFN- γ and TNF response in THP1 cells as compared to H37Rv (23). In animal models, lineage 2 strains inducing a poor Th1 response caused earlier death of mice, compared with comparator strains^{129, 130, 135}. Several human and animal studies have suggested that the propensity of lineage 2 strains to induce lower levels of protective Th1 cytokines (such as TNF, IL-12, IFN- γ) may be, in part, responsible for this enhanced virulence^{137, 206, 216, 219, 289}.

In our study, Lineage 3 strains were found to induce high levels of TNF and low levels of IL-12p40. Our findings are in line with those of Newton and colleagues²⁸⁴ who described high TNF and a low IL-12p40 secretion by MDM infected with a lineage 3 strain. However, other Lineage 3 strains have been reported to either induce similar levels of pro-inflammatory cytokines to lineage 2 strains (low levels) (23) or to induce similar levels as H37Rv (higher levels)^{216, 219, 284}. A recent study by Kanji *et al.* found that all lineage 3 induce higher levels of TNF but that the RD149 deleted sub lineage of CAS1 strains (lineage 3) induce more TNF than the CAS1 strains (lineage 3) without the RD149 deletion in THP1 cells. CAS1 strains (with the RD149 deletion) from extra-pulmonary sites of infection induced lower level of TNF than those isolated from pulmonary sites²⁰¹.

In summary, we have demonstrated that different modern *M. tuberculosis* lineages exhibit lineage-specific patterns of IL-12p40 and TNF induction, with lineage 4 strains inducing high levels of both cytokines, lineage 2 strains low levels of both cytokines and lineage 3 strains high levels of TNF but low levels of IL12p40. This study suggests that modern strain lineages of *M. tuberculosis* may possess lineage-specific patterns of growth and cytokine induction. These in vitro characteristics may reflect different strategies that *M. tuberculosis* employs to exploit ecological niches in different human populations.

Chapter 5

The interaction between strain lineage and host population: cytokine responses in the monocyte-derived-macrophage model of *M. tuberculosis* infection

University of Cape Town

5.1 Introduction

The global distribution of *M. tuberculosis* strain genotypes is not uniform, with particular strain lineages, or combinations of lineages, predominating in certain geographical areas^{111, 112, 117, 141}. One explanation for this finding is that evolutionary selection pressure is a function of both host and bacterial genetics, and a result of long-term host pathogen interaction. Since disease outcome is likely to be determined by the interaction between bacteria with the host immune system, variation in both host and bacterial factors is likely to influence this interaction.

Variation in the host immune response may partly explain variation in the susceptibility to tuberculosis amongst individuals. Several polymorphisms have been reported in genes encoding various innate immune molecules (e.g. NRAMP1, iNOS), macrophage receptors (e.g. TLRs, Mannose receptors [MR, CD 206], Vitamin D receptor [VDR]) and cytokines^{53, 90, 291-293}. For example, Natural Resistance Associated Macrophage Protein 1 (NRAMP1, encoded by *SLC11A1*) is an integral membrane protein, which is found to be present in the lysosome compartment of monocytes and macrophages, and is involved in iron metabolism and macrophage activation. A number of studies have reported that polymorphisms in *SLC11A1* genes are associated with tuberculosis disease susceptibility amongst Asian and African populations²⁹⁴⁻²⁹⁷. Polymorphisms in the gene encoding the vitamin D receptor (VDR) have reported to be associated with hydroxyl cholecalciferol deficiency and susceptibility to tuberculosis in the Gujarati Indian community in the United Kingdom²⁹². Functional defects in genes encoding IL-12p40²⁹⁸ and IFN- γ receptor 1/2^{299, 300} have been associated with

fatal mycobacterial infection in patients, as a result of defective IFN- γ signalling and a poor Th1 response.

On the other hand, despite exhibiting relatively lower DNA sequence variation than many other pathogenic organisms; the role of pathogen variation is emerging as an important determinant of the outcome of infection³⁰¹⁻³⁰³. Decades of animal studies have reported phenotypic diversity in *M. tuberculosis* strains^{129, 131, 133}. Recently, *in vitro* models of human infection have suggested that strain diversity amongst *M. tuberculosis* exists influences growth or cytokine induction^{201, 203, 216, 217, 237}. A number of *in vitro* and *in vivo* studies have reported that different genotypes of *M. tuberculosis* induce distinct patterns of immune responses^{216, 217, 237}. Epidemiological evidence demonstrates that three modern lineages (lineages 2, 3 and 4) are highly successful in different parts of the world. Whilst this may be due to a founder effect and subsequent expansion of human populations in Europe, India, and China, it is also possible that these lineages have selective advantage over other (particularly ancient) lineages¹¹²(Fig 5.01).

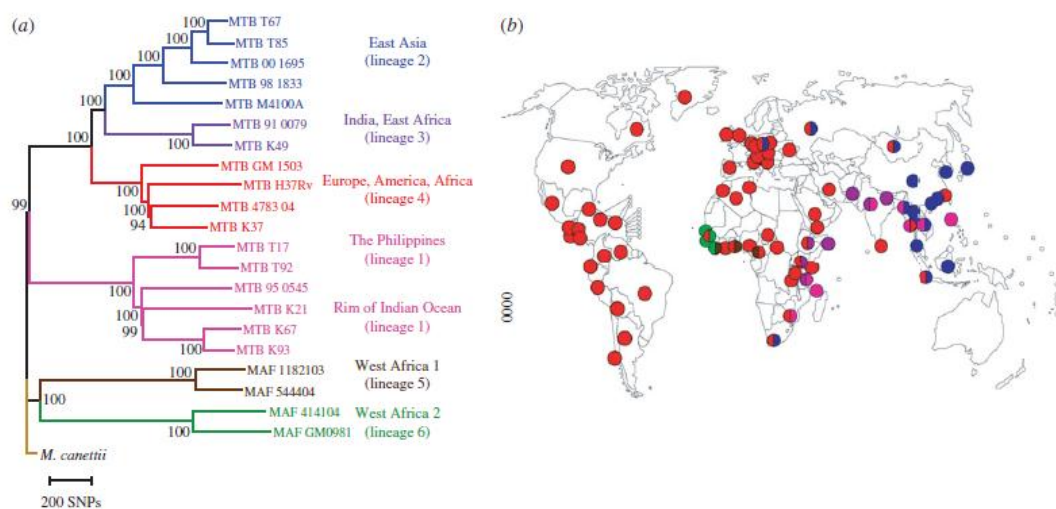


Figure 5.01 Global distribution of MTB Complex based on whole genome sequencing of 22 strains (Taken from Gagneux, *Phil. Trans. R. Soc. B*, 2012).

For example, lineage 2 strains were associated with treatment failure³⁰⁴, relapse, drug resistance^{171, 304, 305}, febrile response³⁰⁶, extrapulmonary tuberculosis and meningeal tuberculosis³⁰⁷. Caws *et al.* reported that lineage 4 strains were associated with pulmonary disease in Vietnamese adults (rather than the extrapulmonary disease)³⁰⁷. Lari *et al.* found that lineage 3 strains are associated with extrapulmonary disease³⁰¹. However, these findings are not often consistent and vary between settings¹⁸⁸. Lineage 2 strains were found to be associated with TBM in immune suppressed HIV patients in a Vietnamese population³⁰², indicating an association between host immune status and a lineage known to be associated with immune subversion, resulting in more severe tuberculosis disease phenotype.

The above evidence suggests that host and bacteria both have an influence on tuberculosis disease outcome. The interplay between different bacterial strains and hosts from different population groups adds an additional level of complexity^{303, 307-310}. For example, studies have reported that polymorphisms in TIRAP (C558T) and TLR2 (T597C) genes are associated with increased risk of tuberculosis meningitis (TBM) in Vietnamese patients^{53, 311}, indicating that polymorphisms in host immune receptors may influence the clinical disease phenotype. Subsequently a study from Vietnam for the first time established an association between *M. tuberculosis* genotype and host genetic background. In this study, Caws *et al.* demonstrated that a polymorphism in TLR2 (T597C) is more commonly found in Vietnamese adult TBM patients infected with lineage 2 strains than those infected with other strains³⁰⁷. Further, a study from Indonesia identified an association between two *SCL11A1/NRAMP1* polymorphisms (D543N G allele and 3' untranslated region insertion/ insertion genotype) and lineage 2 strains in Indonesian tuberculosis patients³⁰⁸.

Immune leukotrienes (LTs) and lipoxins (LXs) are important airway inflammatory mediators (abundant in smooth muscles and small vessels in airway passage) and regulate adaptive and innate immunity by enhancing cytokine production³⁰⁹. LTs and LXs are synthesized by 5-lipoxygenase (ALOX5) from a precursor, arachidonic acid³⁰⁹. LTs function as pro-inflammatory cytokine mediators whereas LXs are anti-inflammatory in nature. LTB₄- (a type of LT) mediated phagocytosis of microorganisms has been reported in alveolar macrophages and polymorphonuclear leucocytes (PMN)³⁰⁹. An exonic non-synonymous polymorphism (g.760A) in ALOX5 was found to be associated with TB in a Ghanaian case control study of 1916 sputum positive TB cases and 2269 healthy controls. Furthermore, the association was strongest in TB cases infected with the *M. africanum* West-african-2 lineage (lineage 6)³⁰⁹, suggesting an association between host and pathogen genotypes.

These findings support the hypothesis that polymorphisms involving host immunity may regulate susceptibility to tuberculosis disease in certain human populations, and that the degree of susceptibility may differ with the genotype of the infecting strain.

Polymorphisms in host and bacterial genotypes have been found to influence innate immune responses to tuberculosis^{53, 137, 138, 307}. *In vitro* infection of monocyte, macrophage and dendritic cells with *M. tuberculosis* strains induces various inflammatory (TNF, IL-12p40, IL-6 etc.), anti-inflammatory (IL-10) and chemokine (MIP-1 α , MIP-1 β , IL-8 etc.) mediators. However, these cytokines are differentially expressed in different donors^{217, 312, 313}. Moreover, various *in vitro* and *in vivo* studies have demonstrated that *M. tuberculosis* genotypes produce different patterns of host immune response^{133, 216, 217, 225}, which may be associated

with variation in virulence, immunopathology and transmission. However, it is not clear how such variation in the immune response to different strains is linked to differences between human population groups. We therefore hypothesized that the immune response to strains from different genotypes would vary depending on the population group of the host.

5.2 Aims of the study

- To compare the early cytokine response in MDM infected *in vitro* with strains of *M. tuberculosis* from different lineages including three modern and one ancient *M. tuberculosis* lineage.
- To determine whether the early cytokine response in MDM infected *in vitro* with strains of *M. tuberculosis* from different lineages differs amongst various population groups.
- To investigate the interaction between *M. tuberculosis* genotype and host population group as determined by cytokine response in the MDM model.

5.3 Methods

5.3.1 Ethical Considerations

The study was conducted with ethical approval from the Faculty of Health Science Research Ethics Committee, University of Cape Town (REC REF 261/2008). Written informed consent was obtained from all the participants enrolled in the study.

5.3.2 Description of MDM donors

Healthy blood donors from five different ethnic groups: Black (Xhosa-speaking), Coloured (mixed ancestry), White (Caucasian), Indian and Chinese from Cape Town were included in the study. The ethnicity of the blood donors was self-defined. Participants were primarily recruited from amongst staff and students at the University of Cape Town. All volunteers gave written informed consent for donating blood and HIV testing. HIV testing was performed in conjunction with pre and post-test counselling by trained nurse counsellor. HIV infected individuals were referred to the appropriate health service for further management and excluded from the study.

Table 5.01 Description of demographic features of blood donors.

Characteristics	Ethnic Groups				
	Black	Coloured	Chinese	White	Indian
Age (mean \pm SD)	40 \pm 9	27 \pm 6	26 \pm 5	28 \pm 6.1	26 \pm 5.24
Gender (n= 69)	15	7	15	16	16
Male	4	0	6	4	8
Female	11	7	9	12	8

5.3.3 *M. tuberculosis* strains

In this study cytokines and chemokine responses were measured in MDM infected with strains from the three modern lineages as described in chapter 2 [lineage 2 (RXH24, RXH248, RXH360), lineage 4 (RXH202, RXH266, RXH6) and lineage 3 (CH and RXH379)] as well as an ancient lineage strain (Lineage 1 MANU strain IND30, kindly provided by Dr Nerges Mistry, Foundation for Medical Research, Mumbai, India).

