Clinical Strains of *Mycobacterium tuberculosis* Induce Strain-Specific Patterns of Cytokine Production, Gene Expression and Pathway Changes in Pulmonary Alveolar Epithelial Cells

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Submitted in fulfilment of academic requirements for the degree of Doctor of Philosophy (PhD) in the Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal (Westville Campus), Durban.

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PREFACE

The experimental work described in this dissertation was carried out in the School of Life Science; University of KwaZulu-Natal (Westville Campus), Durban, South Africa from January 2011 to February 2015 under the supervision of Dr M. Pillay and the co-supervision of Prof. B. Pillay, and at Johns Hopkins University from June-July 2012 in collaboration with Dr B. Bishai.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

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DECLARATION 1- PLAGIARISM

I, Nontobeko Eunice Mvubu, declare that

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DECLARATION 2- PUBLICATIONS

Details of contributions to publications that form part and/or include research presented in this dissertation (include publications in preparation, submitted, in press and published and give details of the contributions of each authors to the experimental work and writing of each publication).

Peer review publications:

I: Mvubu NE, Pillay B and Pillay M. 2015. Virulent *Mycobacterium tuberculosis* strains induce strain specific *in vitro* cytokine and chemokine responses in pulmonary epithelial cells. Manuscript in preparation.

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II: Mvubu NE, Pillay B, Pillay M. 2013. Induction of cytokine production by clinical strains of *Mycobacterium tuberculosis* in pulmonary epithelial cells. South African Society for Microbiology (Limpompo, South Africa). Oral presentation.

III: Mvubu NE, Pillay M, Gamieldien J, Bishai W, Pillay B. 2014. RNA-Seq reveals strain-specific immune gene expression by epithelial cells infected with *Mycobacterium tuberculosis* strains of varying pathogenicity. International Journal of Infectious Diseases 21, Supplement 1:6. Presented at the 16th International Society for Infectious Diseases (Cape Town, South Africa). Oral presentation.

IV: Mvubu NE, Gamieldien J, Bishai WR, Pillay B, Pillay M. 2014. 135: *Mycobacterium tuberculosis* strains induce differential cytokine production and gene expression patterns in pulmonary alveolar epithelial cells. Cytokine 70:60. International Cytokine and Interferon Society (Melbourne, Australia). Poster presentation.

Signed:

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DEDICATION

This thesis is dedicated to my friends and family
who have been there throughout the project and in my life.
You inspire me to be better than yesterday and for that, I am truly thankful.

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ABSTRACT

The epidemiological success of *M. tuberculosis* strains, dominant in different geographic regions globally, may be ascribed to a subversion of the host's protective immune response. The increasing prevalence of F15/LAM4/KZN, Beijing, F11 and F28 *Mycobacterium tuberculosis* strain families, coupled with rapidly evolving drug resistance within the KwaZulu-Natal province of South Africa population has resulted in a need to characterize host response associated with infection by these strains. Therefore, in this study, cytokine/chemokine production and host transcriptomics were investigated in A549 pulmonary epithelial cells infected with the F15/LAM4/KZN, Beijing, F28, F11, Unique and H37Rv strains.

Cytokines/chemokines were quantified using the Bio-Plex Pro Human Cytokine 27-Plex assay at 0, 24, 48 and 72 hr post-infection. Changes in host gene expression were determined by whole genome RNA Sequencing (RNA-Seq) using the Illumina HiSeq 2000 platform. The 50 bp reads were mapped to the human genome (hg19) using Tophat (2.0.10). Differential expression was quantified using Cufflinks (2.1.0) with false discovery rate (FDR) of 0.05 and a log fold change cutoff of ≥2. R commands (Bioconductor), MeV and Ingenuity Pathway Analysis (IPA) were used to generate heat maps, network and pathways analysis.

Twenty-three out of 27 analytes were detected. All strains, except the F28 strain induced an increased production of 18, and a decrease in 5 cytokines/chemokines at 24, 48 and 72 hr post-infection, compared to the uninfected control. Increased production of all 23 analytes by the F28 strain occurred at 48 and 72 hr. Among the 23 cytokines/chemokines that were detected, anti-inflammatory and pro-inflammatory cytokines, as well as chemokines were produced at the different time intervals. Compared to the other strains, high cytokine levels were induced by the F28 strain at 48 hr and F15/LAM4/KZN strain at 72 hr for most analytes. A lower cytokine production was induced by the Beijing and Unique strains at all-

time intervals. In the case of the laboratory strain H37Rv either a higher or lower cytokine/chemokine production was observed, compared to the clinical strains.

RNA-Seq revealed differential gene expression that varied among the strains with respect to both up- and down-regulated genes: F15/LAM4/KZN (1187), Beijing (1252), F11 (1639), F28 (870), Unique (886) and H37Rv (1179). A total of 292 genes were commonly induced by all strains, of which 52 were down-regulated and 240 were up-regulated. Different strain combinations induced different genes that were involved in a variety of pathways, including immune response and apoptosis pathways. Furthermore, strain specific genes were activated by each strain as follows: F15/LAM4/KZN (138), Beijing (52), F11 (255), F28 (55), Unique (185) and H37Rv (125). The F15/LAM4/KZN, Unique and H37Rv were the only strains that had molecular signatures with overlapping functional Kegg and Reactome pathways for their specific genes.

IPA analysis revealed canonical pathways that differed among the strains, with the interferon signalling and hepatic fibrosis/hepatic stellate cell activation pathways being among the top 5 pathways in all the strains. Cholesterol biosynthesis and immune related pathway enrichment was similar in the Beijing and Unique strains whilst the F15/LAM4/KZN strain showed closer relatedness to the F11 strain, and the F28 strain closely clustered to the H37Rv strain. The Beijing and Unique strains highly enriched cholesterol biosynthesis pathways compared to other clinical and laboratory H37Rv strain. The top scoring networks induced by these clinical strains varied among the strains with the associated functions. These gene networks were involved in antimicrobial response, developmental disorder, organismal injury, infectious disease and cellular development. Among the transcriptional factors, only *EHL*, *IRF7*, *PML*, *STAT1*, *STAT2* and *VDR* were induced by all clinical strains, while other factors were strain specific.

In conclusion, low cytokine/chemokine production and activation of immune associated pathways by the Beijing and Unique strains suggest a higher virulence for these strains compared to the F15/LAM4/KZN, F11 and F28 strains. These characteristics may explain the high transmissibility and prevalence of the Beijing strains. A similar pattern exhibited by the less prevalent, non-clustering Unique strain, may suggest some virulence attributes in common with the Beijing strain. Findings in this study have the potential to reveal useful biomarkers that can be used as targets for alternative TB therapeutics including immuno-modulators that take into consideration network regulations and strain-specific pathways and molecular signatures.

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LIST OF ABBREVIATIONS

APCs	Antigen presenting cells
BAL	Bronchoalveolar lavage
BCG	Bacillus Calmette–Guérin
CD	Cluster of differentiation
CFU	Colony forming unit
ChoRE	Carbohydrate response elements
cm2	Square centimetre
CTD	Comparative Toxicogenomics Database
DCs	Dendritic cells
DC-SIGN	Dendritic cell specific intercellular adhesion molecule grabbing nonintegrin
ELISA	Enzyme-Linked Immuno-Sorbent Assay
EMEM	Eagle's Minimal Essential Medium
FCS	Fetal calf serum
FDR	False discovery rate
FGF	Fibroblast Growth Factor
FXR	Farnesoid X receptor
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSEA	Gene set enrichment analysis
Hg	Human genome
HIV	Human immunodeficiency virus
hr	Hour
IFN-γ	Interferon gamma
IL	Interleukin
IPA	Ingenuity pathway analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
LPS	Lipopolysaccharide
LSP	Large Sequence Polymorphism
LXR	Liver X alpha and beta
MAIT	Mucosal Associated Invariant T-cells
MDR	Multi-drug resistant
MeV	Multi Experiment Viewer
MIP	Macrophage inflammatory protein
mL	Millilitre
MMPs	Matrix metalloproteinases

MOI	Multiplicity of infection
MTBC	Mycobacterium tuberculosis complex
NGS	Next generation sequencing
NK	Natural killer
NOD	Nucleotide-binding-oligomerization-domain
OADC	Oleic acid Albumin Dextrose Catalase
OD	Optical density
PBMCs	Peripheral blood mononuclear cells
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PDGF-BB	Platelet-derived growth factor-bb
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RD	Regions of Difference
RFLP	Restriction fragment length polymorphism
RNA-Seq	RNA sequencing
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
RXR	Retinoid X receptor
SEM	Standard error of the mean
TB	Tuberculosis
TCF	Tissue culture flask
TDR	Total drug resistant
TGFβ	Transforming growth factor beta
TH	T helper
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
UCSC	University of California Santa Cruz
USA	United State of America
VEGF	Vascular Endothelial Growth Factor
WGS	Whole Genome Sequencing
WHO	World Health Organization
XDR	Extensively drug resistant

INTRODUCTION

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB) remains one of the major causes of mortality worldwide, causing infections in ~2 billion people worldwide, leading to 1.5 million deaths each year (WHO, 2014; WHO, 2015). Even though TB results from the failure of the host to eliminate the pathogen, there are different protective mechanisms within the human immune system against *M. tuberculosis*. These include cell mediated immunity as well as innate immunity (Bhatt and Salgame, 2007; van Crevel *et al.*, 2002). The success of cell mediated immunity lies in the ability of the innate cells such as macrophages, to interact with the T-lymphocytes ($CD4^+$ and $CD8^+$) that secrete cytokines and other intermediates to kill the infected cells (van Crevel *et al.*, 2002). On the other hand, the success of *M. tuberculosis* as a pathogen lies in its ability to survive and persist within lung macrophages and epithelial cells and avoid killing by the immune system, thus leading to acute disease (Chuquimia *et al.*, 2012; van Crevel *et al.*, 2002).

The effectiveness of the host immune system is driven by the production of a range of cytokines/chemokines by both immune (macrophages, T-cells, dendritic cells, γδ T cells, and natural killer (NK) cells) and non-immune cells, e.g., epithelial cells (Lee *et al.*, 2009; Sharma *et al.*, 2007). Epithelial cells are abundant in the lung, when compared to macrophages (Chuquimia *et al.*, 2012; Lutay *et al.*, 2014), and are likely to be the first cells to encounter *M. tuberculosis* during infection. Epithelial cells and macrophages are part of the innate immune system and they are responsible for recruitment of T-cells that are required for cytokine production. These cells also produce cytokines (e.g., IFN-γ) that may shift the host immune response towards either

bacterial clearance or survival (Sharma *et al.*, 2007). Pulmonary epithelial cells were shown to have the ability to produce a variety of cytokines and chemokines during *M. tuberculosis* infection (Lee *et al.*, 2009; Lin *et al.*, 1998; Sharma *et al.*, 2007).

M. tuberculosis strains of varying pathogenicity have been shown to induce differential cytokine production in monocytes (Manca *et al.*, 2004; Tanveer *et al.*, 2009), macrophages (Koo *et al.*, 2012), epithelial cells (Lin *et al.*, 1998) and mice models (Manca *et al.*, 1999; Manca *et al.*, 2001) after infection. Differential cytokine patterns in macrophages were also associated with ancient and modern *M. tuberculosis* strain lineages (Portevin *et al.*, 2011) indicating an evolutionary relationship between the strains and host inflammatory response. Strains, such as the Beijing and CAS strains, associated with higher virulence, induced lower cytokine levels compared to ancient East African-Indian strains (Rakotosamimanana *et al.*, 2010). Differential cytokine patterns were also observed in a mouse model, and were associated with the reduction of TH1 response leading to more severe disease and significantly reduced time of death (Manca *et al.*, 1999). The susceptibility/resistant characteristics of *M. tuberculosis* strains to anti-tuberculosis drugs can also influence the type and the level of cytokines produced by the host. Higher levels of IFN-γ were shown to be induced by drug susceptible strains compared to drug resistant strains (Cabral *et al.*, 2010). Furthermore, strain specific characteristics were also observed to be important in the host gene expression of different cytokines/chemokines (Koo *et al.*, 2012).

Studies employing different molecular techniques have been used to understand host responses to *M. tuberculosis* infection *in vitro* (Jang *et al.*, 2008; Lin *et al.*, 1998; Sato *et al.*, 2002; Tailleux *et al.*, 2008; Volpe *et al.*, 2006) and *in vivo* (Manca *et al.*, 1999; Manca *et al.*, 2001). The advent of advanced RNA-Seq technology in molecular studies now provides numerous advantages over existing molecular techniques and includes higher resolution, improved dynamic range of detection, genome-wide transcription, identification of novel transcripts, expression of small RNA molecules, analysis of transcribed but not translated regions, and novel isoforms expression during transcription (Nookaew *et al.*, 2012).

Only a limited number of pro- and anti-inflammatory cytokines, as well as chemokines, have been analyzed and detected in previous studies on epithelial cells and mycobacterial infection (Chuquimia *et al.*, 2012; Lee *et al.*, 2009; Lin *et al.*, 1998; Méndez-Samperio *et al.*, 2006; Sharma *et al.*, 2007; Wickremasinghe *et al.*, 1999; Zhang *et al.*, 1997). These studies were further limited to using the laboratory control H37Rv strain and some uncharacterized clinical strains (Lin *et al.*, 1998). The major disadvantage in elucidating inflammatory response with the laboratory strain might be the absence of different virulent factors that may be present in clinical strains, thereby resulting in an inability to detect strain-specific and differential cytokine production and gene expression. Furthermore, transcriptomics has not been used to study gene expression and pathway changes of *M. tuberculosis* which have been shown to play important role in host immune response. A better understanding of strain-specific cytokine/chemokine patterns, molecular signatures and pathway changes would potentially facilitate the identification of novel host biomarkers associated with clinical strains of *M. tuberculosis* and host directed therapies that can be exploited to combat infection by these strains. Therefore, this study

evaluated the host response to infection by the clinically relevant *M. tuberculosis* F15/LAM4/KZN, Beijing, F11 and F28 strains in a pulmonary epithelial cell model by investigating cytokine/chemokine production using the Bio-Plex Pro Human Cytokine 27-Plex Assay (Bio-Plex 200 Multiplex, Bio-Rad), gene expression and pathway analysis using RNA-Seq.

CHAPTER 1: LITERATURE REVIEW

TB epidemiology

World TB statistics

The global TB burden has been increasing over the past decade, with an estimated 2 billion people infected and ~9.6 million new cases estimated in 2014. Low income countries have a high TB incidence that is fueled by high HIV-TB co-infections. In 2014, Africa was estimated to have 28% of the world's TB burden while India, Indonesia and China had 23%, 10% and 10% of the global estimate, respectively. Twelve percent of the new cases with active TB were estimated to be HIV-TB co-infected in 2014 (WHO, 2015).

South African TB statistics

South Africa has been classified as one of the 22 countries with high TB burdens globally, with estimated incidence and prevalence of 380 000 and 450 000 (cases for 53 969 000 population), respectively (WHO, 2015). It is estimated that about 60% of individuals with active TB are also co-infected with HIV in this country. HIV-TB co-infection is the driving force of high mortality (72 000) estimated in 2014. Among the nine provinces in South Africa, the KwaZulu-Natal, Eastern Cape and Western Cape provinces are estimated to have 922; 782 and 730 TB cases per 100 000 population, respectively. The Mpumalanga (467/100 000); Gauteng (388/100 000) and Limpopo (354/100 000) provinces are the three lowest ranking provinces in this country. It is estimated that more than 80% of the South African population is latently infected with *M. tuberculosis* (Health, 2014).

Drug resistant TB

TB is of global public health concern, with the current drug treatment regime failing to eradicate the pathogen, resulting in the emergence of multi drug resistant (MDR) (Chihota *et al.*, 2012), extensively drug resistant (XDR) (Pillay and Sturm, 2007; Udwadia, 2012) and total drug resistant (TDR) (Klopper *et al.*, 2013; Udwadia *et al.*, 2012; Velayati *et al.*, 2009) cases globally, making TB control efforts difficult (Dye *et al.*, 2002; Magana-Arachchi, 2013). MDR is defined as resistance to isoniazid and rifampicin together with or without resistance to other first line drugs. XDR results from resistance to isoniazid, rifampicin and any fluoroquinolones as well as one of the three-second line injectables. TDR-TB results from *in vitro* resistance to all first and second line drugs used to treat TB (Velayati *et al.*, 2009). These dangerous forms of TB have been identified in Iran (Velayati *et al.*, 2009), India (Udwadia *et al.*, 2012), South Africa (Klopper *et al.*, 2013) and Italy (Migliori *et al.*, 2007).

Drug resistant TB was initially recognized shortly after the introduction of anti-TB treatment through the emergence of streptomycin resistance in 1947 (Pyle, 1947). In the early 1990s, emergence of clinical strains of MDR *M. tuberculosis* resistant to at least isoniazid and rifampin was reported in the United States (Frieden *et al.*, 1993). Post 1990, MDR-TB, reported in both HIV-infected and uninfected individuals (Coninx *et al.*, 1999; Espinal *et al.*, 2000; Goble *et al.*, 1993) was recognized as a catastrophic global challenge (Nachega and Chaisson, 2003).

In 2014, MDR-TB had been detected in 3.3% of new and 20% of previously treated TB cases. Out of 480 000 people living with MDR-TB, 190 000 deaths worldwide were caused by drug resistant TB. It was estimated that 9.7% of MDR cases progressed to XDR-TB in 2014 (WHO,

2015). Drug resistant *M. tuberculosis* strains have been identified in both developing and developed countries (Banerjee *et al.*, 2008; Rowland, 2012).

South Africa has been reported as one of the countries with the highest prevalence of MDR-TB cases and drug resistant strains worldwide. In 2014, the number of MDR and XDR cases in South Africa were estimated to be 10,085 and ≥500, respectively (WHO, 2014; WHO, 2015). The first drug resistant case in South Africa was reported in 1985 in the Western Cape region, however, strain identity remained unknown (Chihota *et al.*, 2012). Development and transmission of drug resistant strains in South Africa led to the XDR-TB outbreak such as the Tugela Ferry, in the KwaZulu-Natal province (Gandhi *et al.*, 2006; Pillay and Sturm, 2007). These TB outbreaks are fueled by the high prevalence of HIV-TB co-infections making TB control efforts difficult.

M. tuberculosis lineages and strain families

Mycobacterium tuberculosis complex (MTBC) includes *M. tuberculosis* and *M. africanum* that have adapted to the human host (Smith *et al.*, 2006) and animal adapted *M. bovis*, *M. caprae*, *M. microti*, *M. pinnipedii*, *M. origys*, *M. mungi*, *M. suricattae*, the "dassie", as well as the chimpanzee bacillus (Alexander *et al.*, 2010; Coscolla *et al.*, 2013; Cousins *et al.*, 1994; Parsons *et al.*, 2013). Other members of the MTBC comprise *M. canettii* and "smooth tuberculosis bacilli (STBs) (Canetti, 1970; Supply *et al.*, 2013; Van Soolingen *et al.*, 1997). Among the MTBC, one of the early and distinguishable feature was the deletion in the genomic region known as TbD1 (Brosch *et al.*, 2002). Strains harbouring this deletion are collectively known as "modern" strains while strains without this are known as evolutionary ancient strains (Coscolla and Gagneux,

2014). Changes in these clinical strains over the years have given rise to seven well characterized lineages (lineage 1-7).

MTBC are classified using the *IS6110* RFLP analysis that groups clinical strains according to genotype families (Filliol *et al.*, 2006; Gutacker *et al.*, 2006). The recent Large Sequence Polymorphism (LSP) and Whole Genome Sequencing (WGS) techniques grouped human-adapted clinical strains into seven lineages that are distributed over different regions globally (Homolka *et al.*, 2012; Stucki *et al.*, 2012; Tsolaki *et al.*, 2005). These lineages are named as lineage 1-7 and they are phylogenetically grouped together based on their genomic deletion referred to as region of difference (RD) and TbD1. Widespread distribution of these lineages and their phylogenetic groupings are shown in Fig. 1. Other two animal adapted lineages within the MTBC (which include *M. bovis*, *M. caprae*, *M. microti*, *M. pinnipedii* and *M. orygis*) infect wild and domestic animals (Coscolla and Gagneux, 2014).

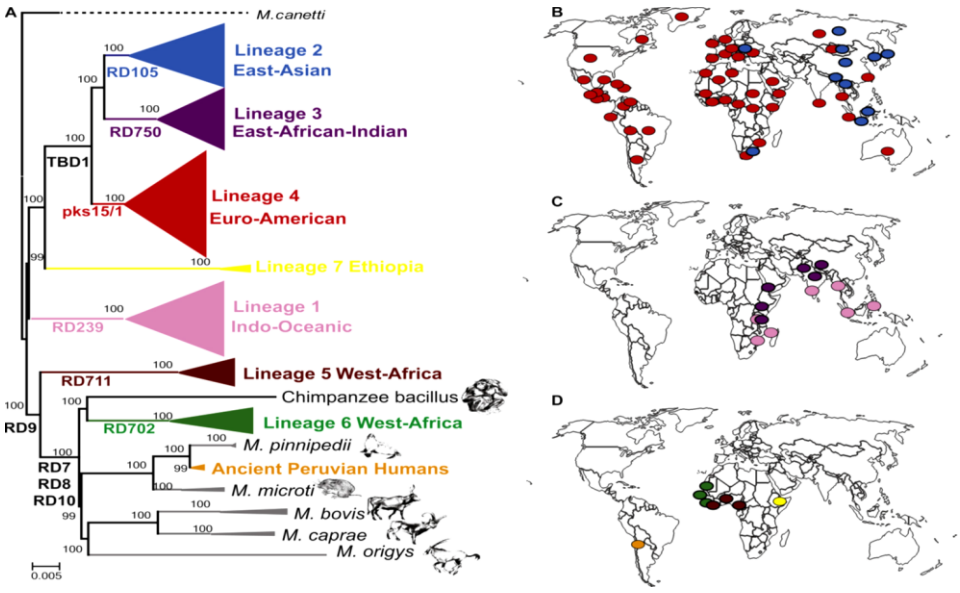


Fig. 1. Phylogenetic groupings of seven major *M. tuberculosis* strain lineages and other members of MTBC with their widespread distributions in different regions globally (Coscolla and Gagneux, 2014).