5.3.4 MDM infection

Blood samples (50 ml) were collected from healthy donors into vacutainer tubes containing preservative-free heparin. Monocytes were isolated and matured to macrophages and infected with different *M. tuberculosis* strains as described in Chapter 3. Culture supernatants were harvested at a single time point 48h post infection and stored at -80°C until assayed. Supernatants were analysed using the MILLIPLEX[®] Multi analytes Profiling human cytokine/chemokine analytes premixed kit (Millipore Corporation, Missouri, USA) on the Luminex system.

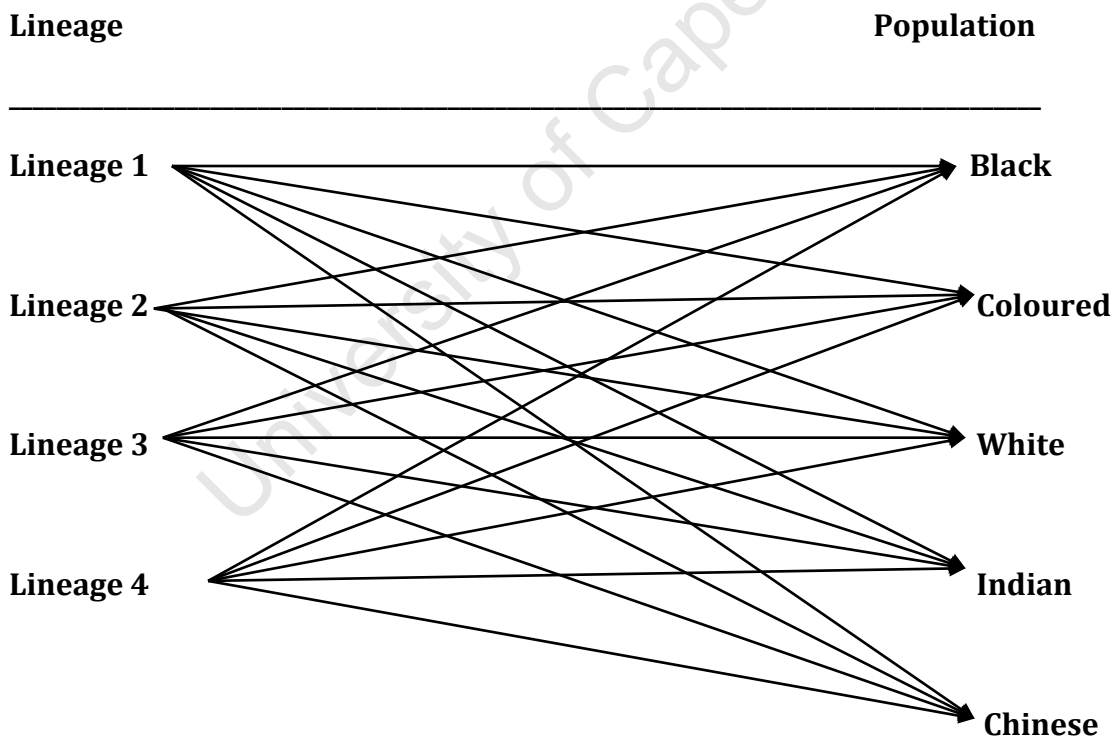


Figure 5.02 Schematic representation of MDM infection model- MDM isolated from different population groups were infected with strains of *M. tuberculosis* from different lineages.

5.3.5 Detection of Cytokines by Luminex multiple cytokine assay

5.3.5.1 Principle

MILLIPLEX™ Multi Analyte Profiling (MAP) is based on Luminex® xMAP® technology. In this technique, microspheres are internally coloured with two fluorescent dyes (red and infrared) in varying concentrations. Up to 100 such distinctly coloured bead sets are prepared, in which each bead is coated with a particular capture antibody. The capture antibody-coated microsphere beads capture multiple analytes from a single test sample. This step is followed by incubation with a biotinylated detection antibody, which is further conjugated with a reporter molecule (streptavidin –PE conjugate) to complete the reaction on the surface of microsphere. The immune complex microsphere is then passed through a laser, which excites the internal dyes marking the microsphere beads. Further, a second laser is used to excite the fluorescent dye (PE) on the reporter molecule. Finally, a high-speed digital-signal processor identifies each individual microsphere and quantifies the results of its bioassay based on the fluorescent reporter signals.

5.3.5.2 Cytokine assessment

Multiple cytokine analysis in *M. tuberculosis*-infected cell culture supernatants was performed using the Milliplex MAP kit. The assay was accomplished in 96 well micro titre plates according to manufacturer's instructions. Briefly, the filter plate was pre-wetted using assay buffer and excess assay buffer was decanted by vacuum filtration. Internal controls and standard were set up ranging from 0 to 10,000 pg/ml for each experiment. After addition of samples to appropriate wells, beads were added to each well and plates were incubated for 1 hour at room temperature. Detection antibodies and streptavidin-Phycoerythrin were

sequentially added for 30 min and the plate was analysed on a Luminex 200 analyser. Results were analysed as described below.

5.3.6 Statistical Analysis

We performed univariate analysis on cytokine data using Graphpad Prism 5. *M. tuberculosis* induced cytokine responses were background subtracted (from uninfected) and, where this resulted in negative values, the negative values were adjusted to zero by adding small positive constant (+1) value assigned to the complete data set. Normality was tested by Shapiro-wilk's normality test. Nonparametric data was analysed by one-way ANOVA and Dunnet's post hoc test.

Linear modelling and Principal Component Analysis (PCA) were used to determine whether *M. tuberculosis* lineages induce specific patterns of cytokine secretion in individual population groups. Analysis was performed using QluCore Omics Explorer 2.2 (QluCore). Prior to analysis, cytokine concentrations were log₂-transformed and normalized to the mean for each analyte with variance -1 to +1. Missing values (resulting from absence of particular sample or inadequate volume for determining concentration of a given analyte) were imputed by K nearest neighbours (k-NN), where a positive constant value (K) is generated to replace the missing value according the level of that cytokine in the most closely correlated sample. Inter-individual variation within population groups was adjusted using the eliminated factors approach applied to the sample annotation 'donor ID' which generated a General Linear Model to normalise baseline differences between donors. Lineage-specific differences were identified by ANOVA, with significance set at p-value and q-value <0.05. The q-value is a measure of false discovery rate, according to the Benjamin Hochberg method³¹⁴, where the distance between

values is a measure of their Pearson correlation. The correlation between significant analytes was analyzed by network PCA. The distance between the analytes in their network represents their Pearson's correlation coefficient and analytes are connected by a line to the analyte to which they are most closely correlated (1st nearest neighbor).

Further, in order to assess possible interactions between *M. tuberculosis* lineage induced cytokine responses and human ethnicity, we performed multivariate analysis using mean rank values (log₁₀) of cytokine concentration analysed using repetitive measure ANOVA.

5.4 Results

5.4.1 Cytokine responses to infection of macrophages from different human population groups by *M. tuberculosis* strains from different lineages

The supernatants obtained after 48 h of *in vitro* MDM infection with various *M. tuberculosis* strain lineages were assayed by Luminex multiple cytokine assay to detect various cytokines and chemokine levels. The initial analysis described below was a simple individual cytokine comparison between lineages (Table 5.02) and between population (Table 5.03) groups without consideration of the interaction between strain and host genotype.

Tumour necrosis factor

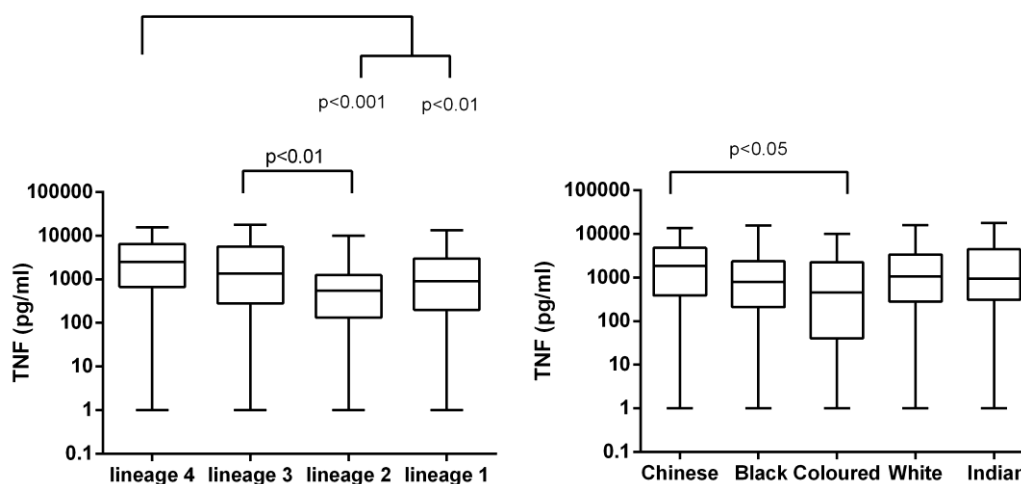


Figure 5.03 TNF response following infection of MDM with *M. tuberculosis* strain lineages (left) and in different population groups (right). Box and whisker plots represent the 25th and 75th quartiles and the horizontal represents the median of TNF response.

Overall, TNF response to infection with lineage 4 strains was higher when compared to lineage 1 ($p < 0.01$) and lineage 2 ($p < 0.001$) strains. Lineage 3 strains induced intermediate responses, which were significantly higher than to lineage 2 strains ($p < 0.001$). We also compared the TNF response in various population groups irrespective of *M. tuberculosis* strain lineage. TNF level was significantly higher in the Chinese population than the Coloured population ($p < 0.001$). There was no significant difference observed in TNF responses between other population groups.

Interleukin-6

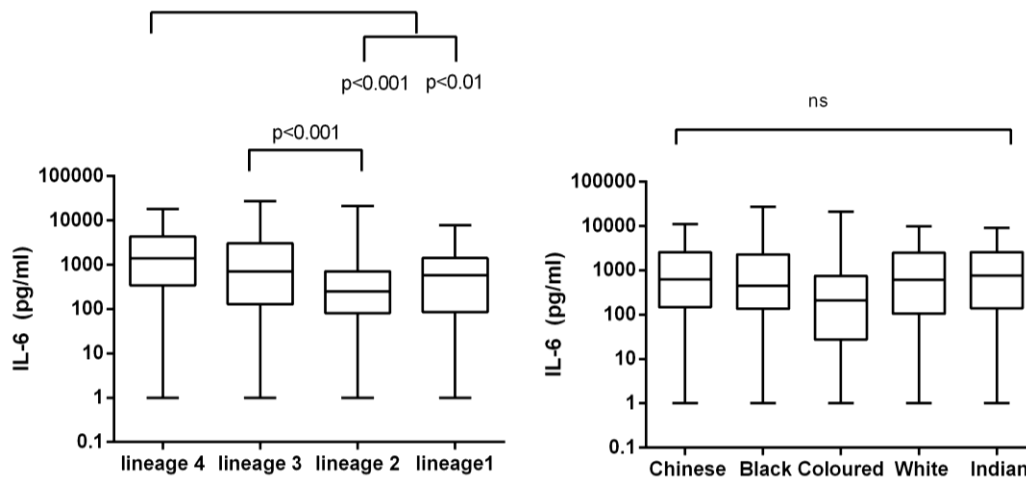


Figure 5.04 IL-6 response following infection of MDM with *M. tuberculosis* strain lineages (left) and in different population groups (right). Box and whisker plots represent the 25th and 75th quartiles and the horizontal represents the median of IL-6 response.

As for TNF, highest IL-6 levels were induced by lineage 3 and 4 strains. Lineage 2 strains induced lower levels of IL-6 as compared to lineage 4 and lineage 3 strains ($p < 0.001$), whilst IL-6 response to the lineage 1 strain was intermediate and lower than that to lineage 4 strains. There was no significant difference in IL-6 levels amongst different population groups.

Interleukin -12p40

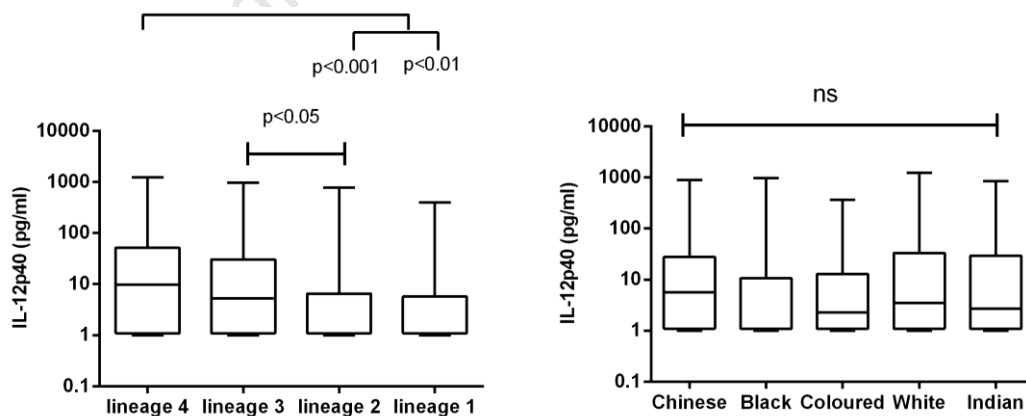


Figure 5.05 IL-12p40 response following infection of MDM with *M. tuberculosis* strain lineages (left) and in different population groups (right). Box and whisker plots represent the 25th and 75th quartiles and the horizontal represents the median of IL-12p40 response.

IL-12p40 responses were low, however the pattern of IL-12p40 responses was similar to the previous two (TNF and IL-6) inflammatory cytokines. Lineage 4 strains induced higher levels of IL-12p40 as compared to lineage 2 ($p < 0.001$) and lineage 1 strains ($p < 0.01$). The lineage 3 strains induced intermediate levels of this cytokine and significantly higher levels than the lineage 2 strains ($p < 0.05$). These results are similar to those described in chapter 4, where we measured IL-12p40 level in MDM with small numbers of donors from blood donor buffy coats. There was no significant difference in IL-12p40 levels amongst the different population groups.

Granulocyte Macrophage-Colony Stimulating Factors

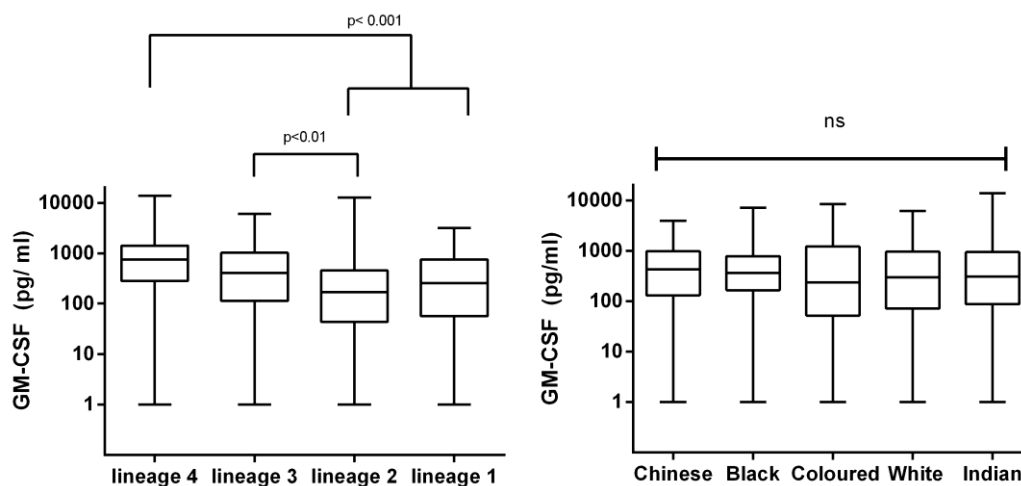


Figure 5.06 GM-CSF response following infection of MDM with *M. tuberculosis* strain lineages (left) and in different population groups (right). Box and whisker plots represent the 25th and 75th quartiles and the horizontal represents the median of GM-CSF response.

GM-CSF showed a similar pattern of responses to the other class I cytokines as described here. Lineage 4 strains induced higher levels of GM-CSF as compared to lineage 2 ($p < 0.001$) and lineage 1 strains ($p < 0.001$). Lineage 3 strains induced intermediate levels of GM-CSF, which were significantly higher than those induced

by lineage 2 strains ($p < 0.01$). There was no significant difference in GM-CSF responses amongst the different population groups.

Interleukin-1 β

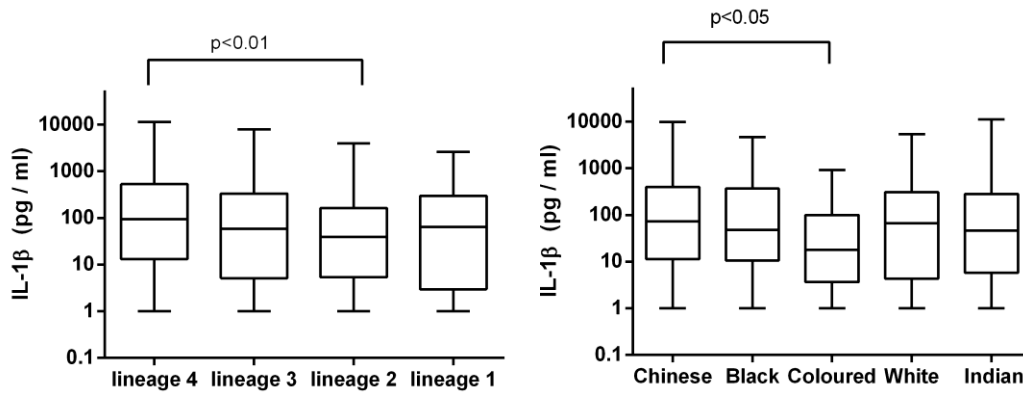


Figure 5.07 IL-1 β response following infection of MDM with *M. tuberculosis* strain lineages (left) and in different population groups (right). Box and whisker plots represent the 25th and 75th quartiles and the horizontal represents the median of IL-1 β response.

IL-1 β levels were significantly higher in response to infection with lineage 4 strains than lineage 2 strains ($p < 0.01$). Lineage 1 strains and Lineage 3 strains induced intermediate levels of IL-1 β . As for TNF, the Coloured population showed lower level of IL-1 β responses than the Chinese population ($p < 0.05$). There was no significant difference in IL-1 β level amongst other population groups.

Interleukin-10

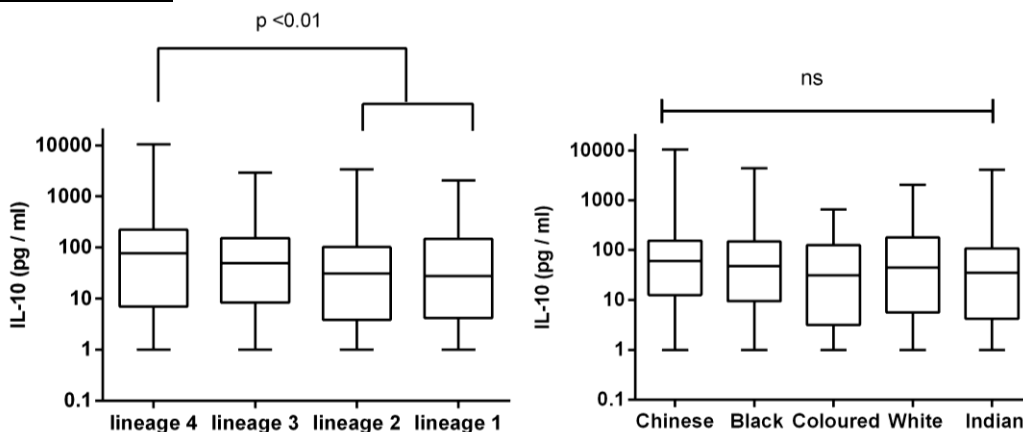


Figure 5.08 IL-10 response following infection of MDM with *M. tuberculosis* strain lineages (left) and in different population groups (right). Box and whisker plots

represent the 25th and 75th quartiles and the horizontal represents the median of IL-10 response.

IL-10 levels were maximum in response to lineage 4 and lineage 3 strains. Lineage 4 strains induced significantly higher levels of IL-10 than lineage 2 and lineage 1 strains ($p < 0.01$). There was no significant difference in IL-10 levels amongst different population groups.

Macrophage Inflammatory Protein -1 α (CCL3)

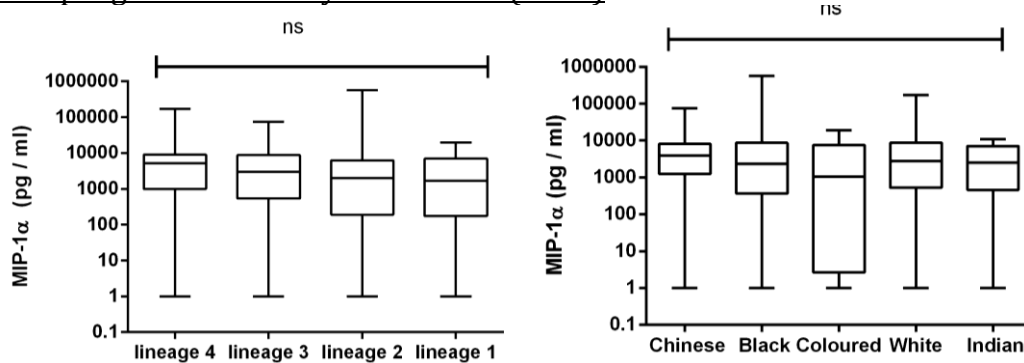


Figure 5.09 MIP-1 α response following infection of MDM with *M. tuberculosis* strain lineages (left) and in different population groups (right). Box and whisker plots represent the 25th and 75th quartiles and the horizontal represents the median of MIP-1 α response.

There was no significant difference in MIP-1 α level in response to different strain lineages or amongst various population groups.

Macrophage Inflammatory Protein -1 β (CCL4)

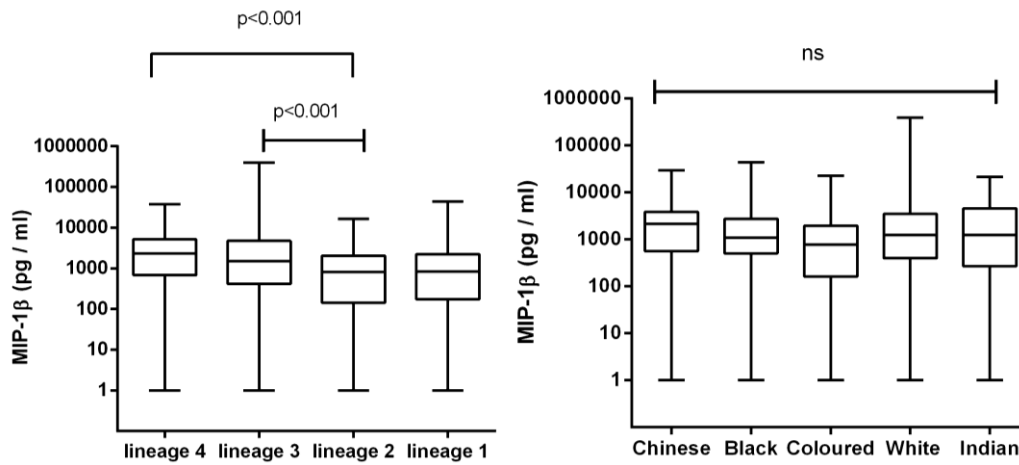


Figure 5.10 MIP-1 β response following infection of MDM with *M. tuberculosis* strain lineages (left) and in different population groups (right). Box and whisker plots represent the 25th and 75th quartiles and the horizontal represents the median of MIP-1 β response.

Lineage 4 and lineage 3 strains induced higher levels of MIP-1 β than lineage 2 strains ($p < 0.001$), whilst lineage 1 strains induced intermediate levels of this cytokine. There was no significant difference in MIP-1 β amongst various population groups.