Among the strain lineages, clinical strains can also be grouped into different strain families using the *IS6110* RFLP genotyping technique. These strain families have been shown to dominate in different regions globally (Coscolla and Gagneux, 2014; Gagneux and Small, 2007). They include the CDC1551 (Valway *et al.*, 1998), CAS (Ali *et al.*, 2014), Beijing (Bifani *et al.*, 2002; Glynn *et al.*, 2002), Haarlem (Bazira *et al.*, 2010), F11 (Victor *et al.*, 2004), F28 (Chihota *et al.*, 2012), and LAM (Soares *et al.*, 2013) strains, respectively. The Beijing is the most dominant family of strains identified in different countries of low, middle and high income resources (Bifani *et al.*, 2002). The distribution of MDR, XDR (Chihota *et al.*, 2012) and TDR (Klopper *et al.*, 2013) strain families within the South African population vary in different provinces. The Western and Eastern Cape provinces reported a high proportion of MDR cases associated with the Beijing isolates, while the KwaZulu-Natal and Gauteng regions are dominated by high prevalence of the LAM4 strain family. The F11 and F28 strain families were also associated with drug resistance in the Western Cape province (Chihota *et al.*, 2012; Victor *et al.*, 2004).

In KwaZulu-Natal, increasing cases of drug resistance have been reported since 1994 with peak MDR cases in 2007 and XDR cases in 2009 (Streicher *et al.*, 2012). In 2005, the F15/LAM4/KZN strain was associated with the highest percentage of both MDR- and XDR-TB in Tugela Ferry (KwaZulu-Natal) with high mortality rates, especially among HIV co-infected patients (Gandhi *et al.*, 2006; Pillay and Sturm, 2007; Streicher *et al.*, 2004). Other strains families such as Beijing, F11 (LAM3) and F28 (S) are also found in this province and associated with either MDR or XDR cases (Fig. 2) (Chihota *et al.*, 2012). Recently, diverse non-Beijing strain genotypes were associated with the MDR- and XDR-TB epidemic in KwaZulu-Natal (Gandhi *et al.*, 2014).

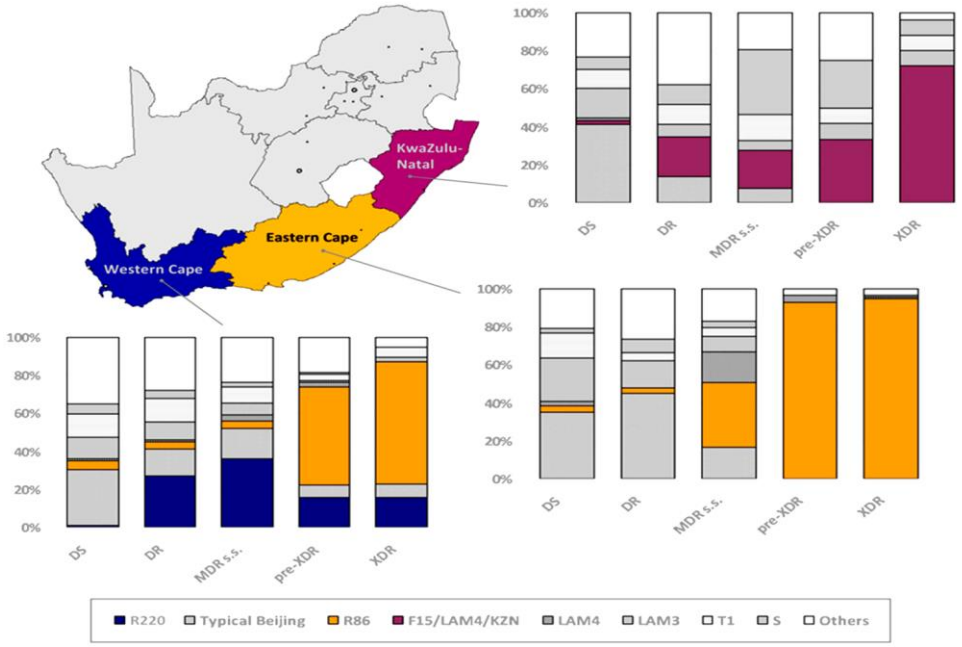


Fig. 2. Population structure of *M. tuberculosis* drug susceptible and resistant strains that are predominantly found in KwaZulu-Natal, Eastern Cape and Western Cape provinces in South Africa. The F11 strains belong to the LAM3 clade while F28 family belongs to the S spoligotype clade (Muller *et al.*, 2013).

Pathogenesis of tuberculosis

Most TB pathogenesis studies are performed in an *in vitro* or *in vivo* model using cell lines, mice, rats, rabbits, guinea pigs and other non-human primates. Despite the fact that these models don't resemble true reflections of human infections, they serve as a good starting point when trying to understand host response and other complex interactions of the "key players" of the immune system which is usually extrapolated into human infections. One of the major limitation in using animal models for TB studies is the failure to address transmission of the pathogen amongst animals (Basaraba, 2008; Young, 2009) and differences in the manifestation of the

disease between humans and animal models (Lenaerts *et al.*, 2015). *In vitro* systems such as cell lines and other *in vitro* lung models can also be used to study TB pathogenesis when trying to isolate and understand the single cell response during infection. This can include trying to elucidate cytokine/chemokine response and other cell specific response that contribute to the outcome of infection. However, this model can be limiting since it is an isolated system lacking the lung structure and full tissue microenvironment which is ideal for understanding TB infection (Guirado and Schlesinger, 2013)

The human respiratory system is divided into the upper (nose, sinus cavities, and pharynx) and lower (larynx, trachea, bronchi and lungs) respiratory tract. Innate defences are present in the upper respiratory system that should prevent colonization of the lower respiratory tract by pathogens such as *M. tuberculosis*. The upper respiratory tract has a microciliary clearance rendered by ciliated epithelial cells that can trap and filter particles that are bigger than 2 μm in a mucus layer during breathing (Ellis, 1998). If these mechanisms fail during TB infection, the lower respiratory tract has abundant epithelial cells in the airway passage to serve as a physical barrier. These epithelial cells are connected by tight junctions, adherens junctions, and desmosomes (Davies and Garrod, 1997) that are relatively impermeable (Eisele and Anderson, 2011). Despite these intrinsic mechanisms to prevent TB, *M. tuberculosis* has adopted different mechanisms to invade and replicate in the lungs of susceptible individuals. TB results from four basic stages due to the failure of the host immune system to eliminate the pathogen (van Crevel *et al.*, 2002).

In the first stage, the primary contact of the *M. tuberculosis* bacilli is with the alveolar epithelial cells (Ahmad, 2010; Lin *et al.*, 1998; van Crevel *et al.*, 2002), alveolar macrophages (van Crevel *et al.*, 2002; Zuniga *et al.*, 2012) and dendritic cells (Cooper, 2009). Pulmonary macrophages and dendritic cells often ingest and digest *M. tuberculosis*. The intrinsic mechanisms of the host to produce reactive oxygen intermediate (ROI) and reactive nitrogen intermediate (RNI) may result in the killing of the *M. tuberculosis* depending on the virulence of the strain, ability of macrophages to effectively respond to infection and the inflammatory microenvironment at the site of the disease as part of the innate immune response (Dannenberg Jr and Rook, 1994; Zuniga *et al.*, 2012). This stage lasts between 3-8 weeks with *M. tuberculosis* being implanted in the alveoli and disseminated by the lymphatic system to the lymph nodes within the lungs to form the Ghon complex (Reece and Kaufmann, 2012).

In the second stage, the intracellular *M. tuberculosis* begins to multiply within macrophages and dendritic cells (Zuniga *et al.*, 2012) leading to disruption of macrophages and attraction of monocytes towards the lungs (van Crevel *et al.*, 2002). This is also referred to as an inflammatory cell recruitment stage due to production of cytokines/chemokines by infected epithelial cells, macrophages and dendritic cells crucial for attracting circulating immune cells (Blomgran and Ernst, 2011; van Crevel *et al.*, 2002; Zuniga *et al.*, 2012). Infection of the resident macrophages and epithelial cells results in the secretion of cytokines and chemokines that effectively recruit immune cells including innate cells such as γδ T-cells, NK-cells, DCs, monocyte derived macrophages as well as neutrophils to the lungs (Guirado *et al.*, 2013; Kang *et al.*, 2011). These cytokines and chemokines ensure effective control of *M. tuberculosis* while initiating both the innate and adaptive immune responses. Despite the beneficial effect of

cytokine/chemokine production in the lung during infection, excessive production and expression of the respective cytokine/chemokine receptors can result in severe lung pathology. This can cause lack of proper lung function which subsequently leads to respiratory failure, thus increasing mortality among TB patients (Sacks and Pendle, 1998). This suggests that production of cytokines/chemokines needs to be a tightly controlled mechanism to avoid immunopathology which can result in progressive TB disease.

The main characteristic of the second stage is the invasion of other organs and infection of most parts of the lungs leading to the granuloma formation (Fig. 3) (Sasindran and Torrelles, 2011; Torrado and Cooper, 2010). It lasts for approximately three months and can be fatal due to the development of TB meningitis or miliary TB. During this period, severe chest pain can be experienced due to pleurisy or inflammation of the pleural surfaces which can last for up to two years (Dannenberg Jr and Rook, 1994; van Crevel *et al.*, 2002).

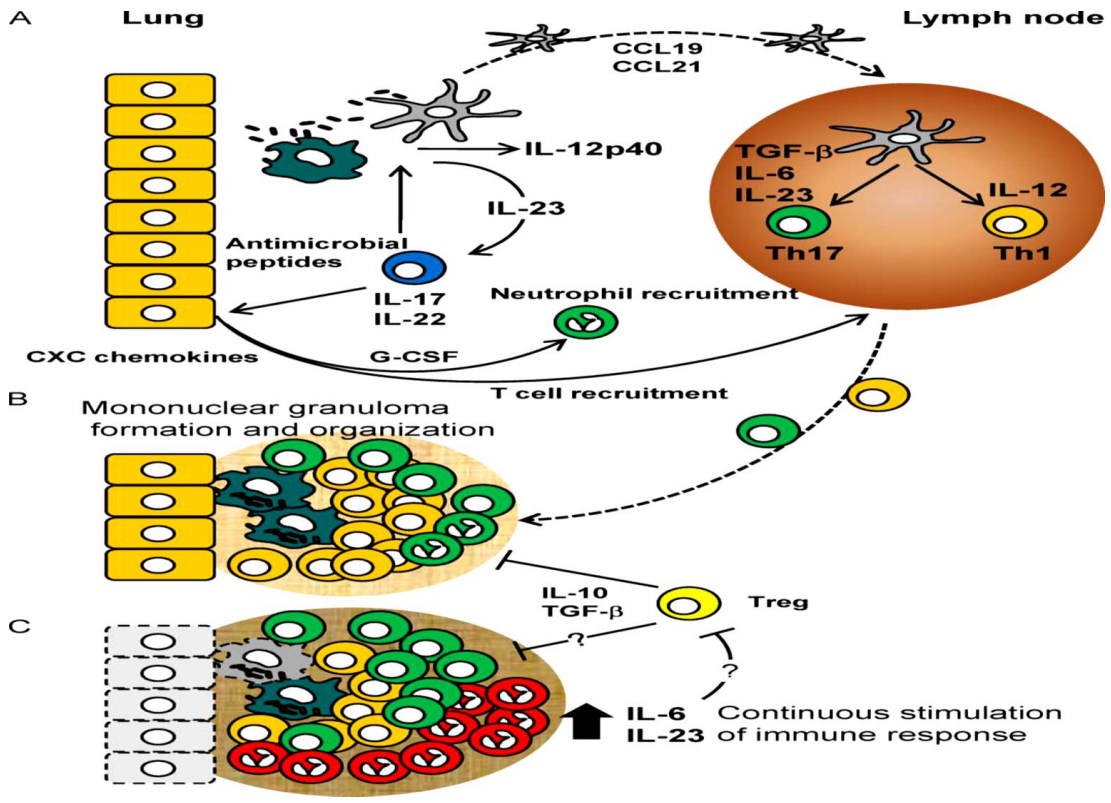


Fig. 3. Formation of the granuloma and involvement of lymph nodes and immune cells (TH1 and TH17) in stages of *M. tuberculosis* infection (Torrado and Cooper, 2010). A: initial stages of infection, B: formation of a mononuclear granuloma, C: setting of immunopathology.

The third stage involves the recruitment of T-lymphocytes that proliferate within the early lesions of tubercles. Macrophages are activated to kill intracellular mycobacteria and produce cytokines. Lesions and T-lymphocytes inhibit the extracellular growth of *M. tuberculosis*, which remains in a dormant stage (Ahmad, 2010; Zuniga *et al.*, 2012). The main characteristic of the third stage is the granuloma containing aggregated T-cells and infected macrophages (Zuniga *et al.*, 2012). When conditions become conducive or if the host is immuno-compromised, *M. tuberculosis* may grow exponentially leading to tissue damage and spread of the bacilli within

the lungs, which characterize the last stage of TB (Smith, 2003; van Crevel *et al.*, 2002; Zuniga *et al.*, 2012).

TB immunology

Innate cells in TB pathogenesis

Pulmonary epithelial cells

Pulmonary epithelial cells are probably the first cells to encounter pathogens such as *M. tuberculosis* because they form part of the lining the lung alveolus (Li *et al.*, 2012). Epithelial cells were long thought to be a passive barrier against pathogens, however, recent evidence has shown that these cells are involved in shaping local inflammatory response during *M. tuberculosis* infection (Lee *et al.*, 2009). The functions of pulmonary epithelial cells range from absorbing substances to the lung tissue (Horvath *et al.*, 2007), protecting the lungs by acting as a physical barrier for pathogens (Eisele and Anderson, 2011), and production of cytokines and chemokines to increase host defence mechanisms (Fig. 4) (Holt *et al.*, 2008). Furthermore, they are known as a "defender of the alveolus" due to variety of functions including modulation of macrophage release of radical oxygen species (Fehrenbach, 2001; Mason and Williams, 1977). They are known to inhibit *M. tuberculosis* replication and attract other cell types (mainly immune cells) to the site of infection (Chuquimia *et al.*, 2012; Lee *et al.*, 2009; Lin *et al.*, 1998; Sato *et al.*, 2002; Sharma *et al.*, 2007).

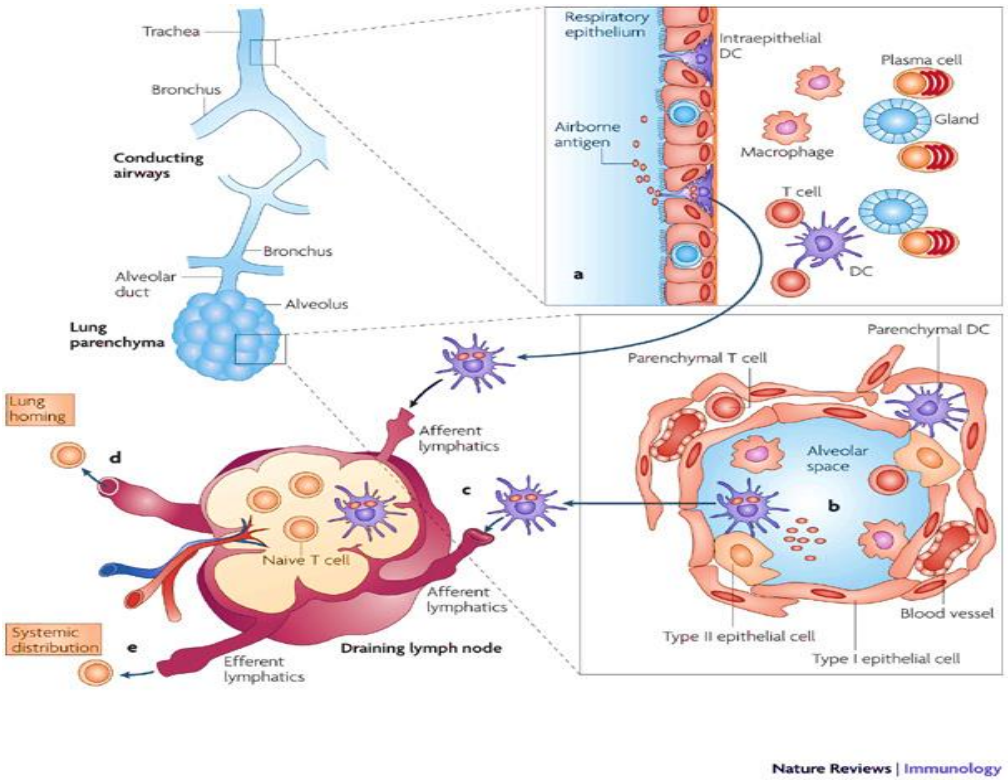


Fig. 4. Overview of pulmonary epithelial cells in the respiratory tract and alveolus with other associated immune cells (Holt *et al.*, 2008). During infection with *M. tuberculosis*, the first point of contact is with pulmonary epithelial cells with residing macrophages. Together, these cells form part of the innate immune response.

Macrophages

Macrophages, which are matured monocytes, also play an important role during *M. tuberculosis* infection (Podinovskaia *et al.*, 2013). These cells are among the largest phagocytic cells, each of which can engulf more than 100 bacteria (Guyton and Hall, 2006). Interaction of *M. tuberculosis* and the macrophages results in different types of actions, i.e., cytokine/chemokine production (Volpe *et al.*, 2006), inflammatory response, sensitization of white blood cells (Leemans *et al.*,

2005), as well as granuloma formation (Flynn *et al.*, 2011). During infection with *M. tuberculosis*, macrophages and epithelial cells respond by producing cytokines and chemokines using different mechanisms. It has been proposed that these cells produce cytokines by the process known as exocytosis (Stanley and Lacy, 2010). The common method of cytokine release in macrophage is constitutive exocytosis. This comes from most constitutively expressed cytokines such as tumor necrosis factor alpha (TNF-α) that resides in the membrane as transmembrane TNF-α precursor until stimulated for its release (Lacy and Stow, 2011). TNF-α is first secreted into the Golgi apparatus and then transported by a carrier protein known as p230 to the cell membrane where it is released to the extracellular space (Stanley and Lacy, 2010).

Dendritic cells

Dendritic cells (DCs) are regarded as professional antigen presenting cells due to their principal role in antigen processing and presentation to naïve T-cells (Wieder, 2003). These cells were first identified by Ralph Steinman and colleagues in the 1970s in spleen tissue (Steinman and Cohn, 1973). DCs are found in the tissue surfaces that are exposed to the external environment. They play a crucial role in the antigen uptake, processing and presentation in both major histocompatibility complex (MHC) I and II molecules, bridging a gap between innate and adaptive immunity (Merad *et al.*, 2013). The lung surface is lined with different cell types, including DCs (Havenith *et al.*, 1993). During *M. tuberculosis* infection, DCs are present in high numbers at the site of infection (Holt and Schon-Hegrad, 1987; Sertl *et al.*, 1986; Van Haarst *et al.*, 1994) and have been shown to increase the strength of cellular response against *Mycobacterium* infections (Henderson *et al.*, 1997; Mohagheghpour *et al.*, 2000; Tascon *et al.*, 2000). Other contradictory reports have demonstrated the ability of *M. tuberculosis* to inhibit the

adaptive immune response through modulating DC adhesion and migration (Roberts and Robinson, 2014).

Natural Killer cells

Natural killer (NK) cells are among the largest granular lymphocytes with both cytotoxicity and cytokine production ability (Trinchieri, 1989). NK cells expressed different ligands such as NKG2D during stress (Lanier, 2005) and toll like receptors (TLRs) during infection (Sivori *et al.*, 2004). They are found in different lymphoid and non-lymphoid tissues, with 10% localized in the mouse lung (Grégoire *et al.*, 2007). These cells play a crucial role in the innate immune response by controlling microbial infections prior to their spreading to other tissues (Vivier *et al.*, 2008). NK cells demonstrated the ability to fight *M. tuberculosis* infection when stimulated with cytokines and N-acetyl cysteine to induce inflammation and contribute to the innate response. This was through an increase expression of cytotoxic ligands (FasL and CD40L) and reduced bacterial growth in monocytes (Guerra *et al.*, 2012). In a mouse model, NK cells were shown to be potent producers of IFN-γ and greatly contribute to early response to *M. tuberculosis* infection without significant contribution to sustaining resistance to infection (Junqueira-Kipnis *et al.*, 2003).

Neutrophils

Neutrophils are the most abundant type of white blood cells in the mammalian body. They are normally found in large blood vessels and axial stream of small vessels. However, they can migrate into tissues for immune surveillance and removal of infectious microorganisms (Witko-Sarsat *et al.*, 2000). These cells express different pattern recognition receptors (May and

Spagnuolo, 1987) including TLR2 in recognition of pathogens such as Mycobacteria (Feng *et al.*, 2003). During *M. tuberculosis* infection, neutrophils were shown to be abundant in the infection site, but failed to control mycobacterial growth contributing to the spread of the bacilli *in vivo*. Therefore, they were thought to contribute to the host tissue pathology and not protection of the host during *M. tuberculosis* infection (Eruslanov *et al.*, 2005). However, other studies reported that neutrophils contribute to the innate control of *M. tuberculosis* (Martineau *et al.*, 2007), and reduced bacterial load (Sugawara *et al.*, 2004), while *M. tuberculosis* colony forming units were increased in granulocyte depleted mice (Barrios-Payan *et al.*, 2005).

T cells

T-cells are among the major players of cell mediated immune response and derived their name from the site of maturing (thymus). They can be differentiated from other white blood cells by the T-cell receptor on the surface (Alberts *et al.*, 2002). Naïve T-cells are involved in the adaptive immune response after encountering a pathogen, and differentiating to become effector T-cells that contribute to the removal of the antigen (Janeway *et al.*, 2001). $CD4^+$, $CD8^+$ and γδT lymphocytes have been shown to be required for control and protection from *M. tuberculosis* (Kaufmann, 2001). Recognition of the *M. tuberculosis* antigens by peripheral T-cells can result in the production of pro-inflammatory cytokines such as IFN-γ and TNF-α (Caccamo *et al.*, 2006; Fenhalls *et al.*, 2002; Peng *et al.*, 2008). The population of γδT T-cells contribute to both the innate and adaptive immune responses during TB (Zuniga *et al.*, 2012), even though the mechanism is not completely understood. Patients with active TB had reduced Vγ9/Vδ2T cells compared to their healthy counterparts (Szereday *et al.*, 2003) indicating the importance of these cells in control of TB infection. These γδ T-cells are induced in abundance in response to

mycobacterial infections. Within this family, Vγ9Vδ2 T-cells are secreted in high quantities in blood in response to *M. tuberculosis* infections and can also be stimulated by mycobacterial antigens (Meraviglia *et al.*, 2011). The γδ T-cells also produce IL-17 and play a role in the early stages of *M. bovis* infection through the production of two major cytokines (IFN-γ and TNF-α) and chemokines, promoting cytotoxicity, and modulate other immune cells as demonstrated in a mouse model (Dieli *et al.*, 2003).