Interleukin-8

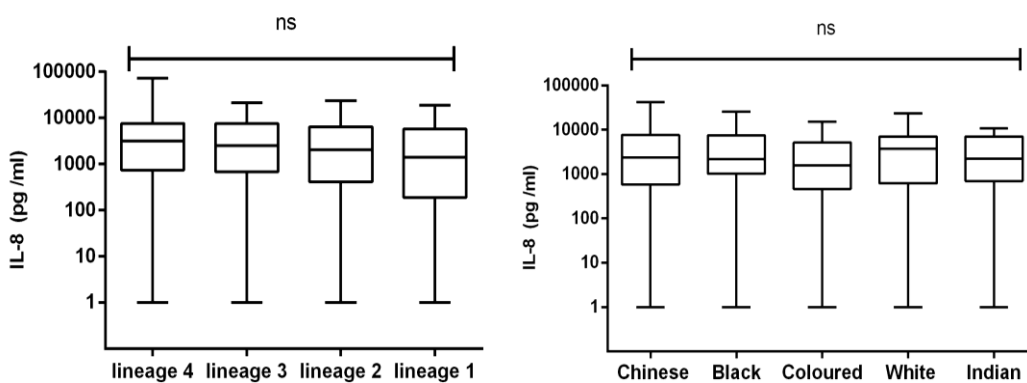


Figure 5.11 IL-8 response following infection of MDM with *M. tuberculosis* strain lineages (left) and in different population groups (right). Box and whisker plots represent the 25th and 75th quartiles and the horizontal represents the median of IL-8 response.

High levels of IL-8 were produced by MDM infected with all the strain lineages of *M. tuberculosis*. However, there was no significant difference in IL-8 levels in response to different *M. tuberculosis* lineages. Similarly, there was no significant difference in IL-8 responses amongst various population groups.

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Table 5.02 Comparisons of cytokine responses following infection of human MDM with different *M. tuberculosis* lineages.

Cytokines	Lineage 4 Median (IQR)	Lineage 3 Median (IQR)	Lineage 2 Median (IQR)	Lineage 1 Median (IQR)	Uninfected Median (IQR)
TNF	2503 (669-6467)	1361 (279-5627)	550 (133-1285)	908.9 (201-2996)	81.58 (24.69-299.7)
IL-6	1405 (343-4361)	708 (128-3073)	276 (103.9-794.7)	588.8 (86-1423)	57.32 (10.87- 182.0)
IL-12p40	9.85 (0.1-52.07)	5.25 (0.1-30.61)	1.16 (0.1-7.8)	1.2 (0.1-6.1)	3.1
GM-CSF	747 (284.6-1413)	404 (113-1016)	168.6 (43.54- 453.2)	256 (56- 749)	21.44 (3.8-97.63)
IL-1 β	93.67 (13.01-524.5)	58.53 (5.0-332.6)	39.27 (3.8-153.1)	63.71(2.94-298.9)	3.46 (3.1- 73.10)
IL-10	77.33 (7-222.1)	49.45 (8.2-152.4)	30.84 (3.8-101.6)	27.60 (4.2-147.9)	6.2 (3.2-48.45)
MIP-1 α	5223 (994.9-9060)	3015 (538.1-8735)	2033 (193.4-6302)	1682 (173.6-6953)	900.9 (93.11-2298)
MIP-1 β	2296 (683.4-5799)	1508 (411.7-4773)	824.2 (142.1-2054)	840.7(173-2235)	338.6 (77.40-1098)
IL-8	3173 (756-7658)	2611 (687.2- 7556)	2060 (460-6479)	1504 (339.8-5879)	4339 (1087 – 9573)

Note:

IQR – Interquartile range between 25th and 75th percentile.
Cytokine responses are background-subtracted values.

Table 5.03 Comparison of cytokine response following infection of human MDM from different population groups with *M. tuberculosis*.

Cytokines	Chinese Median (IQR)	Black Median (IQR)	Coloured Median (IQR)	White Median (IQR)	Indian Median (IQR)
TNF	1845 (384-4833)	797.4 (210.9-2361)	456.8 (40-2245)	1050 (279.9-3350)	949.5 (309.5- 4419)
IL-6	625.5 (147- 2600)	454 (136.3- 2298)	209.1 (27.45 – 758.6)	592 (96.38- 2450)	760.6 (139.5 – 2592)
IL-12p40	5.7 (0.1-27.73)	1.22 (0.1- 18.34)	2.24 (0.1- 12.81)	3.49 (0- 33.39)	2.70 (0-29.12)
GM-CSF	431.7 (130.8-984)	363.3 (165.7-782.4)	235.3(51.99-1215)	297.5 (71.52-960.9)	307.9 (86.68-942.2)
IL-1 β	72.17 (11.33 – 39.4)	47.25 (10.57- 368.3)	15.53 (0-15.53)	65.95(4.2- 309.0)	46 (5.7- 279.4)
IL-10	60.44 (12.65-155.0)	48.30 (9.44-148.7)	19.64 (3.17-124.1)	44.42(5.6-179.3)	34.77 (3.8-104.7)
MIP-1 α	4389 (1847-8389)	2454 (472.1-7588)	1558 (66.25- 7609)	2809 (525.6-8814)	2003 (191.2- 6933)
MIP-1 β	2146(552.2-3847)	1053 (413.7-2641)	654.4 (26.67-1809)	1233 (395-3454)	1053(106-4065)
IL-8	2384 (579.8- 7731)	2005(461.9-7164)	1279 (195- 4853)	4408 (727.3- 8118)	2155 (520-6947)

Note:

IQR – Interquartile range between 25th and 75th percentile.
Cytokine responses are back ground-subtracted values.

5.4.2 The interaction between *M. tuberculosis* lineage-induced cytokine responses and various human population groups

In order to investigate the lineage-specific effect on cytokine patterns both within individual population groups and within the entire study population, principal component analysis and linear modelling with ANOVA was performed. The coloured population was excluded from individual population analysis, as the number of donors was too low to allow robust analysis. The analytes identified by ANOVA to have significantly different patterns of cytokine production in response to different *M. tuberculosis* lineages in each individual population or in the total study population is presented in Table 5.04.

Table 5.04 Analytes identified to be differentially secreted between different lineages during macrophage infection in individual population groups or in all groups considered cumulatively.

Cytokines	All Groups q - value (p<0.05)	Chinese q - value (p<0.05)	Black q - value (p<0.05)	White q - value (p<0.05)	Indian q - value (p<0.05)
IL-12p40	7.71x10 ⁻¹⁴	3.9X10 ⁻⁴	0.01	0.01	9.96X10 ⁻³
GM-CSF	2.60x10 ⁻¹³	7.17x10 ⁻⁰⁷	0.02	0.01	0.03
IL-6	1.59x10 ⁻¹²	1.21 X10 ⁻⁴	0.01	0.03	9.96X10 ⁻³
TNF	6.01x10 ⁻¹³	1.21X10 ⁻⁴	Ns	5.53X 10 ⁻⁴	9.96X10 ⁻³
IL-1β	3.89x10 ⁻⁰⁵	Ns	0.02	Ns	Ns
MIP-1β	9.92x10 ⁻⁰⁹	2.22X10 ⁻⁴	Ns	Ns	9.96X10 ⁻³
MIP-1α	Ns	5.26X10 ⁻⁴	Ns	Ns	Ns
IL-8	Ns	3.04X10 ⁻³	Ns	Ns	0.03

q values represent the false discovery rate, according to the Benjamin-Hochberg method.

Three inflammatory cytokines IL-6, IL-12p40, and GM-CSF were identified to be differentially secreted in response to *in vitro* infection with strains from different lineages in MDM from all four-population groups as well as in the entire cohort. TNF was also differentially produced in MDM from all population groups (and in the total study population) except Black Africans. There were four other analytes which showed differences between lineages in specific populations: MDM from Black Africans showed differential production of IL-1 β , MDM from Chinese donors showed a significant difference in MIP-1 α between lineages, whilst MIP-1 β and IL-8 levels were significantly different between lineages in both the Chinese and Indian populations. Cytokines that showed differential production between different *M. tuberculosis* lineages are depicted in a network diagram, Figure 5.12. In this figure, analytes are connected to their closest neighbour (that which has the most similar pattern of production in response to infection with the various *M. tuberculosis* strain lineages).

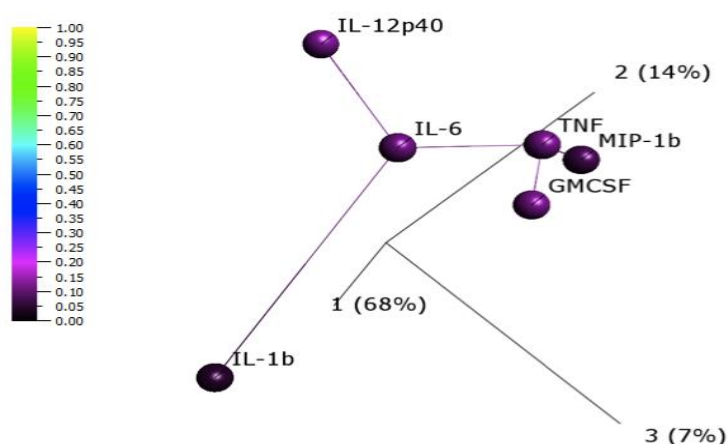


Figure 5.12 Three-dimensional network of Principal Component Analysis plot showing the *M. tuberculosis* lineage-induced innate cytokines that were differentially expressed between lineages. The component vectors are displayed along with a percentage signifying the proportion of the variability in the data

accounted by that component. The distance between the analytes in the network represents their Pearson's correlation coefficient. Analytes are connected with their nearest neighbor (the analyte that displays the most similar pattern in response to infection with the various strain lineages) and they are colored based on their R^2 Statistic.

Analytes identified to have significantly different secretion patterns between lineages were then visualized using PCA analysis to investigate the correlation of patterns between lineages (Figure 5.13), and in different human populations. The combined effects of all cytokines included in the analysis determine the position of each dot in the PCA plot. Tight clustering of samples indicates greater similarity between samples, in terms of the pattern of cytokines produced in response to infection. In the PCA plot, component vectors are displayed along with a percentage signifying the proportion of the variability in the data accounted for by that component. The three first principal (1, 2, 3) components in Figure 5.12 and 5.13 were responsible for 68%, 14% and 7% of the variance in the data subset, respectively. There is no distinct segregation on the PCA plots, however responses to lineage 2 (blue) strains clustered tightly in black and white populations, and, in general, away from lineage 4 (red) strains. In contrast, responses to lineage 4 strains were more dispersed. Responses to all lineages were more tightly clustered amongst black donors. The East-Asian (Chinese) and South-Asian (Indian) populations showed a more dispersed pattern of lineage-induced cytokine responses as compared to the other two population groups.

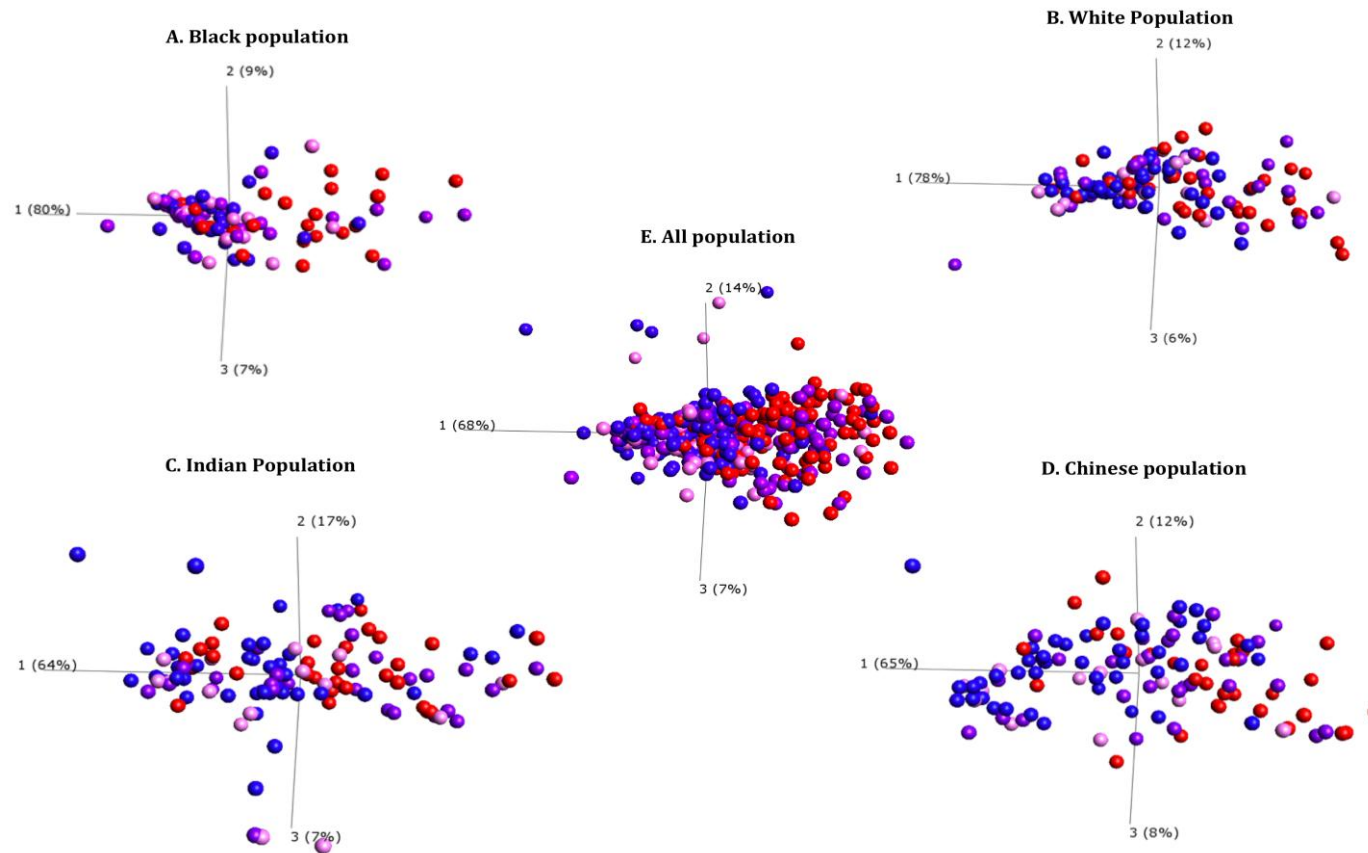


Figure 5.13 Principal component analysis of *M. tuberculosis* lineage induced cytokine responses in individual population groups [A. Black, B. White, C. Indian D. Chinese population, E. All populations combined]. Each dot represents the pattern of cytokine responses in an individual donor. The position of each dot is determined by the combined effects of cytokines identified to contribute to significant differences between each lineage. The distance between points represents correlation between points, measured by Euclidean distance. The first 3 principal components are presented together with the percentage of variance accounted for by that component. Red dots: lineage 4, Blue dots: lineage 2, Purple dots: lineage 3, Pink dots: lineage 1.

In another approach, to investigate the interaction between *M. tuberculosis* strain lineage-induced cytokine responses and various human populations; we expressed the cytokine response in mean rank order across various lineages and population groups. Actual cytokine values were replaced by their rank order values and these ranked values were analysed using repeated measures analysis of variance to compare cytokine levels among population groups (for each individual cytokine), to compare levels in response to different lineages and to investigate whether the differential production in response to different lineages differed amongst the different population groups. The mean rank orders were graphed by population group and lineage, with lineage means connected within population groups to give a visual impression of the lineage-specific responses within each population group (Figure 5.14).

The TNF response showed a clear pattern of lineage effect - mean rank order: (lineage 4 > lineage 3 > lineage 1 > lineage 2; $p < 0.001$). There was no significant population effect ($p = 0.42$), nor was there evidence of lineage-population interaction ($p = 0.38$). Virtually identical patterns to those found in TNF were observed for IL12p40.

There was a possible interaction between lineage and different population groups for IL-6 responses ($p = 0.07$). Lineage 4 strains displayed highest mean rank order across the population groups but responses to lineage 3 strains were variable amongst the population groups, with low rank order in the Black population and high rank order in the Indian population.

The IL-1 β response showed similar pattern of mean rank order of the 3 modern lineages (lineage 4 > 3 > 2, $p < 0.05$) as found for TNF and IL-12p40 across the

population groups. However, interestingly, ancient lineage 1 strains showed a different pattern amongst both Indian and Chinese populations, having high mean rank order in these populations, however the overall interaction between lineage and population group was statistically not significant ($p=0.42$). There was no significant population effect in IL-1 β response ($p=0.60$).

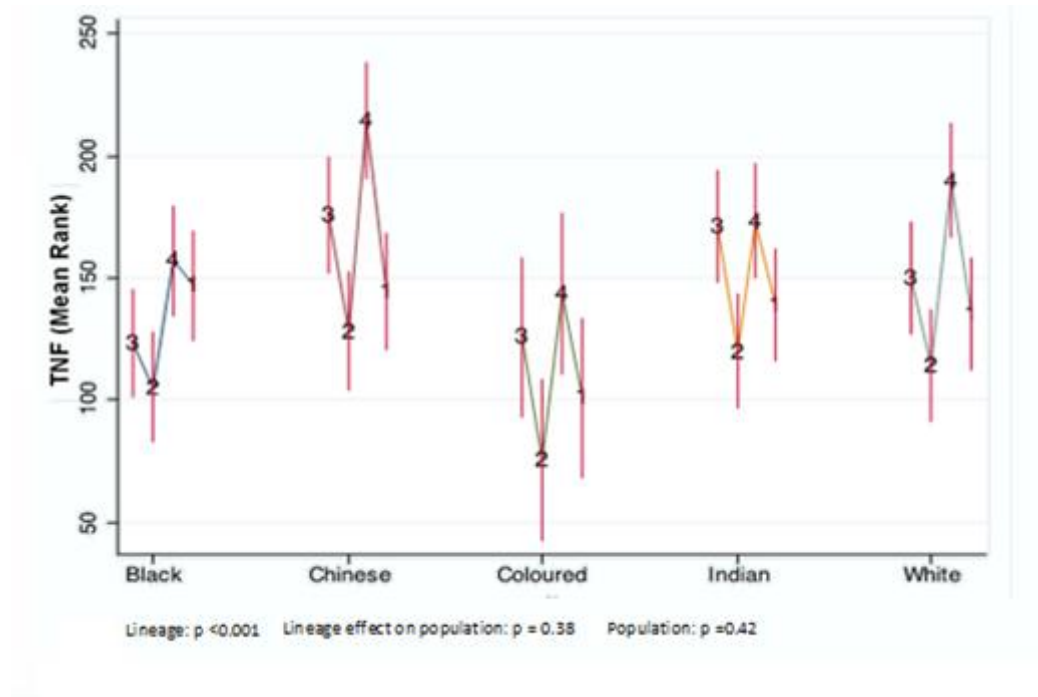
For GM-CSF there was a clear lineage-specific response similar to that for TNF (mean rank order: lineage 4 > lineage 3 > lineage 1 > lineage 2; $p<0.001$) however there were no population or lineage-population specific effects.

The IL-10 response demonstrated a similar pattern of mean rank orders across the Chinese, White and Indian population groups (lineage 4 > lineage 3 > lineage 2/1). However, a different pattern was observed amongst black and coloured populations, where lineage 3 and 4 showed similar rank orders to lineages 1 and 2. However, the interaction between lineage and population group was not statistically significant ($p=0.30$).

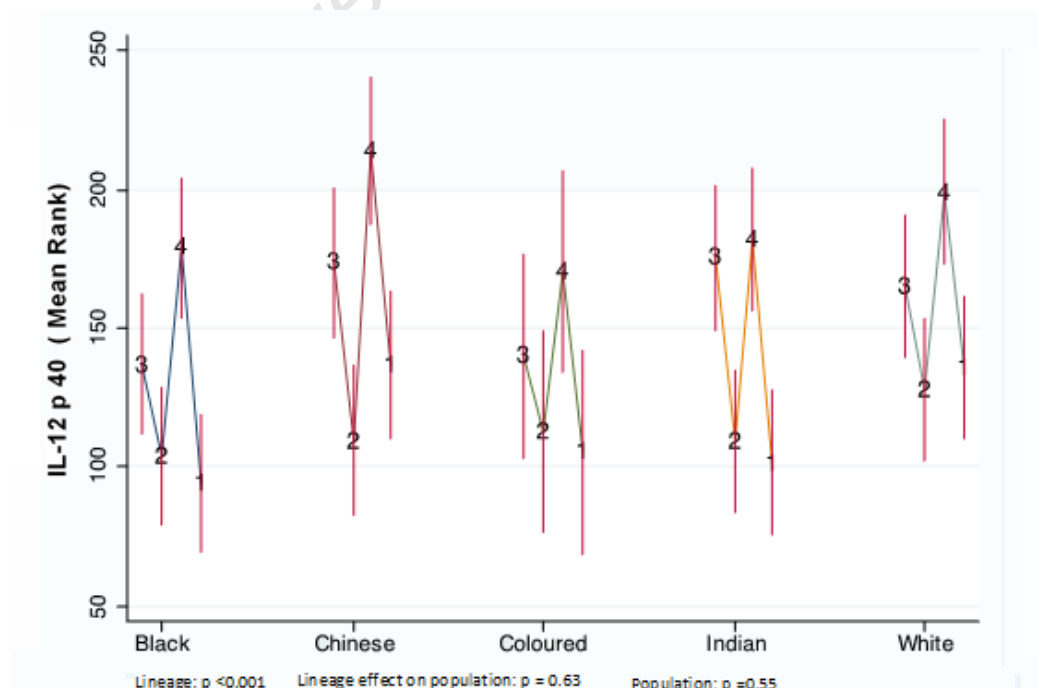
The chemokine MIP-1 α showed large lineage-specific differences in the Chinese population, whilst in contrast, amongst the Black population, there were no differences in response to infection between different lineages. However, these differences in response were not statistically significant ($p=0.32$). MIP-1 β responses followed a similar pattern to TNF, with a clear lineage-specific effect ($p<0.05$) but no interaction between population and lineage. Patterns of IL-8 response differed across all population groups, with no consistent lineage-specific effect, in contrast to most of the other cytokines assayed. No population effect was observed in IL-8 response ($p=0.67$).

Figure 5.14 Graphs showing the interaction between *M. tuberculosis* lineages induced cytokine response and various population groups –