Mucosal Associated Invariant T-cells (MAIT)

There are unique subsets of T-cells that lack $CD4^+$ and $CD8^+$ co-receptors and are monoclonal in their expression of T-cell receptor. One subset within this group is known as invariant natural killer cells while the second one is named Mucosal associated invariant T-cells (MAIT). Both these subsets are known to express a singular TCRα chain with limited sequence diversity (Gold and Lewinsohn, 2011). MAIT cells are known to play a role in early detection of intracellular infection in DCs, macrophages, monocytes and epithelial cells (Gold *et al.*, 2010; Le Bourhis *et al.*, 2010). MAIT cells can be useful in detecting infected epithelial cells that lack MHC-II molecules which cannot be recognized by CD4 T-cells. Infected airway epithelial cells were shown to be lysed by *M. tuberculosis*-reactive MAIT clones via the granule exocytosis pathway that effectively contributes to the clearance of intracellular *M. tuberculosis* infection prior to the acquisition of an adaptive immune response (Gold and Lewinsohn, unpublished). Recognition of infection to non-antigen presenting cells by MAIT cells provides early control of the infection in innate cells at the site of infection (Gold and Lewinsohn, 2011). MAIT cells were decreased in patients with active TB and other lung bacterial infections compared to healthy individuals and cancer patients (Le Bourhis *et al.*, 2010), suggesting migration of these cells to the lungs

resulting in their depletion in blood.

Invasion of the innate cells by *M. tuberculosis*

Even though epithelial cells and macrophages have been shown to increase the host response to infection, *M. tuberculosis* has adopted various mechanisms to invade and even replicate within these cells (Ashiru *et al.*, 2010; Ashiru *et al.*, 2012; Chakraborty *et al.*, 2013; Mehta *et al.*, 1996). The initial crucial step occurs when *M. tuberculosis* interacts with cell receptors "negotiating" an entry into the cells. Complement, mannose and other surface receptors are present on macrophages and allow for phagocytosis of *M. tuberculosis* into these cells (Raja, 2004). In epithelial cells, microfilament and microtubule pathways were proposed to facilitate the entry of *M. tuberculosis* into the cell, via by integrin and vitronectin receptors on the cell surface. Blocking these receptors led to a significant reduction of *M. tuberculosis* uptake by epithelial cells (Bermudez and Goodman, 1996).

Increasing interest and research on pulmonary epithelial cells have led to the discovery of a variety of other surface receptors (Li *et al.*, 2012) that are complementary to the mycobacterial cell wall and other surface appendages. Pathogen associated molecular patterns (PAMPs) can range from lipoproteins (Chambers *et al.*, 2010; Randhawa *et al.*, 2011), cellular appendages such as flagellin (Koff *et al.*, 2008) and nucleic acid (Numata *et al.*, 2011; Torres *et al.*, 2010). These molecular patterns are used by the pathogen to interact with epithelial cell receptors. TLRs (Mayer *et al.*, 2007), Dectin-1 (Lee *et al.*, 2009), nucleotide-binding-oligomerization-domain (NOD), mannose, dendritic cell specific intercellular adhesion molecule grabbing nonintegrin (DC-SIGN), and c-type lectin receptors (Ferguson and Schlesinger, 2000) are known to be expressed by epithelial cells and used for mycobacterial entry into these cells. Activation of

surface receptors results in a series of pathways required for a defensive response and antimicrobial peptide production by epithelial cells (Li *et al.*, 2012).

Some of the receptors used by macrophages are similar to those of epithelial cells, while others are macrophage specific. These receptors are used for phagocytosis of *M. tuberculosis* as a mode of entry into these cells (Hossain and Norazmi, 2013), and include complement receptors (Velasco-Velázquez *et al.*, 2003), TLRs (Harding and Boom, 2010), mannose (Rajaram *et al.*, 2011), scavenger, NOD, surfactant protein A and DC-SIGN receptors (Guirado *et al.*, 2013). After initial interaction with macrophage receptors, *M. tuberculosis* is phagocytosed and subjected to intra-lysosomal hydrolase degradation once the phagosome fuses with the lysosome. However, *M. tuberculosis* survives within macrophages using different mechanisms (Welin, 2011). These include inhibition of phago-lysosome fusion and acidification of the phagosome, resistance to killing by ROIs and RNIs and modification of the bacterial lipid composition, which indirectly alters the efficiency of *M. tuberculosis* to interact with the immune cells (Sasindran and Torrelles, 2011; Vandal *et al.*, 2009; Welin, 2011).

Upon invasion of both macrophages and epithelial cells, *M. tuberculosis* starts replicating depending on the virulence of the strain. Highly virulent Beijing and F15/LAM4/KZN strains were shown to replicate at a higher rate in pulmonary epithelial cells compared to less virulent Unique strains in aerobic (Ashiru *et al.*, 2010) and anaerobic conditions (Ashiru *et al.*, 2012). This replication causes increased cytotoxicity induced by both the F15/LAM4/KZN and Beijing strains in pulmonary epithelial cells (Ashiru and Sturm, 2015). In contrast, the avirulent H37Ra strain induced higher apoptosis levels compared to the virulent H37Rv strain (Danelishvili *et al.*, 2003) in macrophages, demonstrating cell-specific response upon infection by diverse *M. tuberculosis* strains.

Cytokine and chemokine production upon *M. tuberculosis* infection

Involvement of the circulating T-cells during infection can be class specific depending on the cytokines they produce. Increasing understanding and appreciation of the different cytokine families began before 1977 when IL-1 was discovered (Dinarello *et al.*, 1977). Cytokines are classified into different families based on sequence homology, receptor chain similarities and functional properties with other proteins. Cytokine profiles have been used to divide $CD4^+$ T cells into different subsets. Major influences of the cytokine profiles in their microenvironment depends on the presentation of antigens by antigen presenting cells resulting in differentiation of $CD4^+$ naïve T-cells into TH1, TH2, TH9, TH17, TH22 and T-follicular effector cells (Fig. 5). TH2 response and inflammation occurs through the production of IL-4, IL-5, IL-9, IL-13 (Akdis, 2006; Larché *et al.*, 2006) resulting in production of IL-25, IL-31 and IL-33 (Bilsborough *et al.*, 2006; Dillon *et al.*, 2004; Kakkar and Lee, 2008; Kang *et al.*, 2005). TH1 immune response results through the production of IFN-γ by TH1 cells that provide protection from invading parasites (Akdis, 2006; Akkoc *et al.*, 2008). The major characteristic of TH17 is the expression of IL-17A, IL-17F, IL-6, IL-8, TNF-α, IL-22 and IL-26 (Burgler *et al.*, 2009; Harrington *et al.*, 2005). TH9 cells are produced from TH2 by the combination of IL-4 and TGF-β resulting in production of IL-9 and IL-10 (Veldhoen *et al.*, 2008). The largest subsets of T-cells are presented in the lymphoid tissues by T-follicular helper cells while providing helper functions to B cells (King *et al.*, 2008). Other well characterized regulatory T-cells (Treg) are known to regulate and counter balance the immune response. T-reg phenotypes include $CD4^+$ CD25 forkhead box 3 $(FoxP3)^+$ Treg cells as well as type1 (Tr1) cells, with distinct mechanisms of action (Akdis and Akdis, 2009; Klunker *et al.*, 2009). Other contributors of immune regulation are the γδ T-cells

(subset of $CD8^+$ T-cells), IL-10 producing B-cells, IL-10 producing NK-cells, DCs as well as macrophages (Akdis *et al.*, 2011).

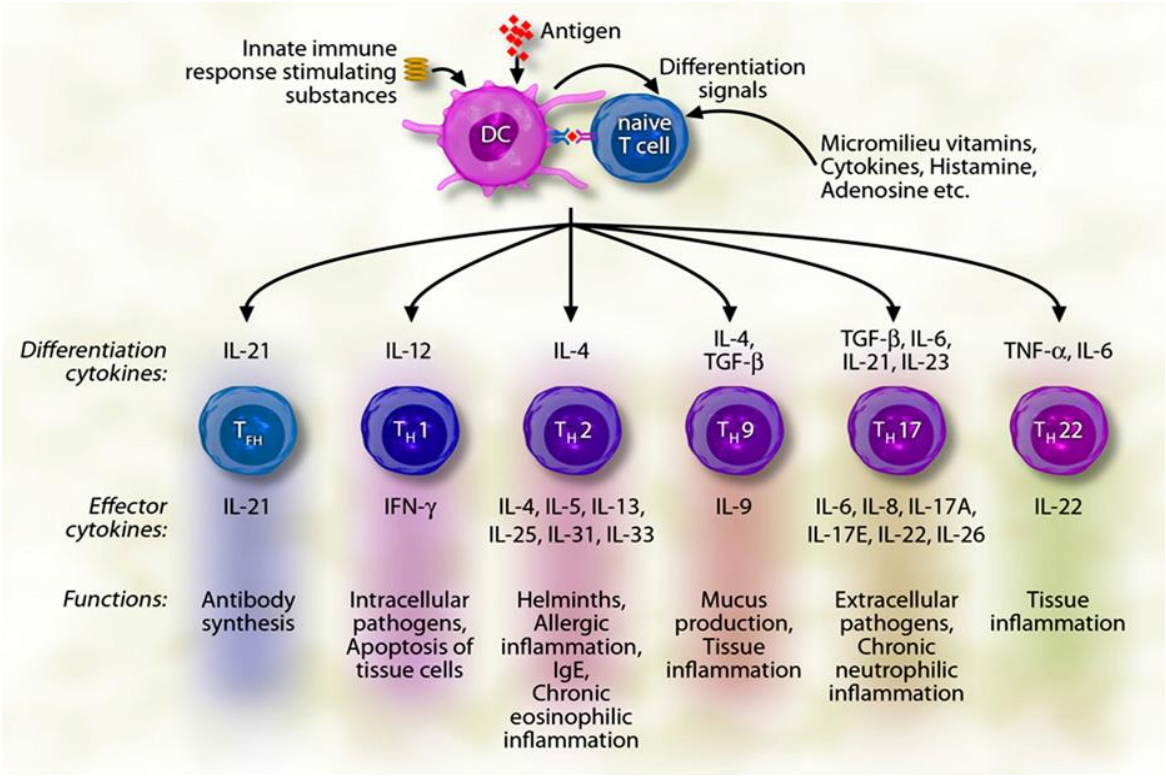


Fig. 5. Differentiation of naïve T-cells into different sub-sets that results in production of variety of cytokines that are involved in both innate and adaptive immune responses during infection or other inflammatory diseases (Akdis *et al.*, 2011).

TH1 cytokines are required for protective immunity against TB. Individuals with mutations in the genes coding for TH1 cytokines are highly susceptible to *M. tuberculosis* infections (Cooke *et al.*, 2006; Correa *et al.*, 2005; Ding *et al.*, 2008; Stein *et al.*, 2008). TH2 cytokines inhibit the production of IFN-γ and activation of macrophages, thus weakening the host defence system. Most TB patients are observed to secrete high levels of TH2 (Barnes *et al.*, 1993; Boussiotis *et*

al., 2000) cytokines that will prolong effective early response during *M. tuberculosis* infection. Both TH1 and TH2 cytokines are required for effective clearance of *M. tuberculosis*, provided they manifest at the right time of infection (Infante-Duarte and Kamradt, 1999). This TH1/TH2 balance is very critical for the outcome of *M. tuberculosis* infection and is required for an effective immune response while maintaining proper lung function.

The different components of bacterial cells that can elicit cytokine production include extracellular fimbriae, lipopolysaccharide and other extracellular components such as ESAT-6 (Rakotosamimanana *et al.*, 2010). These bacterial components provoke the network involved in cytokine production in a way that one cytokine can signal the induction of others. Different immune and non-immune cells produced specific cytokines and chemokines during *M. tuberculosis* infection (Carmona *et al.*, 2013; Lee *et al.*, 2009; Manca *et al.*, 2004) .

There are two major classes, pro- and anti-inflammatory cytokine production that are driven by *M. tuberculosis* infection, which determine the outcome of the infection. The release of pro-inflammatory cytokines leads to an inflammatory response through the cascade of pathways and networks that regulate their release and their action. The anti-inflammatory cytokines are released in order to antagonize the action of pro-inflammatory cytokines and to prevent inflammatory disorders such as severe lung pathology associated with excessive production of pro-inflammatory cytokines (Elenkov and Chrousos, 1999; van Crevel *et al.*, 2002). In addition to pro- and anti-inflammatory cytokines, chemokines are also produced during *M. tuberculosis* infection and these are crucial for a chemotactic effect of other cell types such as circulating granulocytes, macrophages, T-cells and dendritic cells (Algood *et al.*, 2003; Jang *et al.*, 2008; Lin *et al.*, 1998; Slight and Khader, 2013; Vesosky *et al.*, 2010; Yu *et al.*, 2012).

Pro-inflammatory cytokines

Pro-inflammatory cytokines are produced in response to *M. tuberculosis* infection and greatly contribute to the outcome of infection through induction of inflammation and recruitment of immune cells to the site of infection to control infection (van Crevel *et al.*, 2002). Epithelial cells and macrophages lining the alveolar surfaces are the first cells to encounter *M. tuberculosis* (Chuquimia *et al.*, 2012), and their contribution to the local inflammatory response is through the production of pro-inflammatory cytokines (Lee *et al.*, 2009; Lin *et al.*, 1998; Sato *et al.*, 2002). Pro-inflammatory cytokines are also produced by other immune cells such as T-cells, dendritic cells and NK cells which play a significant role during exposure to *M. tuberculosis* (Akdis *et al.*, 2011), however over production can result in severe immunopathology that can compromise normal lung function (Moreira *et al.*, 1997).

Interleukin-1 (IL-1)

IL-1 belongs to a family of pro-inflammatory cytokines that plays an important role in immune response by causing cell proliferation, differentiation and immune response to infection. This family consist of IL-1α, IL-1β as well as IL-18 that have minimal sequence homology but play an important role in the immune response (Akdis *et al.*, 2011). IL-1α is produced as a biologically active molecule while IL-1β and IL-18 need to be activated by caspase-1. IL-1 cytokines are produced by a variety of innate immune and non-immune cells including macrophages, epithelial cells, keratinocytes, dendritic cells, osteoblasts, endothelial cells and others especially those providing protection to underlying tissues. They tend to act on different types of cells causing hypotension, fever, and other immunological responses. They promote differentiation of $CD4^+$ T cells into TH17 cells in synergy with other cytokines (Akdis *et al.*, 2011). Within this family, IL-18 is a potent activator of IFN-γ in synergy with IL-12. High

expression of IL-1β at the site of infection observed in patients with active TB (Bergeron *et al.*, 1997; Law *et al.*, 1996; Schauf *et al.*, 1993) suggest an important role of this cytokine in effective immune response. This cytokine may also be associated with directing T-cells towards a TH1 response as significantly high secretion was observed in TB patients compared to healthy individuals (Katti, 2011). IL-1 production is crucial in development of early phase immune response to *M. tuberculosis* infection, as IL-1α/-β double knockout mice developed a significantly larger granulomatous response compared to the wild-type mice (Yamada *et al.*, 2000). Despite the crucial role of IL-1 in early response, excessive production of IL-1β was suggested to contribute to exacerbated pathology characterized by increased neutrophil influx to the lungs in IL-1 deficient mice treated (Mishra *et al.*, 2013). Decreased TH1 response was observed in IL-18 knockout mice during *M. tuberculosis* infection, resulting in uncontrolled intracellular growth. These findings suggest that IL-18 contribute to protection against mycobacterial infection while the balance is shifted towards TH2-biased immune response in the absence of IL-18 (Schneider *et al.*, 2010).

Interleukin-2 (IL-2)

IL-2 activates T-cells in response to infection and is produced primarily by $CD4^+$ and $CD8^+$ T cells, NK and DCs that have been stimulated by foreign antigens (Akdis *et al.*, 2011). IL-2 functions mainly by increasing T-cell response, even though excess production of this cytokine can lead to induced cell death as part of the protection against antigens. The role of IL-2 in growth and differentiation of B-cells allows for rapid response to previously exposed antigens (Akdis *et al.*, 2011). IL-2 is also intrinsically involved in development and peripheral expansion of $CD4^+$ $CD25^+$ regulatory T cells via CD25, CD122 and CD132 cell receptors (Nelson, 2004).

Mannering and Cheers (2002) showed that in early stages of infection of mice with *M. avium*, there was high expression and production of IL-2 which acts in synergy with IFN-γ to restore immune response mainly by T-cells. However, in the late stages of infection, the mice could not restore an immune response and production of IFN-γ regardless of IL-2, which indicates that the latter is important during early response of the host to mycobacterial infections (Mannering and Cheers, 2002). Individuals infected with Mycobacteria have a reduced ability to respond to IL-2. Their peripheral blood mononuclear cells displayed a defective IL-2 receptor response especially in immune-compromised individuals (Toossi *et al.*, 1986) and those with polymorphisms in IL-2 gene (Sivangala *et al.*, 2014) indicating its importance in cell mediated immunity. In addition to IFN-γ detection, IL-2 can be used as a diagnostic marker to detect and discriminate between active TB cases and latently infected individuals. This may provide better control of the disease due to early detection of latent infections (Biselli *et al.*, 2010)

Interleukin-6 (IL-6)

IL-6 is produced in significant quantities at the site of infection at the early stages of *M. tuberculosis* infection. A variety of cells including T-cells, B-cells, smooth muscles, eosinophils, chondrocytes, osteoblast (Akdis *et al.*, 2011) and epithelial cells (Lee *et al.*, 2009) produce IL-6 that has properties of both pro- and anti-inflammatory cytokines (VanHeyningen *et al.*, 1997). It can be harmful when produced in large quantities since it appeared to suppress production of TNF-α and IL-1β by interfering with their transcription (Schindler *et al.*, 1990) even though both cytokines provides protection during mycobacterial infections. It behaves like an anti-inflammatory cytokine because it binds to a gp130 receptor ligand much like other major anti-inflammatory cytokines (Opal and DePalo, 2000). Pro-inflammatory functions of IL-6 include

promoting B-cell differentiation, proliferation and production of immunoglobulins by B-cells (Akdis *et al.,* 2011; van Crevel *et al.,* 2002) and it ability to induce TH17 cells with other cytokines (Burgler *et al.,* 2009). IL-6 is one of the cytokines produced early during mycobacterial infection at the site of infection (Bellamy *et al.*, 1998; Law *et al.*, 1996; Okamura *et al.*, 1998) while another study suggested that IL-6 can be used as a potential diagnostic marker for TB infection (Singh and Goyal, 2013). IL-6 has also been suggested to have protective roles during *M. tuberculosis* infection as IL-6 deficient mice were more susceptible to infection compared to the wild type mice (Ladel *et al.*, 1997). This increased susceptibility might be related to the failure of early IFN-γ production before the development of adaptive T-cell immunity (Saunders *et al.*, 2000). Patients with large cavities secreted significantly higher levels of TNF-α, IL-1β and IL-6 compared to individual with smaller cavities. These findings suggests an inflammatory role of these cytokines in inducing tissue necrosis at the site of infection (Tsao *et al.*, 1999)

Interleukin-7 (IL-7)

IL-7 is a lymphopoetic cytokine produced by epithelial cells, keratinocytes, macrophages, as well as B and dendritic cells (Akdis *et al.,* 2011). IL-7 promotes the synthesis of other inflammatory cytokines in macrophages (Akdis *et al.,* 2011), and induces *bcl-2* expression in pre-T-cells prolonging their survival (Kim *et al.*, 1998; Niu and Qin, 2013). *M. tuberculosis* infected mice were shown to survive longer than controls when treated with IL-7. This was due to its ability to activate immune effector cells, mainly T-cells, and enhancing antimicrobial activity observed in infected macrophages compared to mice treated with IL-2, IL-4 and phosphate buffer saline (PBS) (Maeurer *et al.*, 2000). Despite the ability of IL-7 to increase

survival, it can also cause a deleterious effect. In mice treated with IL-7, anti-inflammatory cytokines (IL-4 and IL-10) were increased while IFN-γ was decreased in early infection in these mice (Maeurer *et al.*, 2000). Proliferation of memory phenotype $CD8^+$ cells is jointly promoted by IL-7 and IL-15, however, these cytokines were shown not to be required for the development of memory phenotype $CD4^+$ cells (Tan *et al.*, 2002). Mice treated with IL-7 and IL-15 showed significantly improved CD4 and CD8 T-cell memory response, enhanced T-cell proliferation, TH1 type cytokine production and increased production of multi-functional *M. tuberculosis*-specific memory T-cells (Singh *et al.*, 2010) demonstrating the role of both cytokines in T-cell memory functions and differentiation.

Interleukin-8 (IL-8)

IL-8 is a chemokine produced by epithelial cells, monocytes, lymphocytes, neutrophils and other immune and non-immune cells. The cytokine network and interaction is important during IL-8 production, which is stimulated by IL-1α, IL-1β, and TNF-α (Akdis *et al.*, 2011). IL-8 production can be driven by stimulation of both immune and non-immune cells exposed to *M. tuberculosis* lipopolysaccharides. Lin *et al*. (1998) showed that IL-8 production increases significantly and was maximal after day 6, post-infection. It was also shown to be strain specific in pulmonary epithelial cells infected with clinical and laboratory *M. tuberculosis* strains (Lin *et al.*, 1998).

Epithelial cells are important producers of IL-8 which serves as a chemo-attractant of T-cells, neutrophils, NK cells, basophils as well as eosinophils during infections (Akdis *et al.*, 2011; Lin *et al.*, 1998). Song *et al*. (2003) demonstrated increased IL-8 production during early *M. tuberculosis* infection of macrophages. The ability of *M. tuberculosis* to enter both epithelial

cells and macrophages allows for the rapid response by recruiting other immune cells to the site of infection. Intracellular growth rate is also important during IL-8 production as *M. tuberculosis*-infected macrophages produce lower levels of IL-8 compared to the fast growing *M. smegmatis* strain (Song *et al.*, 2003).

Interleukin-12 (IL-12)

Once infected with *M. tuberculosis*, phagocytic cells produce IL-12 that is known to induce IFN-γ production by the lymphoid cells at the site of infection, thereby connecting the innate and the adaptive immune responses. Sources of IL-12 production are antigen presenting cells usually at the site of infection (Hamza *et al.*, 2010). Mutations in *IL-12p40* gene or *IL-12R* gene for IL-12 protein production in humans were associated with increased susceptibility to *M. tuberculosis* infections due to reduced IFN-γ production and T-cell associated response (Raja, 2004). IL-12 is known as one of the immunoregulatory cytokines that induces differentiation of $CD4^+$ T cells into TH1 helper cells (Hamza *et al.*, 2010). Early expression of mRNA for the production of IL-12 compared to other cytokines during the T-cell differentiation has been shown to favour a TH1 over TH2 immune response. This was observed in mice infected with the highly virulent *M. tuberculosis* CDC1551 strain that induced an early production of IL-12 and IFN-γ, which were responsible for the control of mycobacterial growth resulting in longer survival times (Manca *et al.*, 1999).

Interleukin-15 (IL-15)

Activation and recruitment of T-cells and NK cells are stimulated by IL-15 production by monocytes, γδ T-cells and macrophages (van Crevel *et al.*, 2002). IL-15 is produced by

macrophages and non-immune cells such as keratinocytes and skeletal smooth muscles. Different types of stimuli, including bacterial LPS, trigger the activation of innate immunity. It shares some functions with IL-2 such as activation of T-cells indicating its role in the cytokine network (Akdis *et al.*, 2011). One of the important functions associated with IL-15 during *M. tuberculosis* infection is to induce differentiation of γδ T-cells into Vγ9Vδ2 T-cells that later become part of the memory cell pool. The effector memory cells have anti-mycobacterial activities hence the viability of the intracellular *M. tuberculosis* was reduced in their presence (Meraviglia *et al.*, 2010).

Tumor necrosis factor alpha (TNF-α)

TNF-α is released in response to *M. tuberculosis* stimulation of monocytes, macrophage (Valone *et al.*, 1988), dendritic cells (Henderson *et al.*, 1997) and pulmonary epithelial cells (Lee *et al.*, 2009). This suggests an important role of TNF-α in granuloma formation (Kindler *et al.*, 1989; Senaldi *et al.*, 1996), macrophage activation and immuno-regulatory properties (Orme and Cooper, 1999; Thurnher *et al.*, 1997), walling-off of the infection and prevention of dissemination of the pathogen (Raja, 2004). Since granulomatous response plays a crucial role in the control of TB, early production of TNF-α may account for increased survival of *M. tuberculosis* CDC1551 infected- compared to Beijing infected- mice with low TNF-α levels (Manca *et al.*, 1999). Production of TNF-α by epithelial cells stimulated with *M. tuberculosis* peaked at 18 hr and was reduced at subsequent incubation times (Lee *et al.*, 2009). Despite its advantages, and even though it has been thought to be important in preventing latent TB activation, elevated TNF-α in hosts infected with less virulent strains of TB may account for the unwanted inflammatory response such as fever and wasting, (Dheda *et al.*, 2010; Manca *et al.*,

1999; van Crevel *et al.*, 2002) and destruction of the lung tissue (Moreira *et al.*, 1997) that can compromise the lung function. This suggests that even though pro-inflammatory cytokines can be beneficial during infection, their overproduction can result in severe inflammation compromising proper lung function. TB patients have been observed to recover rapidly from deterioration through the reduction of TNF-α in plasma (Hsieh *et al.*, 1999). The negative effect of TNF-α can be limited by down-regulating the TNF-α gene (Friedland *et al.*, 1995) and increasing TNF-α receptors to reduce its level in blood plasma (Juffermans *et al.*, 1998) .

Interferon gamma (IFN-γ)

One of the important pro-inflammatory cytokines produced in response to mycobacterial infection is IFN-γ (also known as type II). IFN-γ is produced by NK cells, macrophages, T-cells bearing γ/δ T-cell receptors (Hedges *et al.*, 1995) and epithelial cells (Sharma *et al.*, 2007). Epithelial cells produce IFN-γ during *M. tuberculosis* infection, suggesting an important role for these cells in both innate (Sharma *et al.*, 2007) and adaptive immune responses (Nagabhushanam *et al.*, 2003). T-cell receptors respond rapidly to mycobacterial proteins and other non-protein components of *M. tuberculosis*. IFN-γ is known to up-regulate expression of the MHC class II antigens, secretory components and other intracellular adhesion molecules which are important for antigen presenting cells (Hedges *et al.*, 1995). It was observed that some *M. tuberculosis* strains have the ability to reduce the response of macrophages to IFN-γ reflecting the survival mechanism that has been adapted by this pathogen to survive in the presence of IFN-γ (Ting *et al.*, 1999). Interferon alpha (IFNI) and Beta (IFNIII) are also produced upon mycobacterial infections in both *in vitro* and *in vivo* infection models. Production of IFN-α was shown to promote *M. tuberculosis* infection, as IFN-α deficient mice had lower bacterial loads compared

to wild type (McNab *et al.*, 2013). IFN-β interferon was suggested to play an important role in epithelial cells immunity during *M. tuberculosis* infection (Bierne *et al.*, 2012).

Other important cytokines produced during *M. tuberculosis* include IL-17 and IL-23. IL-17 is an inflammatory cytokine that can induce production of other chemokines and recruiting cells to parenchymal tissues. IL-23 is the driving force of both IL-17 and Th17 cells through induction of Th17 production from naïve CD4+ T cells (Khader and Cooper, 2008). IL-23 is produced by dendritic cells exposed to *M. tuberculosis* (Khader *et al.*, 2005; Lockhart *et al.*, 2006), and together with IL-17, they contribute to the early control of infection. However it has been reported that over production of IL-17 may result in severe immunopathology of the lungs due to increased tissue damage and reduced fibrin deposition (Dragon *et al.*, 2008) and together with IL-23, they can alter the functional profile of neutrophils (Zelante *et al.*, 2007). This suggests that a crucial balance needs to be reached for production of both cytokines to effectively respond and control *M. tuberculosis* infections without compromising lung function.

Some pro-inflammatory cytokines are beneficial, while others are harmful during mycobacterial infection. Many pro-inflammatory cytokines have been observed to act in synergy forming a cytokine network to provide a more efficient immune response during *M. tuberculosis* infection (Fig. 6) (van Crevel *et al.*, 2002).

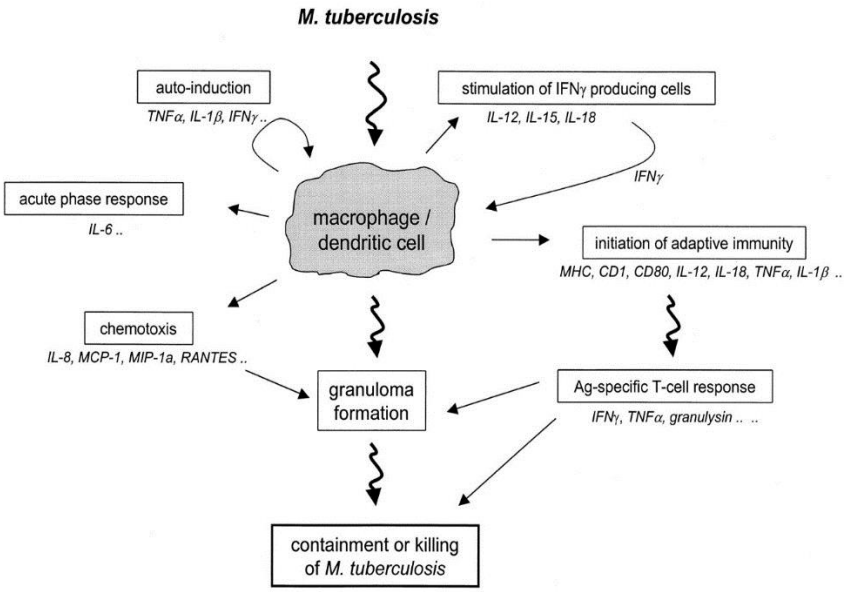


Fig. 6. Response of macrophage and dendritic cells infected with *M. tuberculosis* (van Crevel *et al.*, 2002). When innate cells are infected with *M. tuberculosis*, they secrete cytokines and trigger cascades of events that results in granuloma formation and involvement of other cells to render immunity at the site of infection.

Anti-inflammatory cytokines

Anti-inflammatory cytokines antagonize the action of the pro-inflammatory cytokines, making individuals more susceptible to *M. tuberculosis* infections. These include IL-1ra, IL-4, IL-10, IL-11, IL-13 and transforming growth factor beta (TGF-β) (Opal and DePalo, 2000). Anti-inflammatory cytokines may be produced at the site of infection by macrophages, T-cells, dendritic cells (Jin *et al.*, 2014; Opal and DePalo, 2000) and epithelial cells (Lutay *et al.*, 2014). Some of the pro-inflammatory cytokines such as IL-6 and IL-11 have both pro- and anti-inflammatory properties (Akdis *et al.*, 2011; van Crevel *et al.*, 2002).

Interleukin-1ra (IL-1ra)

IL-1ra belongs to the IL-1 family and its release is activated by the same stimulus that causes IL-1 production. It is commonly known to be effective in inhibiting IL-1 cytokine activity by binding to either IL-1RI or IL-1RII receptors. This causes the failure of IL-1 binding by competitive inhibition, thus reducing its production (Akdis *et al.*, 2011; Arend and Guthridge, 2000) and contribute to the control of increased inflammation caused by IL-1. IL-1ra is secreted by a variety of cells including monocytes, endothelial and epithelial cells (Akdis *et al.*, 2011). To be an effective inhibitor, at least 100-fold should be secreted due to the high number of free IL-1RI and IL-1RII receptors in a variety of cells (Arend and Guthridge, 2000). Maintenance of the IL-1 and IL-1ra in the body is important because an imbalance between these two causes progressive diseases such as lupus and increased susceptibility to viral infections (Akdis *et al.*, 2011). The role of IL-1ra in TB infection was demonstrated by Wilkinson *et al.* (1999) where polymorphism in the gene coding for IL-1ra resulted in an increased production of anti-inflammatory cytokines IL-4 and IL-10 (Wilkinson *et al.*, 1999). Since IL-1ra is secreted at high levels in individuals with TB, it was shown to be one of promising biomarker targets for TB infections (Ruhwald *et al.*, 2009).

Interleukin-4 (IL-4)

IL-4 is predominantly produced by TH2 and other immune cells which include basophils, eosinophils, mast cells and other CD-1 cells (Akdis *et al.*, 2011). Production of IL-4 causes suppression of IFN-γ and macrophage activation, thus suppressing cell-mediated immunity and weakening the host defence mechanism and can also serve as an effector cytokines for TH2 cells to produce IL-4, -5, -13, -25, -31 and -33. The ability of IL-4 to weaken host defence mechanism

was demonstrated in *M. tuberculosis* infected mice with anti-IL-4 treatment that caused reduction in the lungs bacillary load, thus activating TH1 response (Buccheri *et al.,* 2007). Similar results were also seen with the IL-4 knockout mice that had reduced bacterial burden compared to the wild type (Roy *et al.*, 2008). This supports numerous hypotheses that cytokines inducing an anti-inflammatory response such as IL-4can enhance the pathological state of the host, thus reducing the ability to fight infections (Roy *et al.*, 2008). Therefore, in order to successfully fight *M. tuberculosis* infections more pro- instead of anti-inflammatory cytokines should be secreted by the host. However, this needs to be a tightly controlled process since excessive inflammation may cause immunopathology to the host lung tissue (Saunders and Britton, 2007).

Interleukin-10 (IL-10)

Most cells including B-cells, T-cells, macrophages and dendritic cells have the ability to produce IL-10. Compared to other TH2 cytokines, IL-10 is different in that it is produced by TH1, TH2 and TH17 cells and this is beneficial to the body to neutralize the net inflammatory effect of TH1 cytokines. IL-10 prevents immuno-pathology that is associated with the production of high levels of pro-inflammatory cytokines. Gene expression and production of IL-10 in *M. tuberculosis* strain infected monocytes/macrophages was shown to be dependent on the type of infecting strain (Manca *et al.*, 2004; Tanveer *et al.*, 2009; Wang *et al.*, 2010).

IL-10 production provides protection against an uncontrolled immune response during infections to prevent autoimmune diseases. The effectiveness of IL-10 as an anti-inflammatory cytokine lies in its ability to inhibit myeloid cells (which are macrophages and DCs), to activate TH1 subsets that can produce a variety of pro-inflammatory cytokines (such as IL-1α, IL-1β, IL-6, IL-

12, IL-18, TNF-α), as well as beneficial chemokines such as [monocyte chemo-attractant protein-1, macrophage inflammatory protein (MIP)-1 (α and β), Rantes, inducible protein (IP)-10] that contribute to the TH1 response (Akdis *et al.*, 2011; Redford *et al.*, 2010). Though beneficial during inflammation, IL-10 can induce down-regulation of surface expression of class II MHC molecules and co-stimulatory molecules CD80/CD86 on monocytes and macrophages (de Waal Malefyt *et al.*, 1991). Similar to other anti-inflammatory cytokines, IL-10 production during *M. tuberculosis* infection is not beneficial to the host since it was reported to increase susceptibility of the host to infection (Boussiotis *et al.*, 2000). Beamer *et al.* (2008) showed enhanced survival of the mice treated with anti-IL10RI antibodies. This led to an increased production of CD4 and CD8 cells that have the ability to produce beneficial cytokines such as IFN-γ during infection (Beamer *et al.*, 2008). Increased IL-10 production was observed in serum and lungs of individuals with pulmonary TB (Almeida *et al.*, 2009; Deventer *et al.*, 1999), indicating its involvement during *M. tuberculosis* infection.

Failure to respond to TH1 cytokines may be associated with the growth rate of a particular mycobacterial species (Mege *et al.*, 2006). Compared to actively reproducing strains, slow-growing and latent *M. tuberculosis* strains induce diminished production of IL-10, allowing the host to respond and control the infection. This also holds true for HIV-TB co-infected patients with low IL-10 production when compared to patients with advanced AIDS. This indicates that IL-10 production is associated with the host's failure to fight infection due to its anti-inflammatory properties (Mege *et al.*, 2006).

Interleukin-11 (IL-11)

IL-11 has both pro- and anti-inflammatory properties and performs a variety of functions which include immuno-modulation and epithelial cell protection (Kapina *et al.,* 2011). This cytokine is produced mainly by epithelial cells, stromal cells, fibroblasts, osteoblasts, synoviocytes and tumor cell lines. Like other anti-inflammatory cytokines, IL-11 suppresses the production of pro-inflammatory cytokines such as TNF-α, IL-1, IL-12 as well as IFN-γ (Akdis *et al.,* 2011). Despite this, IL-11 is associated with important functions such as neuronal development, adipogenesis, and bone marrow recovery as well as platelet production (Akdis *et al.*, 2011; Kapina *et al.,* 2011). One of the well-known function of IL-11 is involvement in stimulating hematopoiesis by supporting the proliferation of myeloid, erythroid, and megakaryocyte progenitor cells (Paul and Schendel, 1992). In an *M. tuberculosis* infected mouse model, Kapina *et al*. (2011) showed that the lung pathology and other key cytokines (IL-6, TNF-α and MIP-2) diminished when IL-11 was neutralized with anti-IL-11 antibodies. This reduction indicates that IL-11 has both pro- and anti-inflammatory properties and might be involved in the cytokine network for production of both TH1 and TH2 cytokines (Kapina *et al.*, 2011).

Interleukin-13 (IL-13)

IL-13 is a well-known anti-inflammatory cytokine belonging to TH2 family, however it may also be involved in recruitment of eosinophils and mast cells during parasite infection (Akdis *et al.*, 2011) and can serve as effector cytokine through regulating B-cell mediated immunity (Wynn, 2003). It functions by directly inhibiting macrophage activity and inducing down-regulation of the nitric oxide synthesis as well as the Toll like receptor-2 pathway (Ghadimi *et al.*, 2010). The main cells producing IL-13 are basophils, mast cells, eosinophils and a subset of T-cells. The

anti-inflammatory properties of this cytokine was seen in IL-13 deficient mice that secrete low levels of other TH2 cytokines such as IL-4, IL-5 and IL-10 (Akdis *et al.*, 2011). High levels of IL-13 were associated with increased susceptibility of mice to *Chlamydia muridarum*, reduced uptake of the pathogen by epithelial cells and increased intracellular bacterial growth *in vitro* (Asquith *et al.*, 2011). Like other pathogens such as *Chlamydia*, *M. tuberculosis* uses multiple strategies to survive within the host. One such strategy is to inhibit protective autophagy in the immune cells by inducing high levels of TH2 cytokines. This was observed in macrophages derived from peripheral blood mononuclear cells (PBMCs) where *M. tuberculosis* infection inhibited protective cell death with an increased induction of IL-4 and IL-13. This suppressed the TH1 immune response rendering the host more susceptible to infections (Ghadimi *et al.*, 2010).

Chemokines

During infection, there is constant communication of the different cell types resulting in immune cell trafficking into the affected tissues to confer increased immunity. This communication and movement is facilitated by adhesins, selectins, chemokines as well as chemokine receptors (Algood *et al.*, 2003). There are four chemokines subfamilies that are classified according to the location of their cysteine residues. These families are C, CC, CXC and CX3C (Zlotnik *et al.*, 2006), and they bind to their respective receptors that are expressed in different cell types (Slight and Khader, 2013) (Fig. 7). The CC chemokines commonly act on CCR receptors while the CXC chemokines bind to CXCR receptors (Borish and Steinke, 2003; Slight and Khader, 2013). Other chemokine receptors include XCR and CX3CR which are used by chemokines belonging to XCL and CX3C families, respectively (Borish and Steinke, 2003; Zlotnik *et al.*, 2006).

The common stimulus that results in chemokine production by both immune and non-immune cells as well as mice models is an inflammation that occurs when cells are infected (Algood *et al.*, 2003; Chuquimia *et al.*, 2012; Lin *et al.*, 1998). *M. tuberculosis* has antigens to stimulate production of different chemokines in macrophages (Sadek *et al.*, 1998; Saukkonen *et al.*, 2002), pulmonary epithelial cells (Chuquimia *et al.*, 2012; Lee *et al.*, 2009; Lin *et al.*, 1998; Sato *et al.*, 2002) and mice models (Jang *et al.*, 2008; Manca *et al.*, 2004; Vesosky *et al.*, 2010). Production of different chemokines at the site of infection (mostly lung tissue) lined by macrophages and different types of epithelial cells ensures an effective involvement of circulating monocytes (Shi and Pamer, 2011), T-cells (Lee *et al.*, 2009; Lin *et al.*, 1998), as well as formation of the granuloma (Algood *et al.*, 2003; Silva Miranda *et al.*, 2012) during *M. tuberculosis* infection.

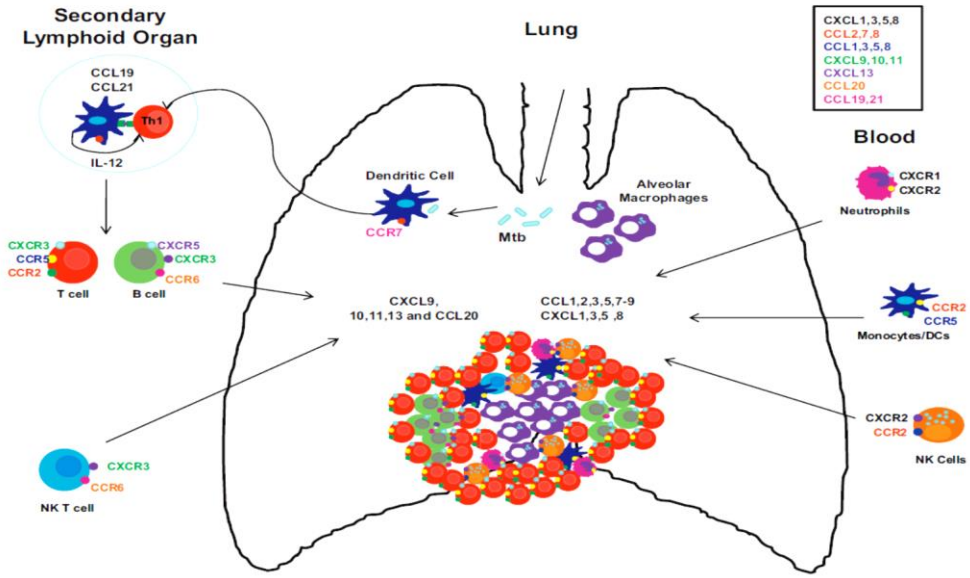


Fig. 7. Communication between the lungs and circulating immune cells using chemokines and their respective chemokine receptors (Slight and Khader, 2013).

Chemokines that significantly contribute to TH1 immune response were produced in murine dendritic cells and macrophages during *M. tuberculosis* infection (Jang *et al.*, 2008) indicating a cell specific chemokine response in different immune cells. Lung epithelial cells were shown to be a source of CCL5 *in vitro* (Nouailles *et al.*, 2014), while in a mouse model, it provided early protection against *M. tuberculosis* infection (Vesosky *et al.*, 2010). This demonstrates a crucial role played by the CCL5 chemokine during *M. tuberculosis* infection both *in vitro* and *in vivo* infection models (Nouailles *et al.*, 2014; Vesosky *et al.*, 2010). Chemokines can also be an indication of *M. tuberculosis* infection and disease occurrence as they were shown to be elevated in individuals with tuberculosis compared to healthy controls (Yu *et al.*, 2012).

Transcriptomics and cytokine/chemokine profiling

Techniques to study gene expression

The use of recent and newly improved molecular techniques has revealed complex molecular mechanisms that are involved in cell differentiation (Harrison, 1990; Lin *et al.*, 2011; Luo *et al.*, 2014; Uskokovic *et al.*, 2009), specific plant molecular markers (Thavamanikumar *et al.*, 2014), single nucleotide mutations (Morin *et al.*, 2008) and more complex isoform switching during gene expression (Trapnell *et al.*, 2010). This began with the introduction of polymerase chain reaction (PCR) (Mullis, 1990; Saiki *et al.*, 1985) that allowed quantifying a single gene in a sample. It was one of the great discoveries in molecular biology that was able to reveal the presence or absence of a gene and its abundance between samples (Garibyan and Avashia, 2013; Saiki *et al.*, 1988).