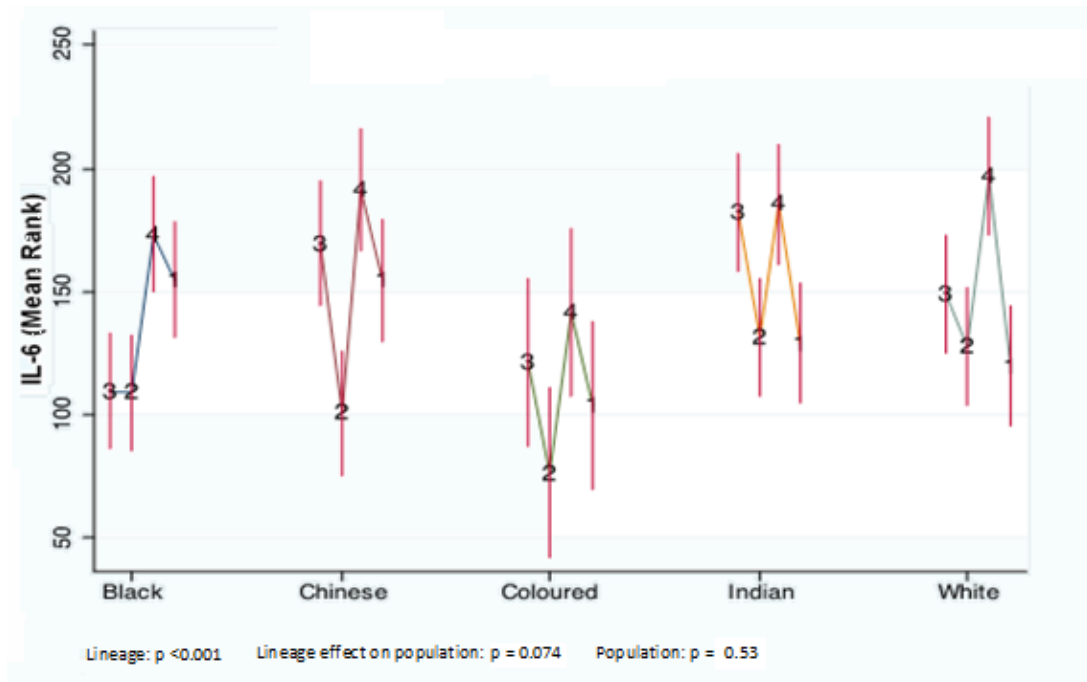
TNF



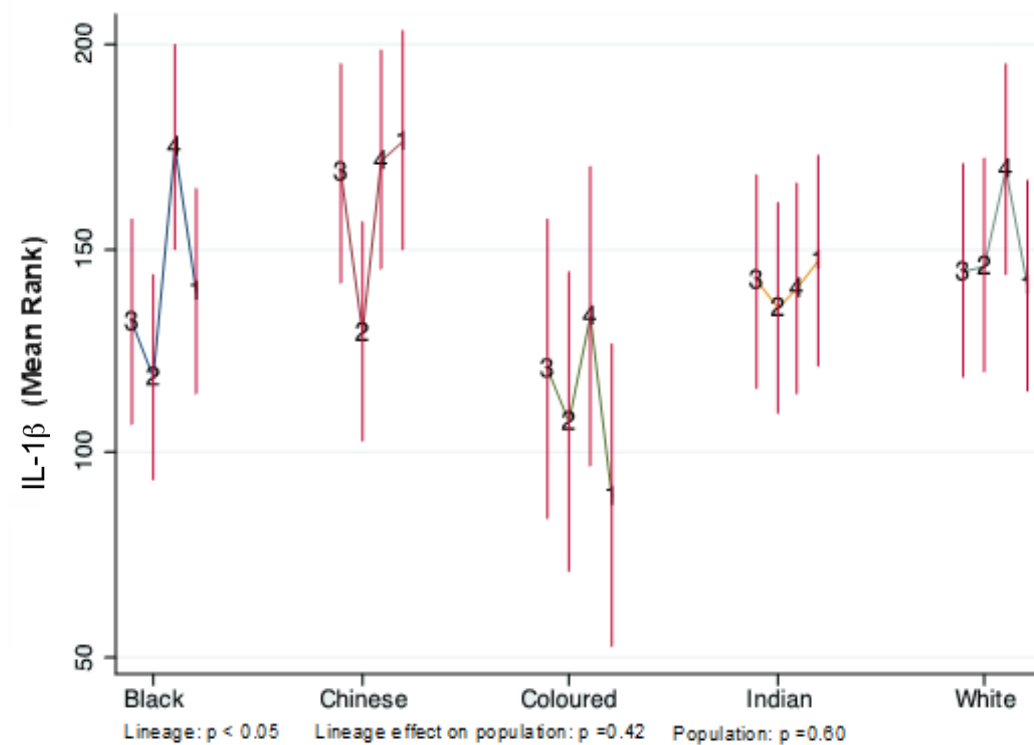
IL-12p40



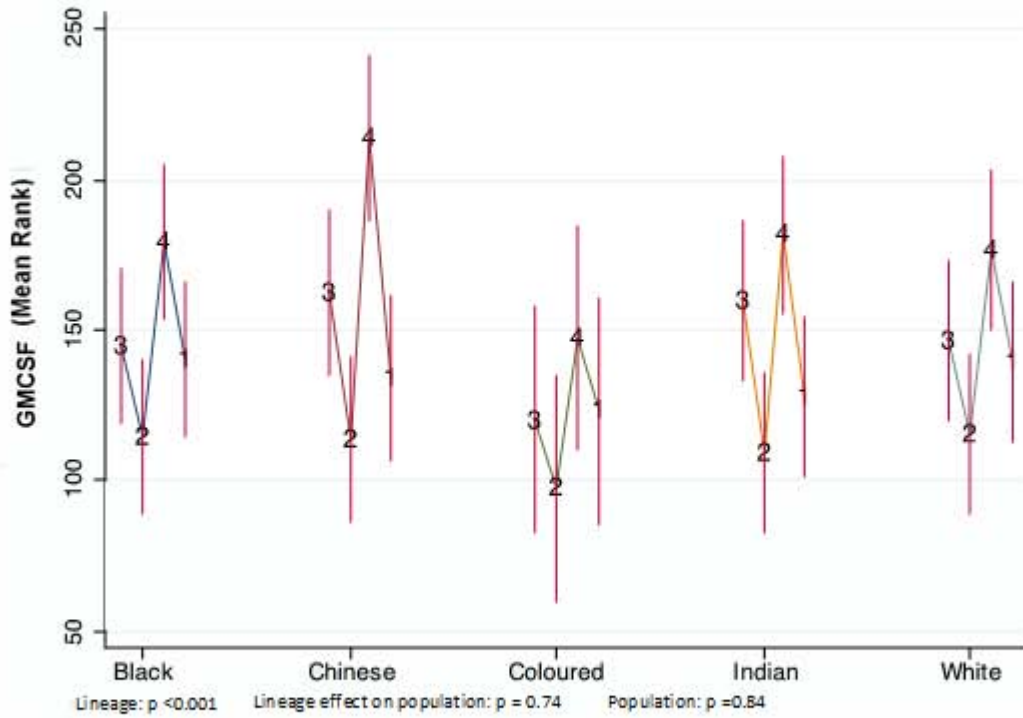
IL-6



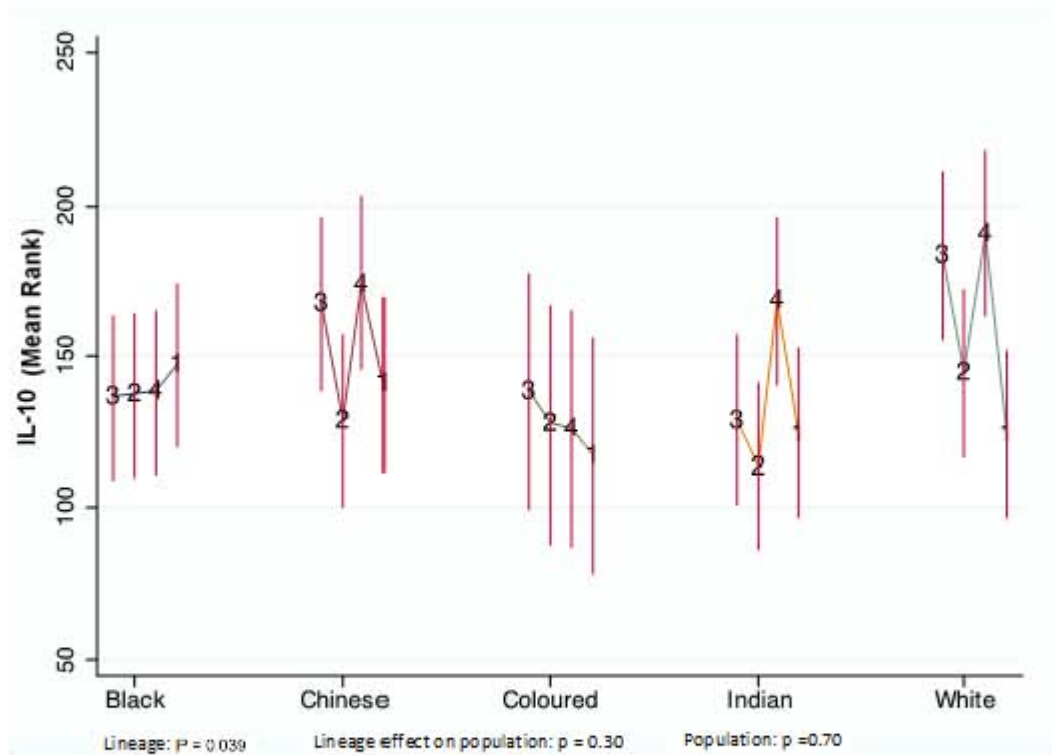
IL-1 β



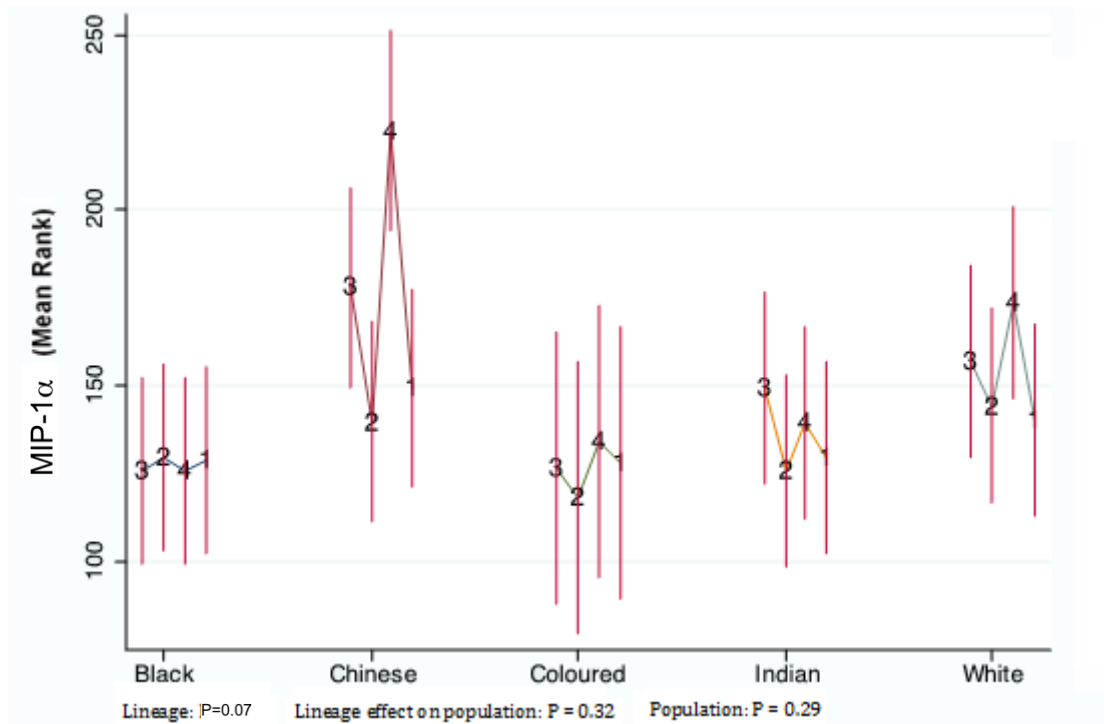
GM-CSF



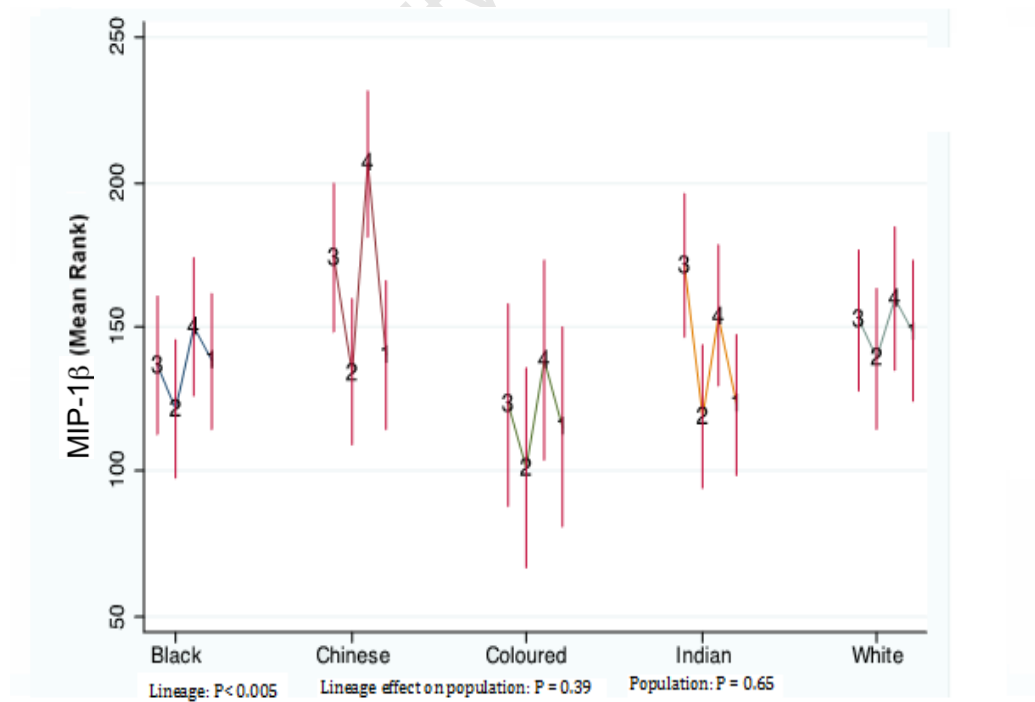
IL-10



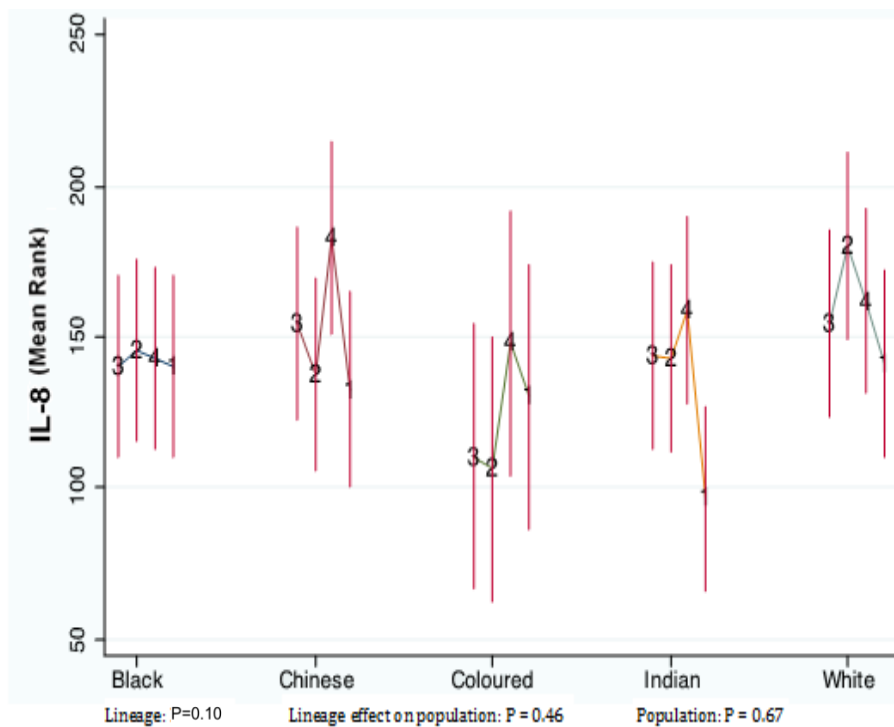
MIP-1 α



MIP-1 β



IL-8



5.5 Discussion

Studies linking epidemiological information with sequence-based phylogenies suggest that *M. tuberculosis* strain lineages are strongly associated with geographical location and that lineages may have adapted to specific human populations¹⁶⁹. Further, different genotypes of *M. tuberculosis* have been reported to exhibit different patterns of host immune response in *in vitro* and *in vivo* infection models^{129, 133, 137, 138, 216, 237}, but the relevance of these findings to the apparent association between strain lineage and host population group is not clear.

Immune triggering commences with interaction between mycobacterial cell surface components and various receptors present on host macrophages and results in secretion of various soluble factors, including cytokines^{315, 316}. These

cytokines play a key role in both enhancing the innate immune response as well as coordinating adaptive responses, which are likely to influence bacterial clearance or persistence in the host. Thus, type-1 cytokines (TNF, IFN- γ , IL-12 and GM-CSF) are inflammatory^{287, 316} and enhance macrophage activity whilst type-2 or regulatory cytokines (eg. IL-10) may suppress anti-microbial activity during *M. tuberculosis* infection⁹³.

Here, we investigated different *M. tuberculosis* strain lineage-induced cytokine and chemokine responses in MDM isolated from a wide range of healthy blood donors, and their interaction, across five human ethnic groups. We measured several pro-inflammatory and regulatory cytokines and chemokine following MDM infection with strains from three modern lineages and a strain from an ancient lineage (lineage 1) by multiple cytokine assays. Additionally, we explored possible interactions between *M. tuberculosis* lineage induced cytokine responses and human ethnic groups.

We observed that MDM infected with lineage 4 strains and lineage 3 strains induced higher levels of pro-inflammatory cytokines- TNF, IL-6, IL-1 β , GM-CSF, IL-12p40 while the pro-inflammatory cytokine secretion by lineage 2 strains was lower than the other two modern lineages, supporting the findings of previous researchers (described in Chapter 2), who studied individual strains in small numbers of donors^{137, 138, 216, 224, 237}. A clinical study reported that Vietnamese adult TBM patients infected with lineage 4 strains have lower mortality than those infected with lineage 2 and lineage 1 strains³⁰⁷. This finding is supported by rabbit infection studies which showed that a lineage 4 strain was highly immunogenic and produced a less severe disease phenotype with comparably

mild focal inflammation of the meninges and minimal infiltrate even in presence of high bacillary loads ¹³¹.

Further, we observed that an 'ancient' *M. tuberculosis* lineage 1 strain induced intermediate levels of TNF and IL-6, lower than lineage 4 and lineage 3 strains, but higher than lineage 2 strains. A clinical study from Vietnam also observed that lineage 1 strains induce low levels of several cytokines (IFN- γ , TNF, IL-10, IL-6, IL-8) in cerebrospinal fluid (CSF) of Vietnamese TBM patients. Lineage 1 and lineage 2 strains were closer in cytokine phenotype when compared with lineage 4 strains³⁰³. Lineage 4 strains were reported to produce elevated levels of these cytokines in CSF³⁰³. In contrast, another study reported higher inflammatory IFN- γ production in PBMC by ancient lineage 1 strains than the modern strain lineages [lineage 3 and lineage 2 strains]²⁸⁹. However, Portevin *et al.* reported that the ancient group of strains exhibit more heterogeneity in inflammatory cytokine response than the modern strain lineages, suggesting ancient lineage 1 strains might also vary in virulence²¹⁷. It should be noted that lineage 1 strains are prevalent in the Indian subcontinent.

Our study was restricted to a limited number of strains from each lineage. One other study has reported that there is variability within lineages with regard to inflammatory phenotype with small numbers of strains exhibiting divergent inflammatory phenotypes²¹⁷. The reason behind such divergence is not yet clear.

In general, absolute levels of IL-12p40 induction were found to be low during *M. tuberculosis* infection in our model. Similarly, we observed low levels of IL-1 β induction following MDM infection with *M. tuberculosis*. We used a low MOI (1:1) in our infection model. Welin *et al.* reported that MDM infected with *M.*

tuberculosis with higher MOI (10:1) induced high levels of IL-1 β whilst low MOI (1:1) induced no or small amounts of this cytokine³¹⁷. IL-1 β induction is facilitated by activation of caspase 1, which causes apoptosis of infected macrophages³¹⁸. The study has found that high MOI triggered the induction of caspase-1 dependent early IL-1 β secretion. This study concluded that virulent mycobacteria are able to multiply to higher numbers inside macrophages by controlling IL-1 β secretion and triggering ESAT-6 dependent necrosis rather than apoptosis³¹⁷.

The cytokine IL-12 is essential for development of Th1-mediated protective immunity in tuberculosis infection^{193, 289, 298}. We found that *M. tuberculosis* infected macrophages produced low levels of cytokine IL-12p40 in our model system, irrespective of the infecting strain or host population, although there was still a clear lineage-specific pattern of responses (lineage 4 > 3 > 2/1).