PCR was a widely used technique over the years (Bustin, 2000; Kotik, 2009; Steffan and Atlas, 1991) and still being used to date (Rak *et al.*, 2014). Quantitative reverse transcriptase PCR (qRT-PCR) was introduced after standard PCR (Higuchi *et al.*, 1993) and used for several applications (Raja *et al.*, 2002; Timken *et al.*, 2005). The major limitation with PCR and qRT-PCR is in the number of genes that can be analysed per sample. This shortcoming was alleviated by the use of microarray that was able to show differential expression of the known genes in the organism's transcriptome (Sinicropi *et al.*, 2007). This is very important when trying to understand not only changes in the genes but their interactions which results in complex pathways during infection. The microarray technique was used for years and is still being used for different applications, however the major drawbacks are the inability to detect small changes in gene expression due to low sensitivity (Czechowski *et al.*, 2004), and not all genes of interest may be immobilized as probes in the microarray tile. In addition, the challenges with microarray data analysis include trying to optimize different brands of library pre-processing methods that giving rise to heterogeneous data formats. Moreover, standard analysis tools cannot be applied to different types of microarrays such as gene expression, SNPs and miRNA arrays tools which requires specific type of data analysis (Guzzi and Cannataro, 2011).

Next Generation Sequencing (NGS) technology is able to overcome some of the microarray limitations, and has allowed better understanding of microbial physiology (Sorek and Cossart, 2010), human genetics (Pickrell *et al.*, 2010), plant diversity (Martin *et al.*, 2013) and also "key players" that are involved during the disease state of the organism (Prasopdee *et al.*, 2014). Furthermore, NGS has several advantages over the microarray technique. These include the

ability to interrogate and quantify the organism's whole transcriptome without background signal that interferes with the down-stream analysis (Nookaew *et al.*, 2012).

The use of NGS has created endless possibilities in biomedical research (Elingarami *et al.*, 2013), drug discovery (Dopazo, 2014), metagenomics (Hu *et al.*, 2011) and human-pathogen interaction (Westermann *et al.*, 2012). RNA-Seq is an NGS based technology that allows quantifying different types of RNA molecules in different tissues (Guo *et al.*, 2014; McGettigan, 2013), detect transcripts that are expressed at a low levels (Zhao *et al.*, 2014), identify novel transcripts, alternative splicing and novel isoforms switching (Park *et al.*, 2013; Trapnell *et al.*, 2010), studying complex transcriptomics (Wang *et al.*, 2009) and analysing of DNA-binding proteins (Kechavarzi and Janga, 2014).

Furthermore, RNA-Seq can detect unannotated exons, determine overall and exon-specific expression, and to assay allele specific expression levels that was not possible with previous molecular techniques (Pickrell *et al.*, 2010). The disadvantages associated with the use of RNA-Seq include the need for high quality RNA samples, high cost of deep sequencing of RNA transcripts and extensive bioinformatics support required to analyse the huge volume of data generated in order to obtain differential gene expression of targeted transcripts (Wang *et al.*, 2009). Gene expression studies are essential to support the data obtained from different protein (e.g. cytokine) assays that may not be sensitive enough to be detected in culture supernatant or blood samples due to being used by cells themselves or produced in an undetectable form (Bienvenu *et al.*, 2000).

Techniques to study cytokine/chemokine profiling

The detection of immunological markers in samples has been previously explored using the well-

known and validated Enzyme-Linked Immuno-Sorbent Assay (ELISA). The use of ELISA is considered as a gold standard for sensitive detection of the cytokine of interest in samples with quantitative and reproducible results. Despite the global use of ELISA for cytokine detection, this technique is limited to detecting only one or very few proteins in samples. Other disadvantages include variability that can be introduced because of differences in antibody quality from different manufacturers and inability to detect cytokines that are out of range in a linear relationship between cytokine concentrations and absorbance reading. Moreover, limited number of cytokines detectable in a single sample by ELISA can limit the understanding of complex interplay of different immunological markers in clinical and biomedical research (Leng *et al.,* 2008).

The recent and highly appreciated cytokine multiplexing technology has been used to detect multiple analytes in the same sample. The Luminex technology apply the use of hundreds of specially prepared micrometer beads internally stained with a mixture of red or infrared fluorescent dyes. The different levels at which these beads are dyed creates hundreds of different fluorescence profiles that can be interrogated individually and classified into different samples (Tighe *et al.,* 2013). Excitation of these beads by a laser from the flow cytometry-based instruments results in emission of light at different wavelengths. The bead surfaces are then conjugated with specific capture antibody for each cytokine which is then differentiated from other beads by its specific spectral light emission. Each of these beads is then incubated with samples (supernatants, blood, etc) for multiplexing purposes in a 96-well plate. These beads can be mixed for detecting up to 100 samples in each well through specific light emission for each cytokine. This is followed by a wash step and thereafter the cocktail of detection antibodies

which are conjugated with fluorescent dyes are added providing distinctions among cytokines of interest. Interaction between cytokines and unique "dyes" within beads allows differentiation between cytokines while the intensity of fluorescence in reporter dye–conjugated detection antibody quantify relative abundance of the cytokines (Khalifian *et al.*, 2015; Tighe *et al.*, 2013). The data analysis plan is usually included within the Luminex software that is used to quantify analytes of interest. Graphical presentations of the data with replicates variations can be used within the software installed on the Bioplex machine (BioRad). The data can also be exported from the software and be analyzed with other statistical software programmes such as SPSS, Genestat and Graph-pad prism.

The advantages offered by the Luminex technology include higher sensitivity and detection ranges (Quinn *et al.*, 2008), less volume of sample needed, high throughput multiplex analysis, reduced time and cost required for data acquisition, evaluation of one analyte in relation to others in a same sample and lastly detecting multiple analytes with dynamic ranges of detection in one sample (Leng *et al.*, 2008; Wang *et al.*, 2005). Therefore, this technology has been used in studying inflammatory response in chronic allergic diseases (Leonardi *et al.*, 2006), immunological changes during mycobacterial infection (Singh and Goyal, 2013) and understanding complex interplays of cytokines in the immune system (Szodoray *et al.*, 2007). One of the major disadvantages of using the Luminex technology is the need for expensive analysis instrument (Khalifian *et al.*, 2015). Variations in sample concentrations can be observed with the use of kits from different manufacturers, product number and assay preparation by different laboratories (Djoba Siawaya *et al.*, 2008; Khalifian *et al.*, 2015).

Host transcriptome changes during *M. tuberculosis* infection

Changes to the overall transcriptional profiles of the immune and non-immune cells occur in response to *M. tuberculosis* infections. These changes may include different molecular networks, transcriptional factors and pathways that are regulated to determine the outcome of the infection. Transcriptomics revealed diverse molecular changes in blood samples between healthy, Bacillus Calmette-Guerin (BCG) vaccinated and patients with active tuberculosis. All three clinical groups were able to show different molecular signatures that can be explored for biomarker discoveries (Lesho *et al.*, 2011). The study by Cliff *et al.* (2012) revealed changes in immune regulation and gene expression in patient's blood under anti-mycobacterial treatment. Their findings demonstrated an early down-regulation of the inflammatory mediators suggested to be linked to killing of the actively dividing bacilli (Cliff *et al.*, 2012). Another i*n vivo* study describing global transcriptome analysis of human blood samples provided evidence of molecular signatures that were promising for vaccine discovery and drug therapies. These interferon inducible signatures were also able to discriminate tuberculosis from other similar infectious diseases (Berry *et al.*, 2010). Molecular signatures identified in pathogenesis studies demonstrate "unique" immunological changes observed during *M. tuberculosis* infection. These signatures can also reveal different stages of infection that can potentially be explored for TB biomarkers or monitoring anti-TB response.

The response of cells that are at the site of infection is important because they alert the host to invasion by a foreign antigen. Broncho-Alveolar Lavage (BAL) fluids (Raju *et al.*, 2008) and Peripheral blood mononuclear cells (PBMCs) (Grassi *et al.*, 2006) have been used to study the host response to infection and overall transcriptional response at the site of infection. Raju *et al.* (2008) compared changes in gene expression between *M. tuberculosis* infected and healthy

individuals. They reported an increase in expression of many immunologically relevant genes in BAL cells. Up-regulated genes included surface markers ranging from CD9-CD68, some belonging to TH1 and TH2 immune response, cytokines, chemokines and immune relevant transcriptional factors. These surface markers are important in increasing the response efficiency of the cells to cytokines and chemokines that are produced during the infection. Most transcriptional factors that were expressed belong to TH1 immunity. These included signal transducer and activator of transcription-4 (*STAT-4*) and *STAT-1* genes, while for the TH2 immunity, only the *STAT-6* gene was expressed and phosphorylated. This reflects the imbalance observed in transcriptional factors that will allow the shift in the type of cytokines that will be produced during TB infection (Raju *et al.*, 2008).

Grassi *et al*. (2006) also analysed the BAL fluid and reported a two-fold increase in expression of a variety of genes coding for cytokine production, cytokine/chemokine receptors, signal transduction, protease and apoptosis related genes in infected patients compared to their healthy counterparts. This indicates that cytokines of the TH1 immunity are expressed during infection (Grassi *et al.*, 2006). Failure of the host to eradicate the pathogen may be due to the inability to express the necessary transcriptional factors required for effective immune response. This was observed in three IFN-γ genes, whose expression was not changed because of the failure to activate interferon regulatory factor-1 (*IRF-1*) and *STAT-1* required for *IFN-γ* expression. This resulted in a lack of responsiveness by the host to the cytokines produced and subsequent failure to eliminate *M. tuberculosis* (Grassi *et al.*, 2006).

Gene expression profiling using real time PCR and microarray analysis have been used to understand the macrophage cell response to *M. tuberculosis* infection. Increased mRNA expression was detected by microarray for genes coding for cytokines, chemokines, binding

proteins, adhesion molecules and different signal transducers in *M. tuberculosis* infected macrophages (Volpe *et al.*, 2006). Pro- (*IFN-γ* and *TNF-α*), anti-inflammatory (*IL-10*) associated genes, and chemokines were also expressed in macrophages in response to *M. tuberculosis* infection (Almeida *et al.*, 2009). Expression levels of *IL-10* increased only for one day and declined in the subsequent days with no interaction observed between IL-10 production and inhibition of the IFN-γ, which is an active and important cytokine to inhibit mycobacterial growth *in vivo* (Volpe *et al.*, 2006).

In an *in vitro* study, DCs had higher expression of immune associated genes compared to macrophages as well as different expression signatures that are specific for these cell types during *M. tuberculosis* infection (Tailleux *et al.*, 2008). To date, only qRT-PCR has been used to study cytokine/chemokine genes in *M. tuberculosis* infected pulmonary epithelial cells. Lin *et al*. (1998) evaluated the expression of five cytokine/chemokine genes by A549 epithelial cells infected with H37Ra strain using qRT-PCR. Increased mRNA levels of *MCP-1* and *IL-8*, while three chemokine transcripts *Rantes*, *MIP-1α* and *MIP-1β* could not be detected. Similarly, Sato *et al*. (2002), showed increased expression of both *MCP-1* and *IL-8* in TB-infected A549 cells. In addition, increased mRNA expression of the pro-inflammatory cytokines, *TNF-α*, and granulocyte-macrophage colony-stimulating factor (*GM-CSF*) were also observed in these studies. Lee *et al*. (2009) observed increased expression of *IL-6, IL-8* and *TNF-α* when epithelial cells where exposed to *M. tuberculosis* using qRT-PCR (Lee *et al.*, 2009), while Sharma *et al*. (2007) detected increase in *IFN-γ* expression using flow cytometry. These findings indicate that epithelial cells respond rapidly to infection by increasing cytokine gene expression and possible production, thereby attracting macrophages to the infected site (Lee *et al.*, 2009; Lin *et al.*, 1998; Sato *et al.*, 2002; Sharma *et al.*, 2007).

Rationale for present study

The prevalence and spread of *M. tuberculosis* drug susceptible and resistant strains within the South African population (Muller *et al.*, 2013; Streicher *et al.*, 2004; Streicher *et al.*, 2012) and globally (de Viedma *et al.*, 2005; Glynn *et al.*, 2010; Glynn *et al.*, 2002; Valway *et al.*, 1998) has motivated an increasing necessity for a better understanding of the host response during infection by these strains. Several studies (Chen *et al.*, 2014; Lin *et al.*, 1998; Manca *et al.*, 1999; Portevin *et al.*, 2011; Rakotosamimanana *et al.*, 2010) have reported differential host response during infection by clinical strains of *M. tuberculosis* belonging to different genotype families and lineages. Moreover, host response has been reported during infection with either a laboratory strain H37Rv or few clinically relevant strains *in vivo* (Lopez *et al.*, 2003; Manca *et al.*, 1999; Schaale *et al.*, 2013) and *in vitro* (Carmona *et al.*, 2013; Chakraborty *et al.*, 2013; Chuquimia *et al.*, 2012; Faksri *et al.*, 2014; Lee *et al.*, 2009; Lin *et al.*, 1998; Sharma *et al.*, 2007).

The clinical manifestation of *M. tuberculosis* complex is highly conserved during infection (Homolka *et al.*, 2010). However, several virulence factors have been associated with hypervirulent clinical strains such as those belonging to the Beijing genotype (Reed *et al.*, 2004), causing differential and strain specific response in *in vitro* and *in vivo* pathogenesis studies (Koo *et al.*, 2012; Manca *et al.*, 2004; Subbian *et al.*, 2013). Despite the extensive work on *in vitro* and *in vivo* studies using clinical strains of *M. tuberculosis*, very limited studies have focused on pulmonary epithelial cells infected with such strains (Lin *et al.*, 1998), despite their abundance in the alveolar space. To date, no study had reported on comprehensive transcriptomics in pulmonary epithelial cells infected with clinical strains of *M. tuberculosis* belonging to different genotype families.

This study investigated the response of pulmonary epithelial cells during infection with clinical strains of *M. tuberculosis* by measuring cytokine/chemokine kinetics over time using the Bio-Plex assay. The epithelial cell global gene expression, pathways, networks and transcriptional factors induced by different strains were elucidated using the highly sensitive RNA-Seq technique and different downstream bioinformatics software programmes. Furthermore, strain specific molecular signatures and other molecular mechanisms induced by these different strain families in epithelial cells at an earlier time of infection were analysed.

Objective

To study host-pathogen interactions induced by different clinical strains of *M. tuberculosis* in an *in vitro* epithelial cell model

Aims

To decipher immune responses in pulmonary alveolar epithelial cells infected with *M. tuberculosis* by measuring cytokine/chemokine kinetics over time;

To measure global gene expression of human, including immune response genes in pulmonary alveolar epithelial cells infected with clinical strains of *M. tuberculosis* at 48 hr post-infection; and

To decipher the datasets in terms of pathways, networks and transcriptional factors in pulmonary alveolar epithelial cells infected with clinical strains of *M. tuberculosis* at 48 hr post-infection.

Chapter summary

Chapter 1: Review of relevant literature

Chapter 2: Virulent *Mycobacterium tuberculosis* strains induce strain specific *in vitro* cytokine and chemokine responses in pulmonary epithelial cells.

Clinical strains of *M. tuberculosis* belonging to 4 genotype families and Unique genotype were used to infect pulmonary epithelial cells and supernatants were collected at 24, 48 and 72 hr post-infection. Cytokine production increased gradually with peak production at 48 or 72 hr after infection. All strains induced a strain specific cytokine/chemokine production at different times post-infection. The Beijing and Unique strain induced lowest concentration of cytokines/chemokines compared to other clinical strains. These findings may suggest the presence of different virulence factors among clinical strains and can explain transmissibility of Beijing strain globally, while similar patterns were unexplained for the Unique strain. These findings also suggest that the F15/LAM4/KZN, F11 and F28 strains may be less virulent than the Beijing strain, and that their enhanced transmission may be due to the lack of stringent TB control measures rather than hypervirulence.

Chapter 3: *Mycobacterium tuberculosis* clinical strains exhibit differential gene expression and strain-specific molecular signatures in pulmonary epithelial cells.

Total RNA was extracted from pulmonary epithelial cells infected with clinical strains of *M. tuberculosis* at 48 hr post-infection. RNA-Seq was performed and differentially expressed genes

were compared among clinical strains. Lowest fold changes were induced by the Unique strain while the highest were induced by the F15/LAM4/KZN and F11 strains. The highly virulent strains of *M. tuberculosis* hierarchically clustered separately compared to the less virulent Unique strain suggesting a relationship between strain virulence and gene expression. Strain specific genes varied among the strains, along with their associated Kegg and Reactome pathways that have a potential to be explored for biomarker applications in future studies.

Chapter 4: Canonical pathways, networks and transcriptional factors induced by clinical strains of *M. tuberculosis* in pulmonary alveolar epithelial cells

Differentially expressed genes in pulmonary epithelial cells were used to investigate different canonical pathways, networks and transcriptional factor changes that are regulated by clinical strains. The interferon signalling and Hepatic Fribrosis/Hepatic Stellate Cell Activation pathways were among the top 5 activated pathway by all strains. The Beijing and Unique strains induced increased enrichment in all cholesterol biosynthesis pathways while other strains induced low enrichment of these pathways. Network activation/down-regulation also differed among the strains indicating diversity in gene expression and network enrichment among clinical strains. There were common transcriptional factors among the strains while others were strain specific and might be responsible for down-stream changes and complex pathway enrichment that occur within the host due to infection by different clinical strains of *M. tuberculosis*. Strain specific molecular mechanisms may be explored for host-directed therapy during infection with *M. tuberculosis* strains.

CHAPTER 2: VIRULENT *Mycobacterium tuberculosis* STRAINS INDUCE STRAIN SPECIFIC *IN VITRO* CYTOKINE AND CHEMOKINE RESPONSES IN PULMONARY EPITHELIAL CELLS

Abstract

The *M. tuberculosis* F15/LAM4/KZN, F11, F28 and Beijing strain families have been associated with high transmission rates within the South African population. The current study focused on elucidating the cytokine/chemokine response induced by these strain families of *M. tuberculosis* in pulmonary epithelial cells. The A549 pulmonary epithelial cell line was infected with the F15/LAM4/KZN, F28, F11, Beijing, Unique and H37Rv strains and the supernatant harvested at 0, 24, 48 and 72 hr for cytokine/chemokine analysis using the Bio-Plex Pro Human Cytokine Group 27-Plex Assay. Among the 23 cytokines/chemokines that were detected, a significant production of anti-inflammatory and pro-inflammatory cytokines, as well as chemokines was observed at different time intervals. All strains induced a gradual increase in cytokines/chemokines that peaked mostly at either 48 or 72 hr post-infection. However, production of each cytokine/chemokine was observed to be strain and time specific. High cytokine levels were induced by the F28 strain at 48 hr and F15/LAM4/KZN strain at 72 hr for most analytes. Lower cytokine production was induced by the Beijing and Unique strains at all-time intervals, suggesting a higher virulence of these strains compared to other strain families. On the other hand, the F15/LAM4/KZN strain induced high protective cytokine production, suggesting a lower virulence for this strain. Cytokine/chemokine production by the laboratory H37Rv was variable with either a higher or lower concentration for some analytes compared to

clinical strains. These findings would suggest that the widespread transmission of the F15/LAM4/KZN strain within SA population may not be directly associated with the virulence of the strain, but rather to the failure of proper control measures. The results of this study make a strong case to conclude that virulent *M. tuberculosis* strains induce specific *in vitro* cytokine and chemokine responses in pulmonary epithelial cells.

Key words: Pulmonary epithelial cells, cytokines, chemokines, *M. tuberculosis*, strain-specific

Introduction

TB remains one of the major causes of mortality worldwide, leading to approximately 1.5 million deaths each year (WHO, 2015). This burden may, in part, be attributed to the presence of highly transmissible strains of the causative agent, *M. tuberculosis*, such as CDC1551 (Valway *et al.*, 1998), CAS (Ali *et al.*, 2009), Beijing (Glynn *et al.*, 2002), Type 1 (Sharma *et al.*, 2003), Haarlem (Mardassi *et al.*, 2005), F11, F28 (Victor *et al.*, 2004) and F15/LAM4/KZN (Pillay and Sturm, 2007) that have the capacity to infect and cause disease in large proportions of the population in different geographic regions globally. In South Africa, the Beijing family of strains is largely associated with drug resistance in the Western Cape, Eastern Cape and Gauteng, whilst the susceptible forms dominate in KwaZulu-Natal (Chihota *et al.*, 2012). The F11 and F28 strain families are also present in Western Cape, Eastern Cape and Gauteng provinces, with the former representing the largest proportion of all isolates in the Western Cape (Victor *et al.*, 2004). In KwaZulu-Natal, the F15/LAM4/KZN strain, largely associated with drug resistance since 1994 (Pillay and Sturm, 2007), was responsible for XDR-TB, and high mortality rates in Tugela Ferry

in 2005 (Gandhi *et al.*, 2006). MDR F11 and F28 strains are also present in significant proportions in KwaZulu-Natal (Chihota *et al.*, 2012).

Highly transmissible M. *tuberculosis* strains are characterized by their ability to infect large numbers of patients in a population within defined geographical settings or globally. Hypervirulent *M. tuberculosis* strains are usually characterized by rapid growth of the strain in the mice lungs followed by diminishing TH1 immunity over time (Ordway *et al.*, 2007). A positive correlation was observed between highly transmissible and hypervirulent strains of *M. tuberculosis* in mouse models. Virulent strains induced lower-protective immune responses. This was further correlated with other community markers of transmission such as tuberculin reactivity among contacts, rapid progression to disease and cluster status associated with highly transmissible and hypervirulent strains (Marquina-Castillo *et al.*, 2009). The Beijing family of strains has been shown to be both hypervirulent (Manca *et al.*, 2001) as well as highly transmissible compared to other clinical strains (Bifani *et al.*, 2002). Studies have linked the hyper-transmissibility of the Beijing strains with the constitutive expression of the DosR regulon (Reed *et al.*, 2007) resulting in early death of infected mice (Manca *et al.*, 2001).

M. tuberculosis strains have been shown to invade and replicate in pulmonary epithelial cells that were previously regarded as non-immune cells. Epithelial cells may not be hematopoietic, however they act as physical barrier against pathogens since they are present in large proportions in the lungs compared to other cells (Chuquimia *et al.*, 2012; Lutay *et al.*, 2014). Strains of different genotypes adhere to, invade, and replicate in these cells under both aerobic (Ashiru *et al.*, 2010; Bermudez and Goodman, 1996) and anaerobic conditions (Ashiru *et al.*, 2012). In

addition to their protective barrier functions (Chuquimia *et al.*, 2012), epithelial cells produce a variety of cytokines and chemokines (Chuquimia *et al.*, 2012; Stadnyk, 1994) and play a role in granuloma formation during TB infection (Lin *et al.*, 1998). This reflects their importance in modulating the host innate immune response during TB infection (Stadnyk, 1994). Several cytokines and chemokines, including interleukin (IL)-4 and interferon gamma induced protein 10 (IP-10) (Méndez-Samperio *et al.*, 2006), IL-6 (Lee *et al.*, 2009), IL-8 (Lin *et al.*, 1998; Wickremasinghe *et al.*, 1999), IFN-γ (Sharma *et al.*, 2007), TNF-α (Lee *et al.*, 2009; Zhang *et al.*, 1997), and monocyte chemotactic protein 1 (MCP-1) (Lin *et al.*, 1998) were shown to be produced by epithelial cells infected with Mycobacteria. Uncharacterized *M. tuberculosis* strains have been shown to induce differential cytokine/chemokine responses in pulmonary epithelial cells (Lin *et al.*, 1998). Cytokine responses have been inversely associated with strain virulence in various infection models (Koo *et al.*, 2012; Manca *et al.*, 2004; Manca *et al.*, 1999).

Previous studies on *in vitro* (Lee *et al.*, 2009; Lin *et al.*, 1998; Manca *et al.*, 2004; Sharma *et al.*, 2007) and *in vivo* (Manca *et al.*, 1999; Manca *et al.*, 2001) host response have reported on only a limited number of cytokines/chemokines. These studies were limited to only using the laboratory H37Rv strain and a few characterized and uncharacterized clinical strains. Understanding a wide range of other cytokines/chemokines produced by epithelial cells might provide a better understanding of role played by these cells in both innate and adaptive immune response. Despite major advances in the understanding of TB pathogenesis, there is still a lack of knowledge on the range of cytokines/chemokines induced by highly transmissible strains of *M. tuberculosis* in KwaZulu-Natal. Furthermore, no report is available on cytokine/chemokine response of pulmonary epithelial cells infected with virulent strains of *M. tuberculosis* that

dominate within the South African population. We therefore hypothesized that different virulent strains of *M. tuberculosis* elicit differential and strain specific host cytokine/chemokine response in epithelial cells at different time intervals. In this study we evaluated the response of 27 cytokines/chemokines in pulmonary alveolar epithelial cells infected with 4 well characterized, dominant strains of *M. tuberculosis* in KwaZulu-Natal (South Africa) compared to the laboratory H37Rv strain.

Materials and methods

Ethics approval

The study was approved by the Biomedical Research Ethics Committee, University of KwaZulu-Natal (reference no. BF109/11).

Bacterial isolates

Representatives of *M. tuberculosis* F15/LAM4/KZN, Beijing, F11, F28 and a Unique strain were isolated in our previous studies from sputum specimens of patients in KwaZulu-Natal, South Africa and characterized in Medical Microbiology and Infection Control, University of KwaZulu-Natal. Strain identity had been previously established by means of IS6110-restriction fragment length polymorphism analysis (RFLP) and spoligotyping. The Unique strain represented a genotype that was identified in only a single patient, in contrast to the other 4 strains that were detected in large numbers of patients in the same population investigated. They

were selected for this study as they represented the 4 major strain families isolated from patients in KwaZulu-Natal and other provinces in South Africa. Their high transmissibility inferred higher virulence, whereas the Unique strain was inferred to be less virulent. Drug susceptibility testing established that these representative F15/LAM4/KZN, F11 and F28 were XDR strains while the Beijing and Unique were MDR strains. The laboratory strain *M. tuberculosis* H37Rv (ATCC 25618) was obtained from UKZN (Medical Microbiology and Infection Control) culture collection and included in the study as a virulent control. Stock cultures of all strains had been stored in storage media containing 40% glycerol (Larsen *et al.*, 2007) at -80^{o}C, prior to retrieval and subculture.

Growth and maintenance of bacterial isolates and infection of A549 cells

Culturing of *M. tuberculosis* clinical strains and infection of pulmonary epithelial cells were carried out under biosafety level 2+ (BSL2+) conditions in the TB laboratory, Medical Microbiology and Infection Control, UKZN. Strict safety precautions, such as gloves, N95 mask or PAPYR were utilized during experiments involving *M. tuberculosis*.

Preparation of *M. tuberculosis* stock cultures

Clinical and laboratory strains of *M. tuberculosis* were cultured aerobically in 10 ml Middlebrook 7H9 broth (Becton, Dickinson and Company, South Africa) supplemented with 50% glycerol (Merck, South Africa), 20% Tween-80 (Merck, South Africa) and 10% Oleic acid Albumin Dextrose Catalase (OADC, Becton, Dickinson and Company, South Africa) at 37 ºC

for 3-6 weeks to a mid-log phase (OD 0.8-1_{600nm}). Thereafter, cells were centrifuged for 20 min at 4 000 g at 4 ºC. The pellet was washed thrice with PBS and re-suspended in 1 ml of the Middlebrook 7H9 broth medium containing 40% glycerol (Larsen *et al.*, 2007) and stored at -70 ºC in order to prevent loss of virulence through multiple passages. Stock culture solutions and media were prepared according to the manufacturer's instructions (Becton, Dickinson and Company, South Africa).

Culture of *M. tuberculosis*

Stock cultures were revived in supplemented 7H9 broth and allowed to grow to a mid-log phase with an OD_{600nm} of 0.8-1 (OD_{600nm} of 1 = ~$1X10^8$ Cfu/mL, (Larsen *et al.*, 2007). The Cfu/mL of the cultures was confirmed by plating 10-fold serial dilutions onto Middlebrook 7H11 agar and incubation at 37^oC for 3-4 weeks.

Culture and infection of A549 pulmonary epithelial cells

Stock cultures of A549 human pulmonary alveolar epithelial cells (ATCC CCL 185) were grown until they were ≥90% (2.2 to 2.6 x 10^6) confluent in 25 cm^2 tissue culture flasks (TCFs) containing 5 ml of Eagle's Minimal Essential Medium (EMEM) (Lonza, South Africa), supplemented with 10% heat inactivated fetal calf serum (FCS) (Lonza, South Africa). The confluent A549 cells were counted using the Neubauer counting chamber to determine the accurate number of cells. Thereafter, 4-5 ml of *M. tuberculosis* culture, grown to a log phase was added into confluent A549 cells to achieve the multiplicity of infection (MOI) of ~10:1 in 25 cm^2

TCFs containing 5 ml of serum free EMEM medium. The inoculum was plated to confirm the initial Cfu/ml used to infect A549 cells. The flask containing infected cells were sealed and incubated at 37^{o}C in 5% CO_2. Nine to ten milliliters of supernatant samples, collected from separate flasks at 0, 24, 48 and 72 hr post-infection, were filtered and stored at -70^{0}C in 0.5% bovine serum albumin (BSA) as a carrier protein prior to cytokine analysis. In a separate flask, the infected epithelial cells were lysed with triton-X and 10-fold serial dilutions were plated out on 7H11 agar plates to determine Cfu/ml in order to calculate the number of invaded/replicating cells at each time point post-infection.

Cytokine analysis

Supernatant samples were analyzed using the Bio-Plex Pro™ Human Cytokine 27-plex Assay (Bio-Rad) with the Bio-Plex® 200 (Bio-Rad) in a standard microbiology laboratory in Microbiology, University of KwaZulu-Natal (Westville campus). The 27-Plex assay (Bio-Rad) was chosen because it contained a combination of analytes that were shown previously to contribute to the innate immune response together with some analytes that had not been tested and detected in the epithelial cell line that was used in the current study. The detection limits (pg/ml) of these analytes were: IL-1β (2.05-2283.67), IL-1ra (8.91-30032.7), IL-2 (3.92-15286.77), IL-4 (0.91-3987.72), IL-5 (1.86-2088.53), IL-6 (1.15-4727.29), IL-7 (2.77-45398.65), IL-8 (1.95-22091.85), IL-9 (2.96-10739.4), IL-10 (1.5-6774.42), IL-12 p70 (9.18-35207.24), IL-13 (1.96-24456.64), IL-15 (6.19-5992.8), IL-17 (5.6-24741.17), Fibroblast Growth Factor (FGF) (4.77-4214.15), Rantes (6.38-1772.28), Eotaxin (8.79-23748.07), Granulocyte colony-stimulating factor (G-CSF) (8.8-33201.65), Granulocyte macrophage colony-stimulating factor

(GM-CSF) (12.41-14225.07), TNF-α (5.04-60209.41), IFN-γ (8.46-30645.43), MIP-1α (1.34-1408.05), MIP-1β (4.75-1314.77), MCP-1 (1.42-1529.68), IP-10 (10.13-33863.61), Platelet derived growth factor (PDGF)-BB (6.19-25944.25) and Vascular Endothelial Growth Factor (VEGF) (8.23-28877.51).

Analytes were measured by multiplexed microbead analysis as follows:

Fifty microliters of magnetic beads in a 96-well plate were washed twice with 100 µl of assay buffer. After the addition of 50 µl of standards, blanks and a 1 in 4 diluted culture supernatant samples in their respective wells the magnetic beads were incubated with shaking at 850 rpm for 1 hr. Following three washes with 100 µl of assay buffer, 25 µl of detection antibody were added followed by incubation at room temperature for 30 min with shaking at 850 rpm. The wells were again washed as previously followed by the addition of 50 µl streptavidin-PE and incubated for 10 min with shaking at 850 rpm. Prior to reading, beads were again washed three times and re-suspended in 125 µl of assay buffer. The concentrations of the standards and samples were read using EMEM medium to normalize concentrations of the different analytes against the background by a five-parameter logistic regression algorithm (5PL) provided by the Bio-Rad software (Bio-Plex Manager 6.1).

Statistical analysis, hierarchical clustering and Principal Component Analysis (PCA)

Statistical analysis and graphical representation of the data was carried out using the Graph-Pad prism software (v5). The cytokine data at each point is presented as heat maps of fold changes between infected and uninfected cells and as ± standard error of the mean (±SEM) at different

time intervals. The unpaired student t-test was used to compare the level of cytokine production induced by the clinical strains with the uninfected control and among the strains. A $P<0.05$ was considered significant. Cytokine production patterns among clinical strains were compared using hierarchical clustering within the MeV software (v4.9) and principal component analysis (PCA) was performed using the Canoco software (v4.56). Multiple regression analysis following Pearson correlation between analytes was used to test if our dependent variable (cytokine/chemokine) is controlled by independent variable (strain identity, cfu/ml and sampling intervals). Regression analysis was performed using Genestat (v2.0) and SPSS (v16.0) softwares.

Results

The level of infection achieved in pulmonary epithelial cells varied between 8-9.8 CFU/mL. This was determined by plating initial inoculum and intracellular bacteria and at 24 hr post-infection. The coefficient of variance for the MOI was between 0 and 0.580756495. Therefore, the inter-experimental variations were between 0 and 58%. Due to differences in MOI as well intracellular replication of the clinical strains, Multi-regression analysis was used to test whether different variables influence cytokine production in epithelial cells.

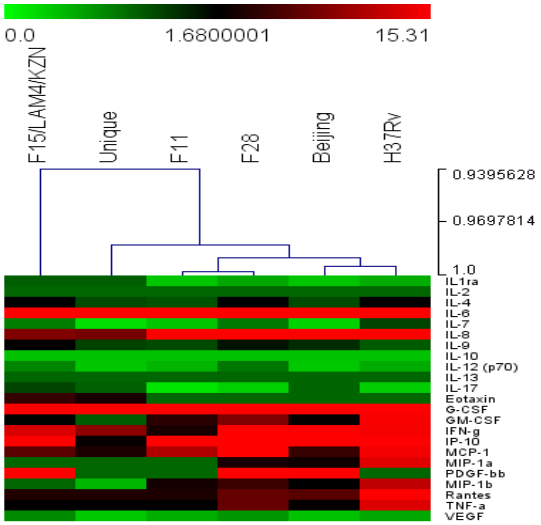
Twenty three out of twenty seven analytes were detected by the Bio-Plex Pro Human Cytokine 27-Plex assay in the *M. tuberculosis* infected A549 supernatants: IL-1rα, -2, -4, -6, -7, -8, -9, -10, -12p70, -13, -17, TNF-α, IFN-γ, Rantes, Eotaxin, G-CSF, GM-CSF, MIP-1α, MIP-1β, MCP-1, IP-10, PDGF-bb and VEGF (Appendix A, Supplementary Table 1). IL-1β, -5, -15 and FGF were below the detectable level of the assay. Varying levels of the cytokines/chemokines were

detected at 10 min and are expressed as a fold increase relative to the uninfected A549 cells (Appendix A, Supplementary Table 1).

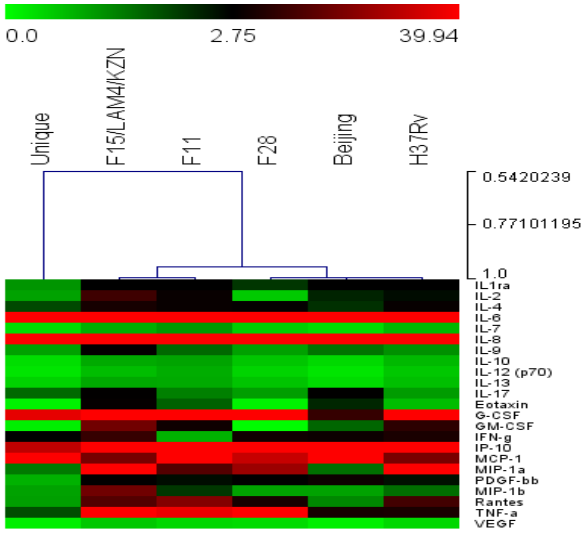
Cytokine/chemokine production patterns induced by clinical strains of *M. tuberculosis*

Four out of 27 analytes were not detected at all intervals. The concentration of the 23 analytes measured in the detectable range in the supernatants of infected epithelial cells were compared to the uninfected epithelial cells and depicted as fold changes for each strain at 24, 48 or 72 hr post-infection. These fold changes are represented as heat maps at the different time intervals and the relationship among the clinical strains is shown by hierarchical clustering (Fig. 1). At 24 hr, Beijing and H37Rv cluster closely together, F11 and F28 closer to the Unique strain, while the F15/LAM4/KZN induces a pattern that differs from other strains. At 48 hr, the F11 strain induced lower fold changes for most analytes with patterns that differ compared to other strains. Diverse cytokine production patterns were observed for the Unique strain compared to other strains at 72 hr post-infection. Furthermore, the F15/LAM4/KZN and F11 induced similar cytokine/chemokine patterns, while the Beijing, F28 and H37Rv closely clustered together (Fig. 1).

24 hr



72 hr



48 hr

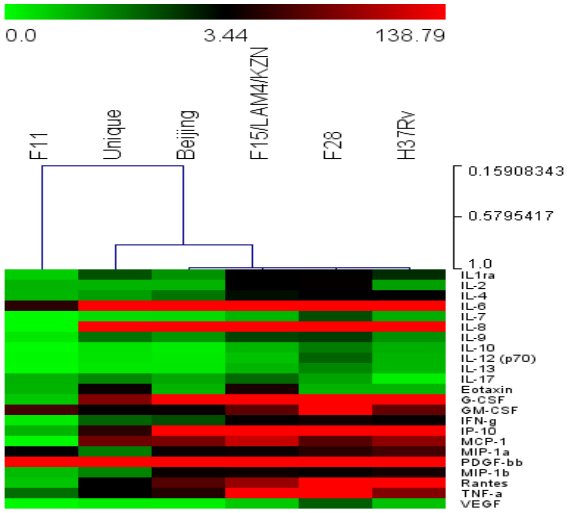


Fig. 1. Heat map and hierarchical cluster analysis comparing fold changes of cytokine/chemokine production by A549 pulmonary epithelial cells infected by the *M. tuberculosis* F15/LAM4/KZN, Beijing, F11, F28, Unique and H37Rv strains at 24, 48 and 72 hr interval. A549 pulmonary epithelial cells were infected at an MOI~10:1 and supernatant was collected at 24, 48 and 72 hr post-infection. Fold changes

between uninfected and infected cells were calculated and compared among clinical strains at 24, 48 and 72 hr after infection. Hierarchical clustering among the strains shows diverse patterns of cytokine/chemokine production induced by the F15/LAM4/KZN strain at 24 hr, F11 strain at 48 and Unique strain at 72 hr after infection. Clustering analysis and heat maps were created using the MeV software.

In addition to hierarchical clustering, PCA was performed to display patterns of cytokine/chemokine production at all-time intervals combined (Fig. 2). The most obvious distinguishable outliers were cytokine/chemokine patterns induced by the F15/LAM4/KZN strain at 48 and 72 hr, F28 strain at 48 and H37Rv strain at 48 hr. The 24 hr samples showed closer relatedness among strains compared to the 48 and 72 hr patterns with the exception of the F11 strain at 48 hr that was closer to the 24 hr samples. Other 48 and 72 hr patterns were specific among other clinical strains, showing strain specific patterns of cytokine production in pulmonary epithelial cells. There was no functional relationship observed for cytokines/chemokines as shown by closeness of PCA arrows for different analytes. Closer arrows indicate related production patterns of those analytes whilst distant arrows indicate differences among analytes. This is observed for the production of the anti-inflammatory cytokines IL-4, IL-9 that is closer to chemokines (MIP-1α, MIP-β, IP-10) and the pro-inflammatory cytokine, IL-6. Despite closer patterns in the production of these cytokines/chemokines, they have diverse functions when produced in the host during infection. Compared to other cytokines/chemokines, IL-17, Eotaxin, IFN-γ and IL-13 showed distinctive patterns when compared to other cytokines/chemokines detected shown by their distance from other analytes (Fig. 2).

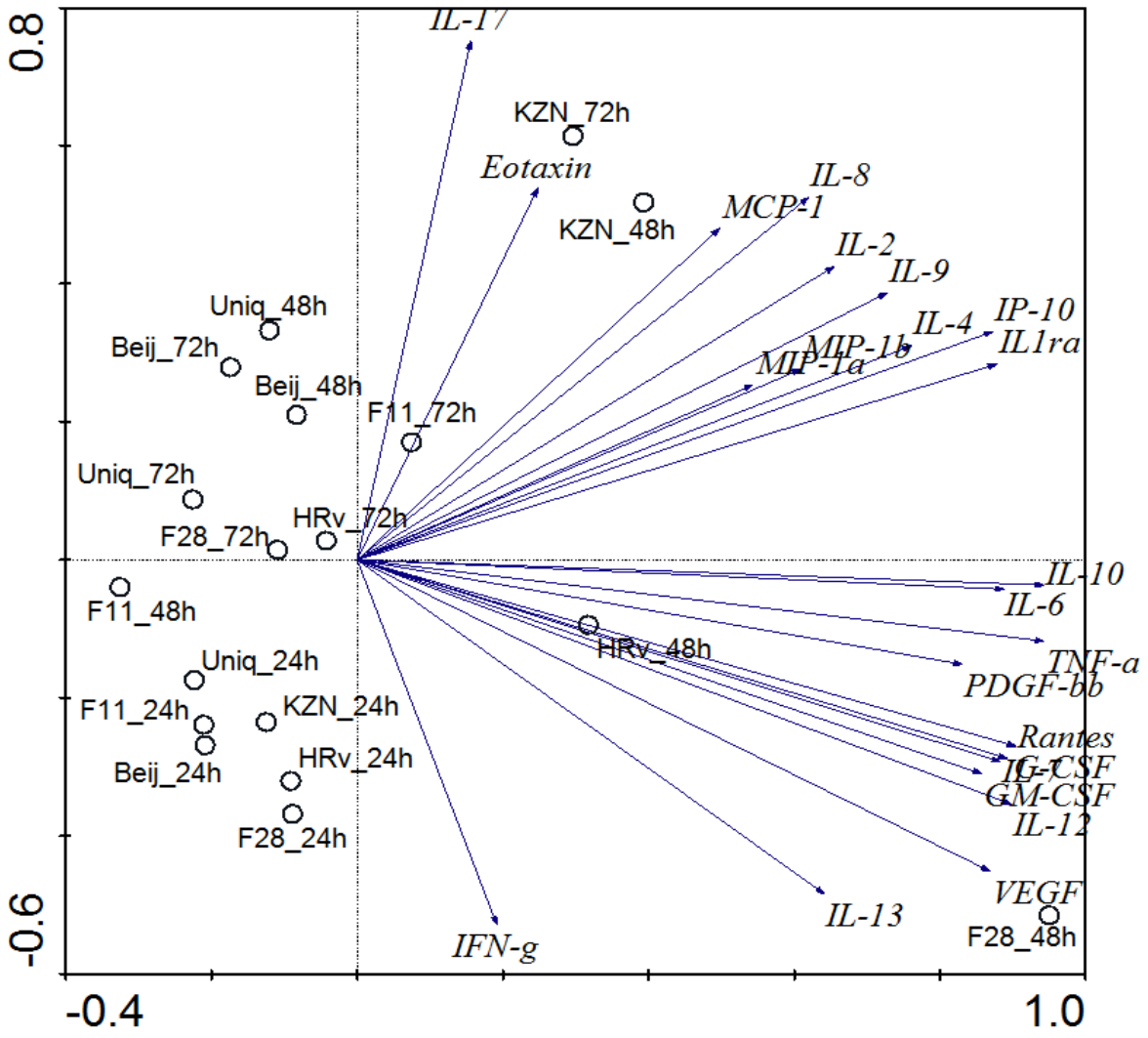


Fig. 2. Principal component analysis of 23 cytokines/chemokines produced at 24, 48 and 72 hr post-infection. A549 pulmonary epithelial cells were infected at an MOI of ~10:1 and supernatant was collected at 24, 48 and 72 hr post-infected. Cytokine production was detected at all three time intervals and cytokine/chemokine patterns among clinical strains were analysed and plotted in a PCA diagram using Canoco software.

We then compared the anti-inflammatory, pro-inflammatory, and chemokine responses induced by the different strains in details.