IL-10 is an important regulatory cytokine involved in suppression of function of macrophages and dendritic cells and inhibition of TNF and IL-12 production, resulting in reduced Th1 response^{319, 320}. IL-10 knock-out mice show reduced bacterial load in mice and increased CD4⁺T cells as well as various Th1 cytokine and chemokines (IFN- γ , TNF, IL-6, GM-CSF, IP-10, IL-17)³²¹, suggesting that IL-10 could regulate the early Th1 response and that neutralization of IL-10 during vaccination may improve protective immunity. We found that lineage 1 and 2 strains induced lower levels of IL-10 than lineage 4 strains, however these strains also induced relatively low levels of Th1 cytokines, so there was no clear evidence of an inverse relationship in this study. We also evaluated various chemokine responses in MDM infection with various *M. tuberculosis* strain lineages. Granuloma formation is important to

contain mycobacterial proliferation and is considered an essential event in the host immune response. Chemokines facilitate granuloma formation by recruiting monocytes, naive lymphocytes, and Th1 cells into the infected tissues from the blood stream. These chemokines are chemo-attractant proteins, which are produced by lymphocytes, macrophages, and neutrophils. The beta chemokines (MIP-1 α and MIP-1 β) are important in macrophage activation and proliferation of T cells. Another chemokine, IL-8 is produced by macrophages and facilitates recruitments of neutrophils, lymphocytes, and monocytes to sites of infection^{322, 323}. The virulence of *M. tuberculosis* strains has been found to be associated with beta chemokine (eg. MIP-1 α and MIP-1 β) induction in infected macrophages: insufficient induction of chemokines during *M. tuberculosis* infection may cause deficiency in the acquired immune response, and MIP-1 β and RANTES in combination enhance killing of *M. tuberculosis*³²⁴. We observed differential induction of MIP-1 β , which was lower in MDM, infected with lineage 2 strains than lineage 3 or 4 strains. The depression in chemokine response may impair Th1 cell migration to infection sites, resulting in poor granuloma formation and failure in bacterial containment³²⁴. This finding correlates with animal models showing disseminated infection with lineage 2 strains^{129, 131, 135}.

The cytokine network from PCA plots further confirmed that the inflammatory cytokines (IL-6, IL-12p40, GM-CSF and TNF) are differentially secreted by MDM infected with different lineages and share the closest patterns of production across the various population groups. Earlier studies reported that successful outbreak strains induce certain inflammatory cytokine responses poorly, which might cause impaired adaptive immunity and rapid disease progression^{138, 225}.

Therefore, variation in inflammatory cytokine response between lineages may be relevant for tuberculosis disease outcomes. Such divergence in inflammatory response across the lineages is likely to be a combined effect of genetic polymorphisms in strain lineages and host innate immune related genes.

Studies examining the interaction between *M. tuberculosis* and the human host have predicted that certain strain lineages may acquire a selective advantage within particular population groups, which may result in increased severity of disease or high prevalence within certain human populations³⁰⁷⁻³¹⁰. Polymorphisms in host innate immune genes have been reported to be associated with higher susceptibility to certain *M. tuberculosis* lineages³⁴⁻³⁶. For example, polymorphism in the *TIRAP* (C558T) and *TLR2* (T597C) genes were found to be associated with TBM rather than the pulmonary tuberculosis in Vietnamese tuberculosis patients³⁰⁷. This effect was modified by strain genotype, TBM patients with polymorphism in 597C allele of *TLR2* gene were more likely to have tuberculosis caused by lineage 2 strains³⁰⁷. However, further studies are needed to demonstrate interactions between host population and *M. tuberculosis* lineages in other population groups.

In order to investigate whether inflammatory responses differ between population groups, we compared cytokine responses in *M. tuberculosis* infected MDM between population groups (irrespective of *M. tuberculosis* strain lineages) as well as the interaction between lineage-induced cytokine responses and the population groups.

In comparison to variation associated with different lineages of *M. tuberculosis*, there was less variation associated with differences in host population. TNF

levels were lower in the Coloured population as compared to Chinese population. TNF plays an important role in maintenance and control of tuberculosis by granuloma formation³²⁵. Previously, studies have been reported that a functional single nucleotide polymorphism (-360 G > A, rs1800629) in the TNF promoter is associated with tuberculosis disease susceptibility^{326, 327}. A case control study has reported that variation in the 3' untranslated region of the *TNFR1B* gene (GTT haplotype) is associated with tuberculosis disease susceptibility in South African Coloured and Ghanaian female populations³²⁸. Further, it has been reported that GTT haplotype is involved with TNFR2 mRNA decay, causing destabilization of TNF and increased susceptibility to tuberculosis³²⁸. There was no evidence of interaction between *M. tuberculosis* lineage and population group in our infection model, with regard to TNF production.