M. tuberculosis strains induce differential anti-inflammatory cytokine response in pulmonary epithelial cells

Varying concentrations of the anti-inflammatory cytokines IL-1ra, IL-4, IL-9, IL-10 and IL-13 were detected in epithelial cells infected with a laboratory and clinical strains of *M. tuberculosis* (Fig. 3; Appendix A, Supplementary Table 1). All strains, except the F15/LAM4/KZN and the Unique strains, induced a significant reduction ($P < 0.05$) in IL-1ra at 24 hr compared to the uninfected cells. Production of IL-1ra was significantly increased by all strains ($P < 0.05$) except the Beijing and F11 strains at 48 hr and the Unique strain at 72 hr after infection. At 24 hr, highest concentrations of IL-1ra were induced by the F15/LAM4/KZN and Unique strains and these were significantly ($P < 0.05$) higher than the Beijing, F28 and H37Rv strains. At 48 hr, significantly higher concentrations were induced by the F28 while the highest concentrations by the F15/LAM4/KZN at 72 hr were only significant against the Unique ($P = 0.0244$) and F28 ($P = 0.0378$) strains.

IL-4 production at 24 and 48 hr after infection was insignificant for all strains (Fig. 3; Supplementary Table 1) compared to uninfected A549 cells and among clinical strains. At 72 hr, Beijing ($P = 0.0118$), F11 ($P = 0.0270$), F28 ($P = 0.0404$) and H37Rv ($P = 0.0036$) induced higher amounts of IL-4 production compared to the uninfected A549 cells, with no significant differences in concentrations between clinical strains (Appendix A, Supplementary Table 3).

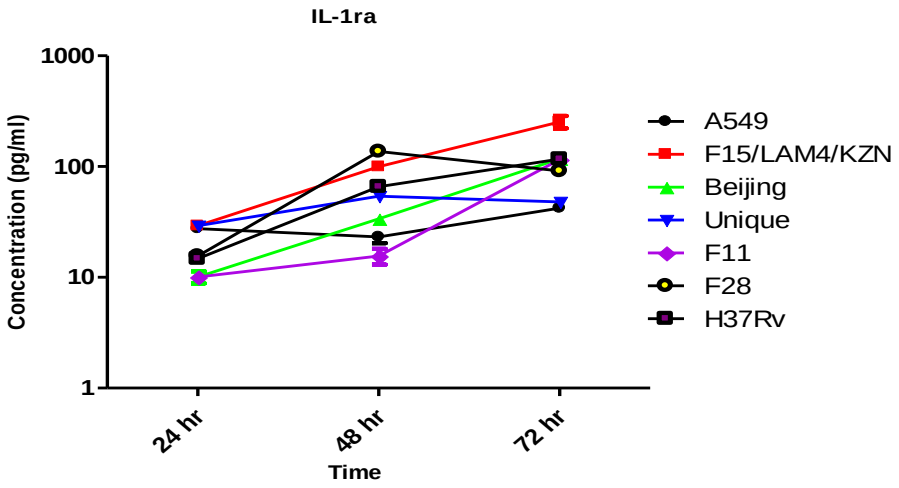
There was no significant increase in the production of IL-9 by any of the strains at 24 or 72 hr compared to the uninfected cells and among clinical strains with the exception of the higher concentrations induced by the F28 strains compared to the Beijing ($P = 0.0100$), and Unique

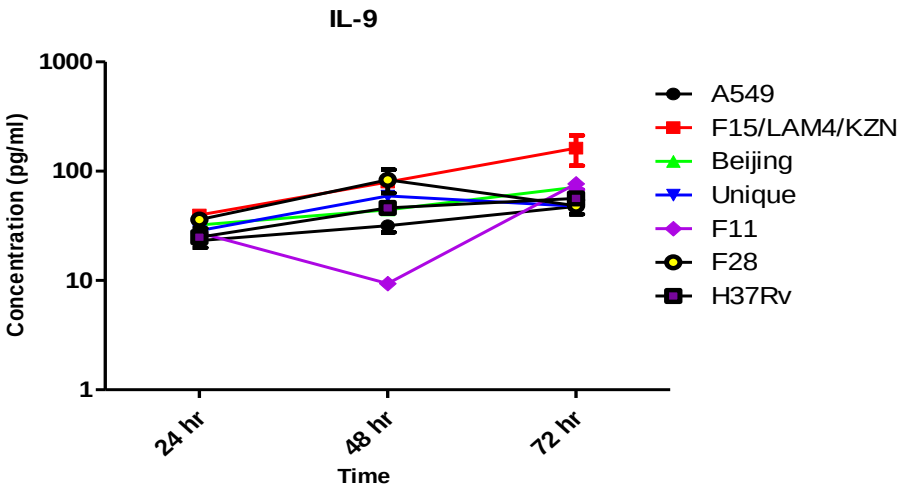
compared to the Beijing (P = 0.0190) strain at 72 hr. IL-9 production by F15/LAM4/KZN (P = 0.0155), Unique (P = 0.0055) and H37Rv (P = 0.0024) increased significantly only at 48 hr, whilst this was significantly reduced by the F11 strain (P = 0.0010). IL-9 concentrations were significantly higher by the F15/LAM4/KZN strain compared to Beijing (P = 0.0459), F11 (P = 0.0073) and H37Rv (P = 0.0312) strains at 48 hr after infection.

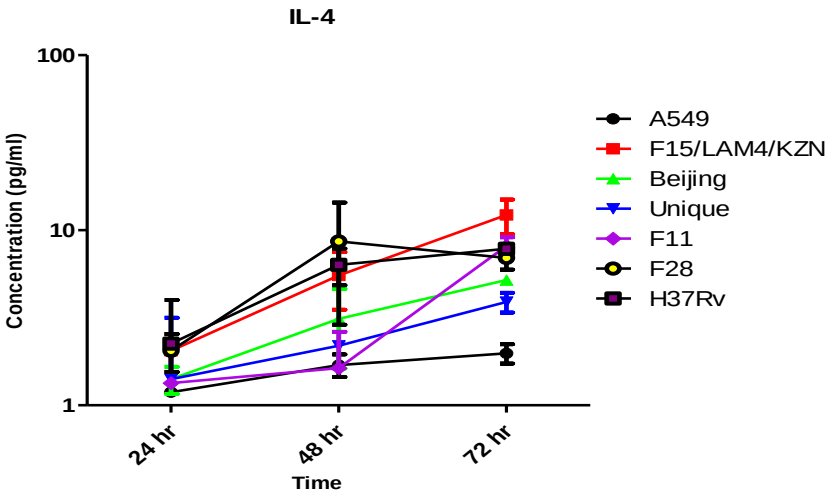
There was no significant difference in production of IL-10 between the uninfected and the infected cells at all-time intervals (Fig. 2; Supplementary Table 1). However, H37Rv induced significantly higher IL-10 compared to the Beijing (P = 0.0445) and F11 (P = 0.0197) strains, respectively, at 48 hr (Appendix A, Supplementary Table 3).

IL-13 production was not induced by any of the strains at 24 hr. Production was reduced at 48 hr by the Beijing, Unique and the F11 strains but reached significance only for the latter strain (P = 0.0395). Significantly higher IL-13 was induced by the H37Rv compared to F11 (P = 0.0257) strain at 48 hr. IL-13 production was reduced by all strains at 72 hr, although this was not significant.

All strains induced a gradual increase in anti-inflammatory cytokines that peaked mostly at 72 hr post-infection, the exception being the Unique strain in the production of IL-1ra and IL-9 and F28 strain for all anti-inflammatory cytokines detected which peaked at 48 hr (Fig. 3; Appendix A, Supplementary Table 1).


IL-1ra
1000
100
10
1
Concentration (pg/ml)
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv


IL-9
1000
100
10
1
Concentration (pg/ml)
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv


IL-4
100
10
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Concentration (pg/ml)
24 hr
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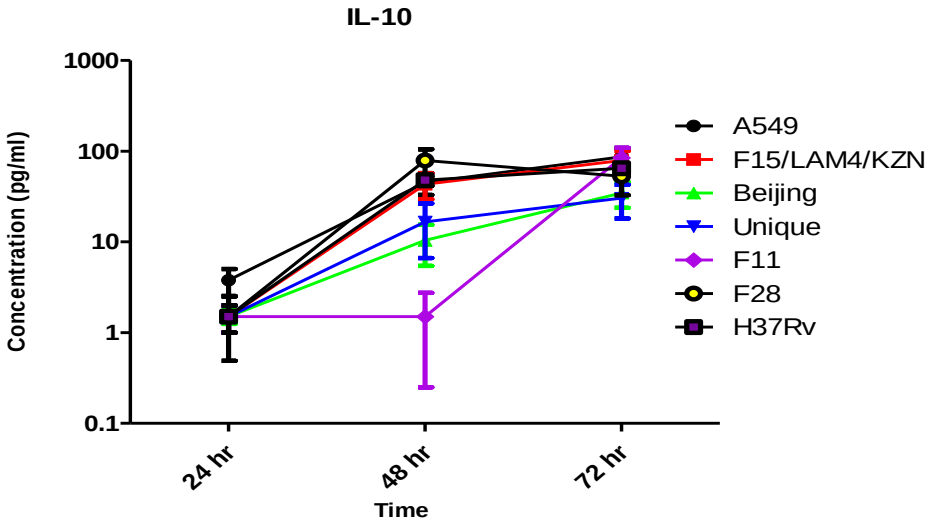
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv


IL-10
1000
100
10
1
0.1
Concentration (pg/ml)
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv

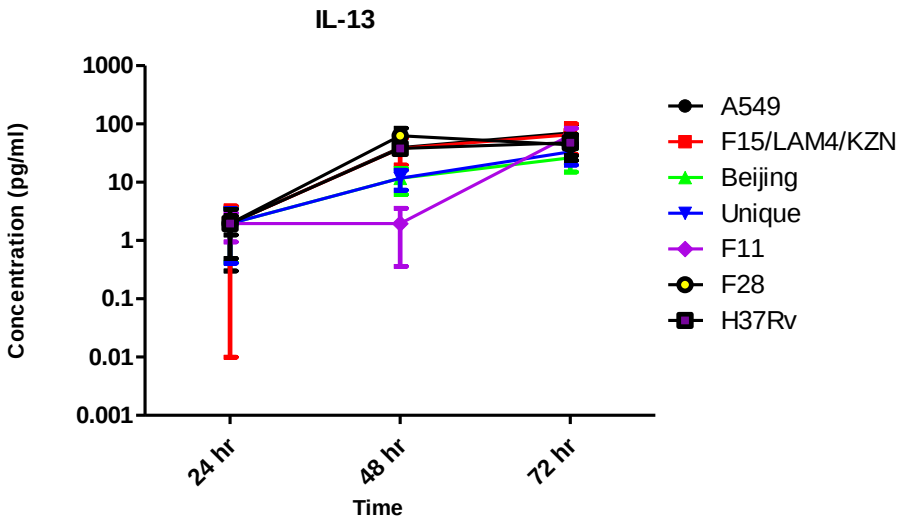


Fig. 3. Kinetics curves of anti-inflammatory cytokines produced by epithelial cells infected with the F15/LAM4/KZN, Beijing, Unique, F11, F28 and H37Rv strains. A549 cells were infected with clinical strains of *M. tuberculosis* and laboratory strain H37Rv at an MOI~10:1. Supernatant was collected at 24, 48 and 72 hr post-infection. Cytokines and chemokines were measured by multiplexing 27-analytes using the Bio-Plex Manager 6.1. The data is presented as ±SEM of two independent experiments. Unpaired student t-test was used for statistical analysis ($P<0.05$ significant).

M. tuberculosis strains induce a differential pro-inflammatory cytokine response in pulmonary epithelial cells

Varying concentrations of the pro-inflammatory cytokines, IL-2, IL-6, IL-7, IL-8, IL-12, IL-17, IFN-γ and TNF-α were produced by epithelial cells infected by the different strains (Fig. 4, Supplementary table 1). IL-2 production increased from 24 48 hr and peaked at 72 hr for all the strains except F28 at 48 hr and Unique at all-time intervals. Significant productions of IL-2 compared to the uninfected was induced by the F15/LAM4/KZN (0.0158) at 72 hr while significant differences between the strains were only observed between the F15/LAM4/KZN and F28 strain ($P = 0.0221$) at 72 hr. IL-6 production by infected cells was significantly higher than the uninfected cells ($P < 0.05$) at all-time intervals for all strains except at 48 hr for Unique and F11 strains. Significant differences in the concentrations were observed among most strains at most time intervals. Significant induction of IL-6 peaked at 72 hr for all strains except the Beijing strain, where the maximum levels were attained at 48 hr. The IL-6 concentration at 24 hr was significantly higher in cells infected with H37Rv and the F28 strain compared to the other strains. F28 strain significantly induced the highest IL-6 concentrations compared to other clinical strains at both 48 and 72 hr after infection (Appendix A, Supplementary Table 3).

There was no significant difference in the production of IL-7 by infected compared to the uninfected A549 cells at 24 hr (Fig. 4). IL-7 production was reduced by the Beijing, F11 and Unique strains at 24 hr, but this did not reach significance. At 48 hr and 72 hr, Beijing and Unique strains significantly reduced production of IL-7, while an insignificant increase was observed for the F28 strain at 48 hr. F11 significantly reduced IL-7 at 48 hr while significant reduction by F28 strain was at 72 hr. At 48 hr, significantly higher IL-7 was induced by the

F28 strain compared to the Beijing (P = 0.0362), Unique (P = 0.0358) and F11 (P = 0.0249) strains. At 72 hr H37Rv induced significantly higher IL-7 compared to the Unique (P = 0.0486) and F28 (P = 0.0288) strains, respectively.

IL-8 production was increased significantly by F15/LAM4/KZN (P = 0.0323), Beijing (P = 0.0254), F28 (P = 0.0258) and H37Rv (P = 0.0173) at 24 hr, and by all strains at 48 and 72 hr with the exception of F11 at 48 hr, Beijing and H37Rv strains at 72 hr (Fig. 4; Supplementary Table 1). At 24 hr, F28 induced significantly higher IL-8 compared to the F15/LAM4/KZN (P = 0.0310), Beijing (P = 0.0400) and Unique (P = 0.0324) strains, while F15/LAM4/KZN peaked significantly at 48 and 72 hr after infection compared to the other strains.

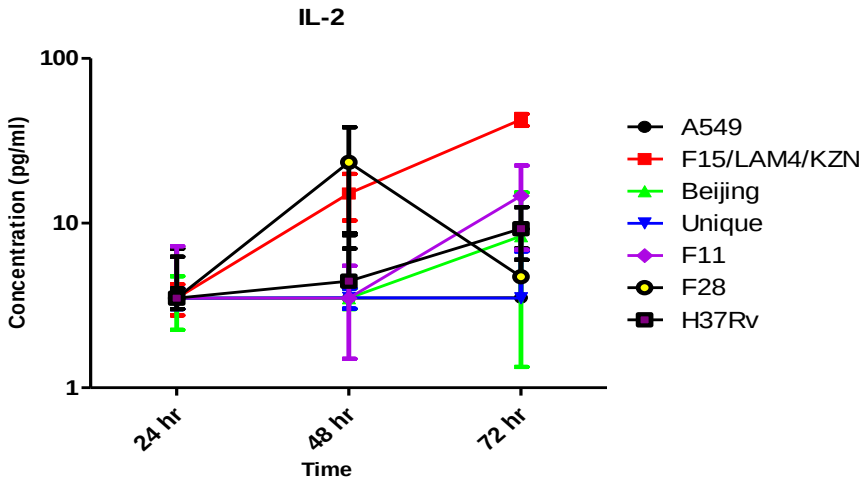
All strains induced gradual increase in IL-12p70 that peaked at 48 hr for the F28 and 72 hr for other strains. IL-12p70 was constitutively produced by the uninfected A549 cells with significant reduction induced by the Beijing (P = 0.0158), Unique (P = 0.0418) and F11 (P = 0.0108) strains at 48 hr. Significant differences of IL-12p70 among the strains was observed between the Beijing vs F15/LAM4/KZN (P = 0.0144), F11 vs F15/LAM4/KZN (P = 0.0090), Beijing (P = 0.0198) and H37Rv (P = 0.0299) strains at 48 hr. At 72 hr, significance was only observed between the Beijing compared to the F28 (P = 0.0306) and H37Rv (P = 0.007) as well as between the F28 and H37Rv (P = 0.0457).

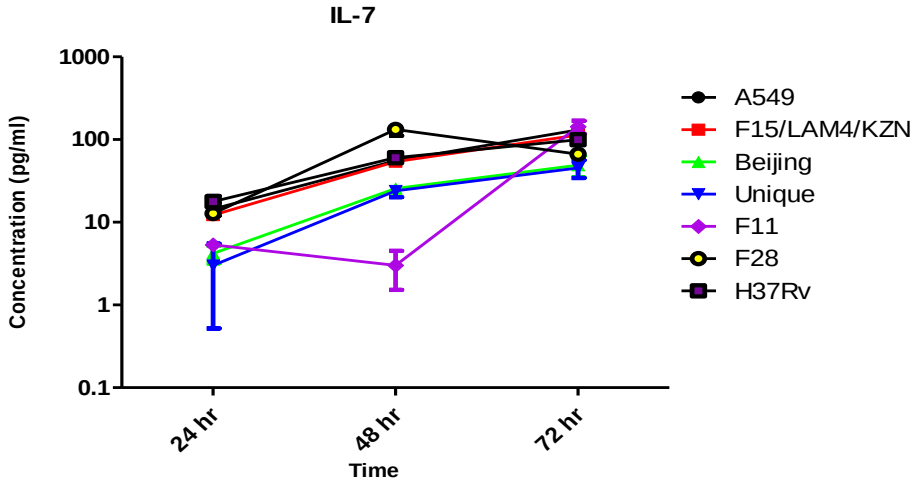
IL-17 was also constitutively produced by the uninfected cells with highest concentration observed at 24 hr for A549 cells and at 72 hr for all the strains except F28 that peaked at 24 hr. Significant reduction of IL-17 was induced by the F11 (P = 0.0285), F28 (P = 0.0361) and H37Rv (P = 0.0307) strains at 24 hr. Significantly higher concentrations was induced by F15/LAM4/KZN and Beijing strains compared to the F11 and H37Rv. F28 induced

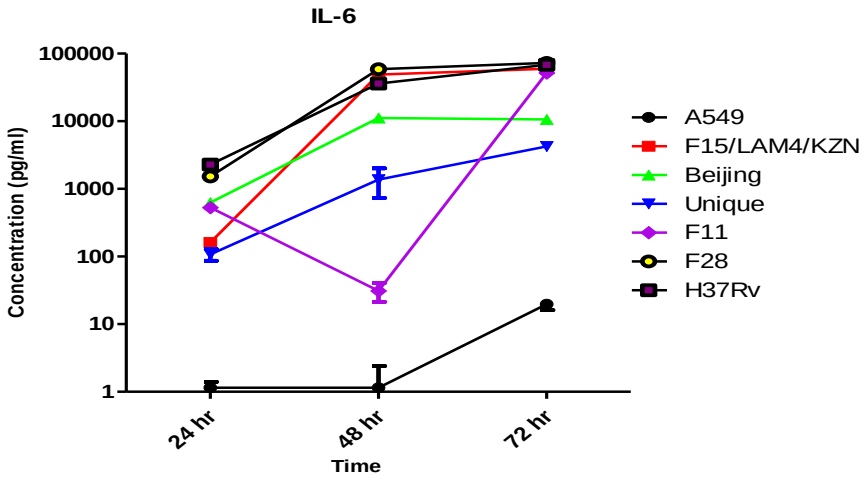
significantly lower than IL-12p70 compared to the F15/LAM4/KZN (P = 0.0251) and Beijing (P = 0.0183) strains, respectively. At 48 hr, significant differences were observed between H37Rv and F15/LAM4/KZN (P = 0.0307), Beijing (P = 0.0397), F11 (P = 0.0472) and F28 (P = 0.0257) strains, respectively.

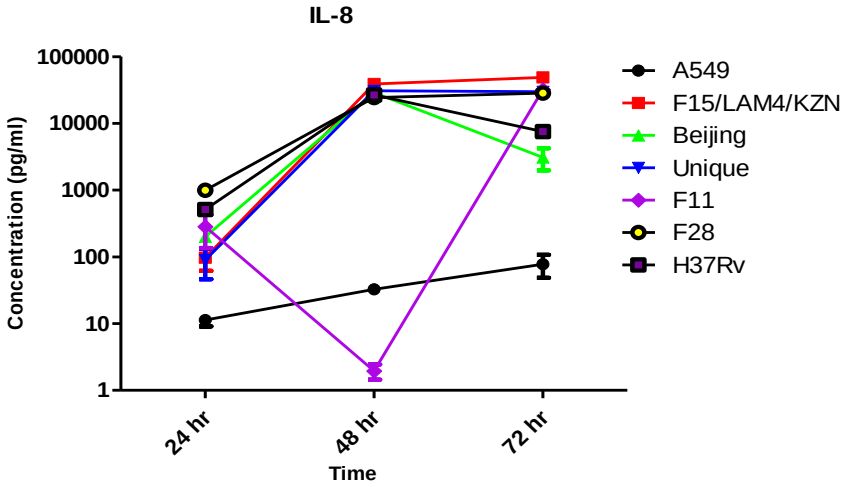
Production of IFN-γ was significantly higher in infected cells compared to the uninfected cells at all-time intervals except for the decreased induction by the F11 strain at both 24 and 72 hr that did not reach significance for both time points (Fig. 4; Supplementary Table 1). Peak production of IFN-γ was significantly induced by the F28 strain at both 24 and 48 hr and by the F15/LAM4/KZN at 72 hr compared to other strains (Appendix A, Supplementary Table 3).

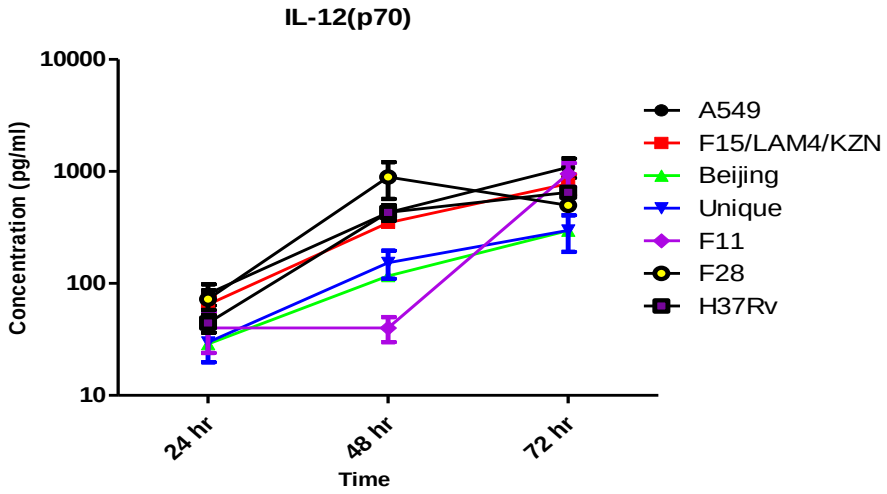
Compared to the uninfected cells, TNF-α production was significantly higher only for F28 (P = 0.0063) and H37Rv (P = 0.0047) at 24 hr, F15/LAM4/KZN (P = 0.0008), Beijing (P = 0.0029), F28 (P = 0.0006) and H37Rv (P = 0.0003) at 48 hr, and for F15/LAM4/KZN (P = 0.0037), F11 (P = 0.0007), F28 (P < 0.0001) and H37Rv (P = 0.0008) at 72 hr. The highest TNF-α concentrations were induced significantly by H37Rv at 24 hr, F28 strain at 48 hr and F15/LAM4/KZN at 72 hr, compared to other strains.