IL-6 is produced by varieties of cells such as macrophages, T cells, endothelial cells, and fibroblasts. IL-6 is a pro-inflammatory cytokine that induces acute phase proteins during infection with *M. tuberculosis*. IL-6 also has inhibitory effect on macrophages and inhibits production of LPS-induced TNF in peripheral blood monocytes³²⁹. We found lower (but not significantly so) levels of IL-6 in the Coloured population as compared to other population groups. Hawn *et al.* reported that a heterozygous state for the 558C-T SNP was associated with low levels of IL-6 induction and TBM in a Vietnamese population⁵³. Recently, a case control study on South African mixed ancestry (Coloured) and Xhosa populations demonstrated that the 558C-T allele was associated with TBM in South Africans mixed descent but the study found no association with TBM in the South African Black (Xhosa speaking) population³³⁰. It should be noted that the Coloured population is heterogeneous with East Asian ancestry common³³¹. The

mechanism that is responsible for the association of TBM with the 558C-T polymorphism in certain human populations is unknown. The 558 polymorphism is synonymous hence, is unlikely to result in amino acid modification. However, synonymous SNPs have been reported to cause alteration in mRNA folding resulting in structural alteration and change in protein expression levels³³².

Our study may not have been sufficiently powered to fully demonstrate interactions between lineages and population groups. However several possible interactions were identified. Interestingly, MDM infected with lineage 3 strains showed higher mean rank order of IL-6 in the Indian population than the South African black population. This finding is consistent with previous findings that a lineage 3 outbreak strain (CH) induced more anti-inflammatory cytokine IL-10 and IL-6 resulting in reduction in IL-12p40 secretion¹³⁸. Lineage 3 strains are prevalent on the Indian subcontinent and uncommon in South Africa. Studies have demonstrated a significant increase of IL-6 level in active pulmonary tuberculosis and tuberculosis pleurisy³³³ and in bronchoalveolar lavage of active tuberculosis patients and alveolar macrophages isolated from tuberculosis lesions³³⁴. IL-6 interferes with IFN- γ mediated signals in virulent mycobacterial (H37Rv) infection. THP1 cells infected with H37Rv induced IL-6 in a time dependent manner and blocking of IL-6 by anti-IL-6 antibody significantly enhanced IFN- γ mediated killing of bacteria. IL-6 inhibits autophagosome formation by inhibiting the Atg12-Atg5 complex³³⁵ (autophagy-related protein). Thus, increase in IL-6 response in MDM infection with lineage 3 strains of *M. tuberculosis* may responsible for skewing of the innate immune response and increased susceptibility to lineage 3 strains in individuals of Indian origin. On the other hand, a polymorphism in the human IL-6 promoter region (G-174-C) has

been reported to be associated with differential expression of IL-6 in tuberculosis and other inflammatory diseases³³⁶. Mutation in the IL-6 promoter region (-174, change of G>C allele) has been found to cause reduced IL-6 levels in *M. tuberculosis* infection³³⁶ in Iranian patients. Studies of other diseases (e.g., chronic arthritis) have reported that the G allele is associated with higher levels of IL-6 response³³⁷ and that C allele frequencies were found to be extremely low in Asian populations (Chinese and Indian). As a result these population groups produce elevated IL-6 responses, which is consistent with our findings^{337, 338}.

IL-10 responses in African Black and Coloured populations were remarkably similar across all strain lineages; suggesting uniform IL-10 induced anti-inflammatory activity for all *M. tuberculosis* lineages in these two population groups (in contrast to the other population groups, where a more clear effect of lineage was observed).

In our study, there was a particularly clear effect of lineage on the induction of MIP-1 α in the Chinese population. We also observed that MIP-1 α secretion during *in vitro* *M. tuberculosis* infection was higher in Chinese population. MIP-1 α polymorphisms have been found to be important in global susceptibility to HIV infection³³⁹. A bi-allelic dinucleotide microsatellite repeat (TA) has been identified in the promoter region (-906) of MIP-1 α gene in Chinese Population. This polymorphism (MIP-1 α -906 (TA) 6/(TA) 6) is associated with higher level of MIP-1 α secretion in several inflammatory diseases³⁴⁰⁻³⁴². This allele is common in the Chinese population, and is associated with increased susceptibility to various inflammatory diseases such as ulcerative colitis and Alzheimer's disease³⁴⁰⁻³⁴². However, no association between tuberculosis and MIP-1 α has yet

been reported in tuberculosis patients. The distribution of another functional single nucleotide polymorphism of MIP-1 α (MIP-1 α +459 C/T) was tested in TB and healthy control subjects of Hong Kong Chinese population but there was no association found³⁴³.

IL-8 responses were interesting in that, unlike most of the other cytokines, there was no clear lineage-specific pattern across the different population groups. In black and white populations there were similarities in the mean rank order in which IL-8 response was slightly increased with lineage 2 strains. In addition, as with MIP-1 α responses, IL-8 responses in the black population were remarkably homogeneous across different strain lineages. It has been reported that a polymorphism in IL-8 promoter region (IL-8-251T>A) is responsible for variation in IL-8 level. The IL-8-251A homozygous allele was found to be associated with increase in IL-8 response in tuberculosis cases in white and African-American TB patients when compared with healthy controls³⁴⁴. It has been reported that the IL-8251A allele is more prevalent in the South African Black population than the South African white population (allelic frequency 0.41 in White and 0.85 in Black)³⁴⁵, which was proposed as a possible reason for increased susceptibility to tuberculosis in the South African Black population. However we did not demonstrate increased IL-8 response in the Black population.

Black donors in this study were significantly older than donors from other ethnic groups. Although we are not aware of any specific literature describing differences in age-specific host responses to *M. tuberculosis*, it is possible that such differences may have affected our findings.

It should be noted that the median levels of TNF and IL-12p40 detected in experiments described in Chapters 4 and 5 differ substantially. This is likely due to a number of differences in experimental design. Experiments in chapter 4 were performed with a small number of donors (n=8) whereas chapter 5 included large number of donors (n= 69). The experimental sets up of two chapters were also quite different as the donors selected in chapter 4 were anonymous and it was from buffy coats supplied by South African Blood Transfusion Services. In Chapter 5 monocytes were isolated from defined blood donors of differing ethnicity. Further, in chapter 5, monocytes were isolated immediately after collection of blood. Moreover, the difference in median cytokine level in TNF and IL-12p40 could be also due to different assay sensitivities as the measurements of cytokine response were performed using two different platforms (Luminex vs. ELISA).

In Summary, we observed that lineage 4 strains and lineage 3 strains induced higher levels of pro-inflammatory cytokines- TNF, IL-6, IL-1 β , GM-CSF, IL-12p40 when compared to lineage 2 and lineage 1 strains We also observed that three inflammatory cytokines (IL-6, IL-12p40, and GM-CSF) were differentially secreted in response to *in vitro* infection with strains from different lineages in MDM from all four-population groups (Chinese, Black, White and Indian), suggesting that this is a conserved pattern of response unrelated to host ethnicity. TNF and IL-6 response was lower in the coloured population. IL-6 responses were particularly variable after infection with lineage 3 strains, with responses was highest in the Indian population group and lowest in the black population group. Possible strain lineage-host ethnicity interactions were also observed for MIP-1 β and IL-8.

In conclusion, polymorphism in either the bacterial genome or in the host immune system may influence the induction of cytokine responses in *M. tuberculosis* infection. The variation observed in *M. tuberculosis* lineage-induced immune responses in specific populations might partly explain the observed association between host population and bacterial genotype.

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Chapter 6

Summary and future work

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Host and bacterial genotypes are both important determinants of tuberculosis disease progression, with the interaction between *M. tuberculosis* and the host innate immune system playing a central role^{53, 292, 346}. The first part of the work presented in this thesis focuses on how strain variation in *M. tuberculosis* influences different virulence-associated characteristics including intracellular growth in macrophages, growth in axenic media and early cytokine response in an experimental model of intracellular infection. The second part of the thesis focuses on the interaction between *M. tuberculosis* strain lineages and different human ethnic groups by exploring induced cytokine and chemokine responses during *in vitro* infection of human MDM.

The first experimental chapter, chapter 3, describes experiments comparing the fitness of different strains of *M. tuberculosis* as measured by *in vitro* growth phenotypes in axenic media and MDM. We compared the laboratory reference strain (H37Rv) with eight strains from the three modern lineages [Lineage 4 (3 strains), Lineage 3 (2 strains), Lineage 2 (3 strains)]. In axenic media, lineage 4 strains demonstrated growth to a higher plateau than strains from the two other lineages. Lineage 3 strains grew relatively poorly, whilst lineage 2 strains showed an intermediate growth phenotype. The laboratory reference strain (H37Rv) grew more rapidly and to a higher level than all clinical strains, suggesting adaptation to growth in axenic media. Intracellular growth patterns were somewhat different, with lineage 2 strains and lineage 4 strains following very similar patterns of growth. Lineage 2 strains grew significantly more rapidly than lineage 3 strains. In summary, the findings from these *in vitro* experiments

suggest that modern lineages of *M. tuberculosis* appear to vary in terms of their growth phenotype in both intracellular and cell free systems.

In chapter 4 we measured early induction of two key pro-inflammatory cytokines, TNF and IL-12p40 in human MDM infected with different modern strains of *M. tuberculosis*. Patterns of cytokine induction by the three modern lineages were distinct and different, with lineage 4 strains inducing high levels of both cytokines, lineage 2 strains low levels of both cytokines and lineage 3 strains high levels of TNF but low levels of IL-12p40. In order to further investigate whether the immune response to strains from different genotypes was influenced by the population group of the host, we performed a cross sectional study in chapter 5 in which MDM from donors of varying ethnicity were infected with *M. tuberculosis* strains from different lineages. We firstly found that the inflammatory cytokines response (TNF, IL-12p40, IL-6, IL-1 β and GM-CSF) differs across the lineages (independent of host ethnicity), where lineage 4 and lineage 3 strains induced higher levels of these cytokines as compared to lineage 2 and lineage 1 strain. We also found that three inflammatory cytokines (IL-6, IL-12p40, and GM-CSF) were differentially secreted in response to *in vitro* infection with strains from different lineages in MDM from all four-population groups (Chinese, Black, White and Indian), suggesting that this is a conserved pattern of responses unrelated to host ethnicity. TNF and IL-6 levels were lower in the Coloured population, which supports previous findings^{328,330}. We also observed high levels of induction of MIP-1 α , which has previously been shown to be increased in Chinese patients with other inflammatory conditions³⁴⁰⁻³⁴². Although the study may have been underpowered to detect interactions between lineages and population groups we observed trends suggesting the possible presence of

such interactions. For example, IL-6 induction in response to infection with lineage 3 strains varied by population group, being highest in MDM from Indian donors, and lowest in MDM from Black African donors. In contrast, IL-6 induction in response to infection with other strain lineages was independent of host population. This finding is of interest, since lineage 3 strains predominate on the Indian subcontinent.

In conclusion, our findings suggest that there are *M. tuberculosis* lineage-specific patterns of growth and cytokine induction and that pattern of cytokine induction may be influenced by host ethnicity. These findings may have important implications for rational vaccine design, since innate immune responses to BCG vaccine itself are likely to differ according to ethnicity of the recipient, which may, in turn, affect acquired memory responses. In addition, differences in such patterns of response may have important implications for disease pathogenesis, affecting important disease outcomes such as the proportion of *M. tuberculosis* infections that progress to active tuberculosis.

Our study was limited to selected strains from particular lineages. Further studies with collections of strains from elsewhere will be important to confirm and extend our findings. It would further be important to explore in more detail whether sub-lineages (within these major lineages) differ in terms of the virulence-associated characteristics assessed in these studies.

The relatively small number of donors limited our study exploring the interaction between host and bacterial genotype. However, studies of this kind are very labour and resource intensive. We have identified several potential interactions, which can be explored in larger similar studies in different geographical settings,

and which may provide further clues as to the reasons for the strong phylogeographic signal in the global distribution of *M. tuberculosis* lineages and the apparent association between particular lineages and host population groups. Such studies may identify effective immune pathways for control of *M. tuberculosis* infection, and assist in rational vaccine design and evaluation.

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