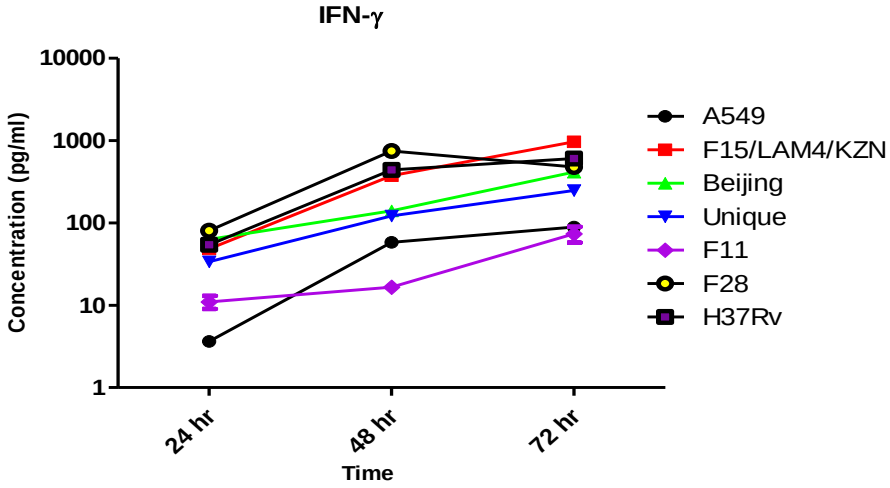

IL-2
Concentration (pg/ml)
100
10
1
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv

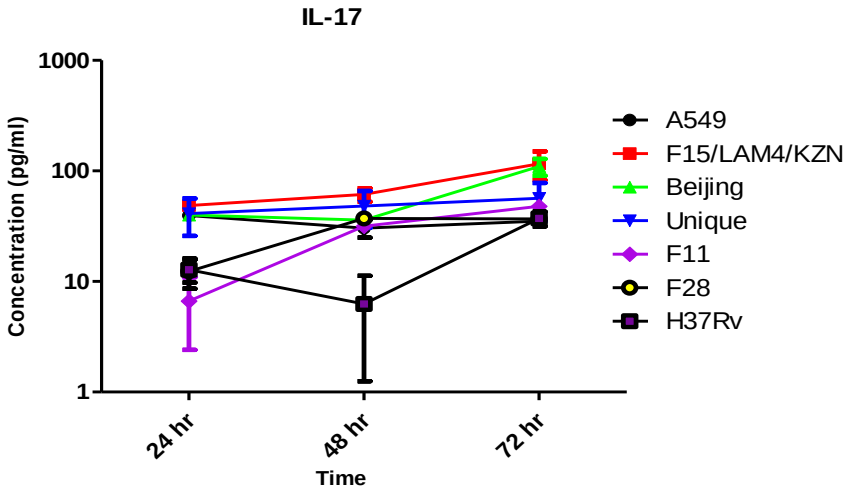

IL-7
Concentration (pg/ml)
1000
100
10
1
0.1
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv


IL-6
Concentration (pg/ml)
100000
10000
1000
100
10
1
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv


IL-8
Concentration (pg/ml)
100000
10000
1000
100
10
1
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv


IL-12(p70)
Concentration (pg/ml)
10000
1000
100
10
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv


IFN-γ
Concentration (pg/ml)
10000
1000
100
10
1
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv


IL-17
Concentration (pg/ml)
1000
100
10
1
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv

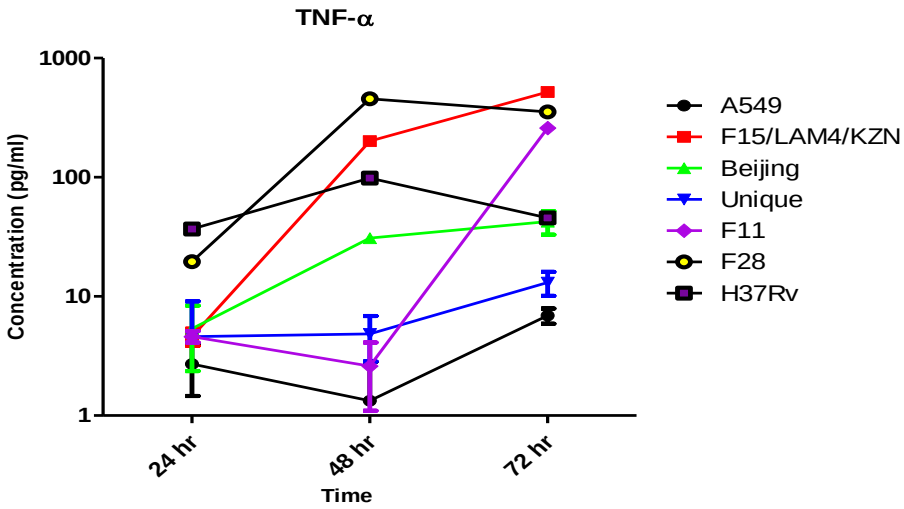

TNF-α
Concentration (pg/ml)
1000
100
10
1
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv

Fig. 4. Kinetic curves of pro-inflammatory cytokine production by epithelial cells infected with the F15/LAM4/KZN, Beijing, Unique, F11, F28 and H37Rv strains. A549 cells were infected with clinical strains of *M. tuberculosis* and laboratory strain H37Rv at an MOI~10:1. Supernatant was collected at 24, 48 and 72 hr post-infection. Cytokines and chemokines were measured by multiplexing 27-analytes using the Bio-Plex Manager 6.1. The data is presented as ±SEM of two independent experiments. Unpaired student t-test was used for statistical analysis ($P<0.05$ significant).

M. tuberculosis strains induce differential chemokine response in pulmonary epithelial cells

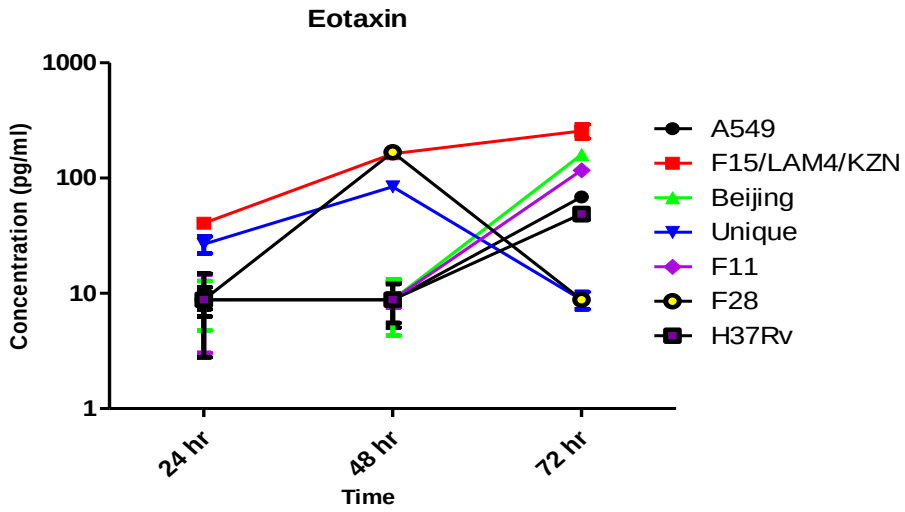
Strain specific production of the chemokines Eotaxin, GM-CSF, G-CSF, IP-10, MCP-1, MIP-1γ, PDGF-bb, MIP-1β, Rantes and VEGF was observed at 24, 48 and 72 hr post-infection (Fig. 5; Appendix A, Supplementary Table 1). The following strains induce significantly higher Eotaxin production compared to the A549 controls: F15/LAM4/KZN (P = 0.0072) at 24 hr; F15/LAM4/KZN N (P = 0.0018), Unique (P = 0.0043) and F28 (P = 0.007) at 48 hr and lastly F15/LAM4/KZN (P = 0.0355), Beijing (P = 0.0031), Unique (P = 0.0075) and F11 (P = 0.007) at 72 hr, respectively.

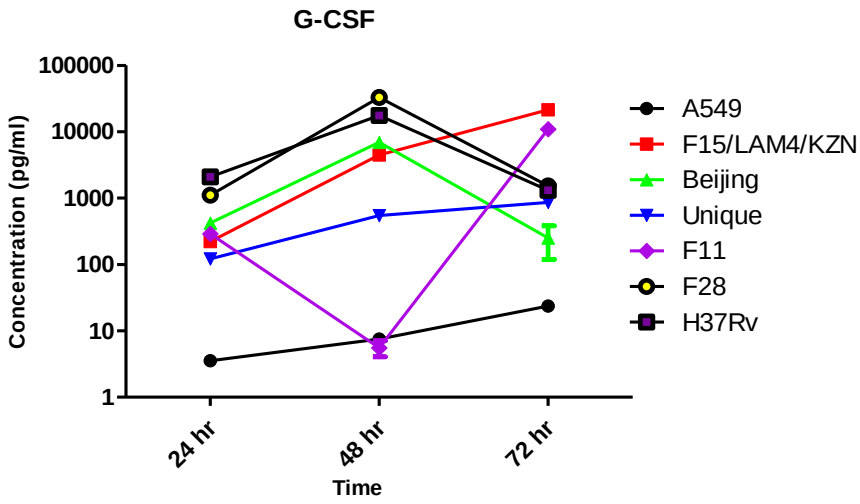
A significant increase in IP-10 and G-CSF production at the different time intervals was induced by all strains with the exception of the Unique at 24 hr and F11 at 48 hr for the former, and F11 at 48 hr and Beijing strains at 72 hr for the latter, respectively. Highest IP-10 and G-CSF at 24 hr was induced by F28 and H37Rv, respectively, and both were significantly higher than other strains. At 48 hr, the highest levels of IP-10 and G-CSF were induced by the F15/LAM4/KZN and F28 strains, respectively. The F15/LAM4/KZN strain significantly induced the highest levels of IP-10 and G-CSF among clinical strain at 72 hr (Appendix A, Supplementary Table 3).

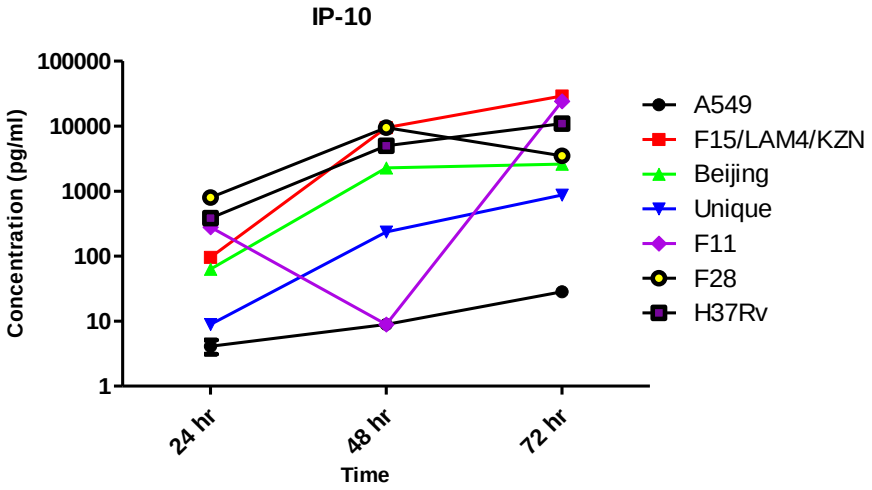
GM-CSF production by all strains was insignificant at 24 hr. However, significantly elevated concentrations were induced by the F15/LAM4/KZN (P = 0.0290), F11 (P = 0.0027), F28 (P = 0.0002) and H37Rv (P = 0.0327) strains at 48 hr and F15/LAM4/KZN (P = 0.0275), F11 (P = 0.0285) and H37Rv (P = 0.0018) strains at 72 hr (Fig. 5; Appendix A, Supplementary Table 1). Significantly higher GM-CSF was induced by the F28 strain compared to other clinical strains at 48 hr, and by the F15/LAM4/KZN at 72 hr compared to the other strains except H37Rv.

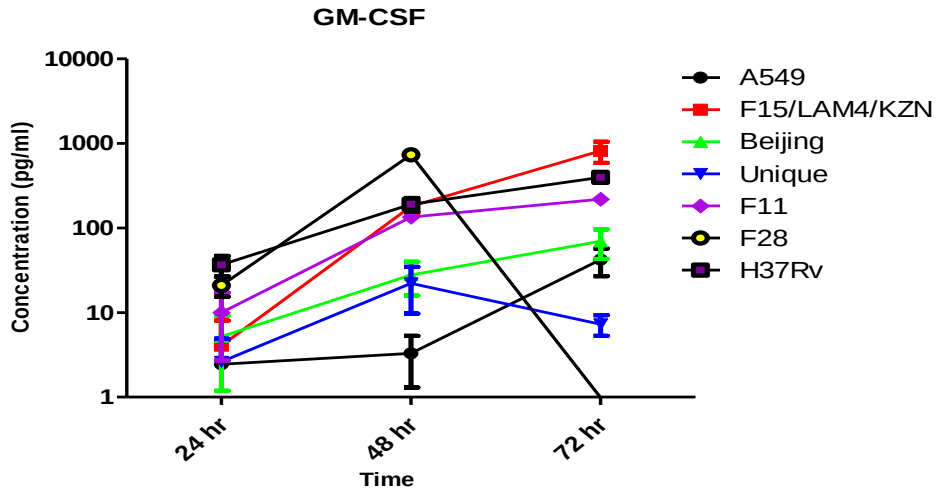
MCP-1 was produced in high concentrations (range 100.48±2.5 to 243.12±68pg/mL) by un-infected epithelial cells compared to other chemokines. However, significant increases in MCP-1 production were induced by the Beijing (P = 0.0002), F11 (P = 0.0434) and H37Rv (P = 0.0141) strains at 24 hr, by F15/LAM4/KZN (P = 0.0001), Beijing (P = 0.0016), Unique (P = 0.0349), F28 (P = 0.0326) and H37Rv (P = 0.0041) at 48 hr, and by all strains at 72 hr, respectively, compared to the uninfected cells. The F11 strain induced significantly higher MCP at 72 hr compared to the other clinical strains. Only H37Rv (0.0051) induced significant MIP-1α at 24 hr, all strains at 48 hr and F15/LAM4/KZN (0.0444) and H37Rv (0.0111) at 72 hr compared to the controls. PDGF-bb increased significantly for the F15/LAM4/KZN (0.0270) and F28 (0.0418) at 24 hr, all strains at 48 hr and 72 hr except for the Unique strain at 72 hr. MIP-1β concentrations were significant compared to the uninfected controls for the following strains: F11 (0.0278), F28 (0.0135) and H37Rv (0.0015) at 24 hr; F15/LAM4/KZN (0.0023) and Beijing (0.003) at 48 hr and F15/LAM4/KZN (0.0421) at 72 hr.

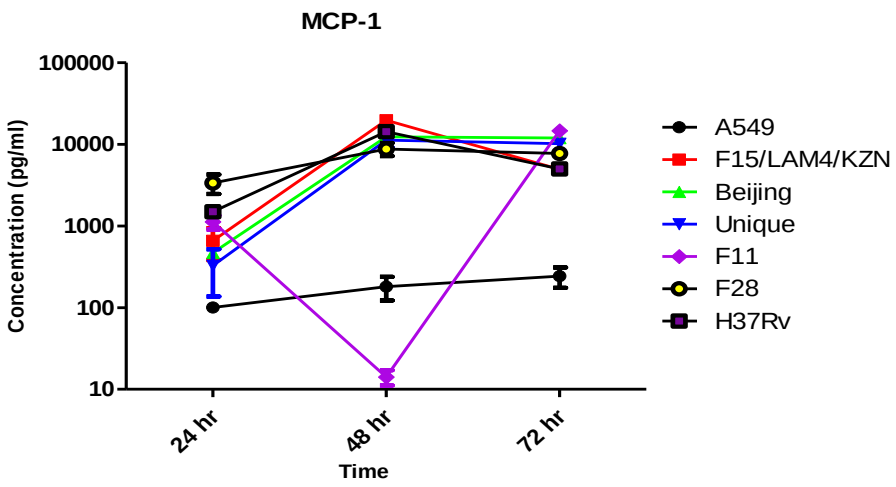
Rantes production was significantly increased for all strains with the exception of Unique at 24 hr, F11 at 48 hr, and Beijing and Unique strains at 72 hr after infection (Fig. 5; Appendix A, Supplementary Table 1). The highest Rantes concentration was induced by the H37Rv strain at 24 hr, F28 strain at 48 hr (significant compared to the F11 strain) and by F11 strain at 72 hr (significant compared to the Beijing (0.0222), Unique (0.0216) and F28 (0.0421) strains, respectively). VEGF was significantly reduced by the strains compared to the control as follows: Beijing (0.006), Unique (0.0389), F11 (0.0148) and H37Rv at 24 hr, all strains at 48 hr and 72 hr except F28 at 48 hr.

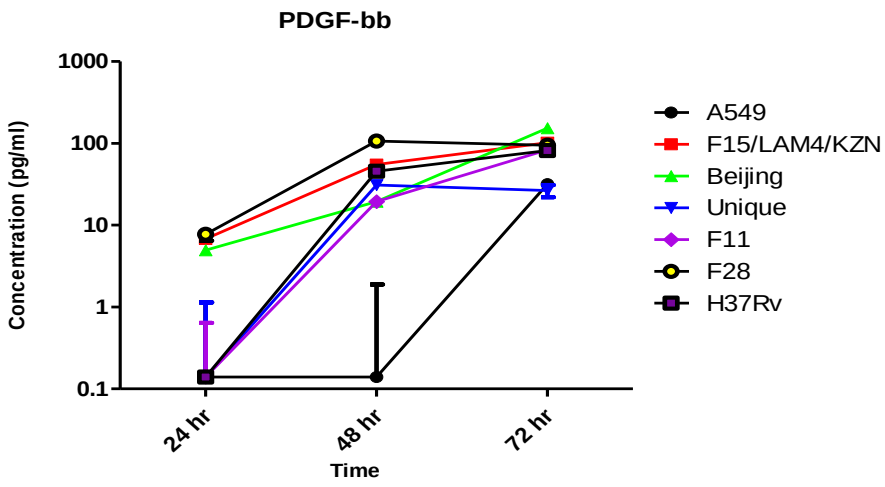

Eotaxin
1000
100
10
1
Concentration (pg/ml)
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv

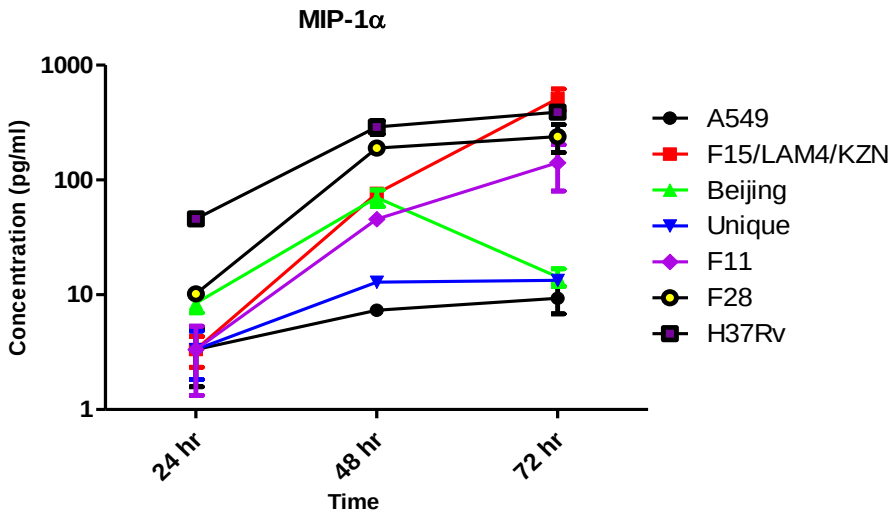

G-CSF
100000
10000
1000
100
10
1
Concentration (pg/ml)
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv

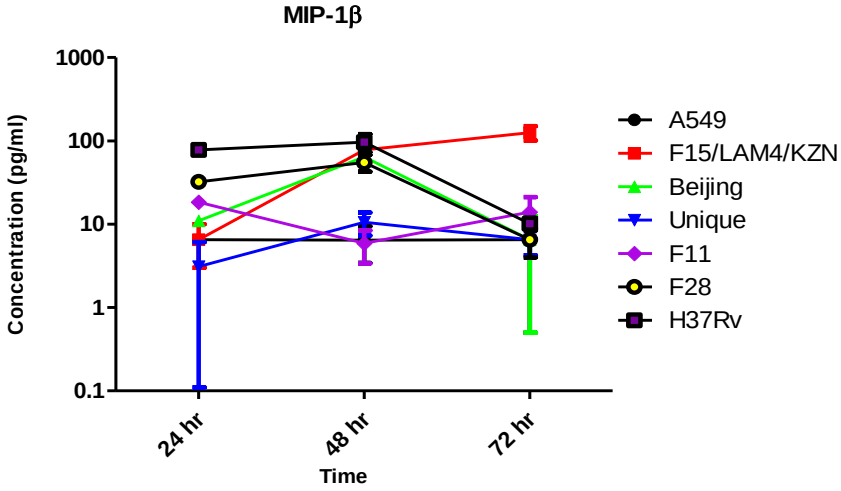

IP-10
100000
10000
1000
100
10
1
Concentration (pg/ml)
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv


GM-CSF
10000
1000
100
10
1
Concentration (pg/ml)
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv


MCP-1
100000
10000
1000
100
10
Concentration (pg/ml)
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv


PDGF-bb
1000
100
10
1
0.1
Concentration (pg/ml)
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv


MIP-1α
1000
100
10
1
Concentration (pg/ml)
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv


MIP-1β
1000
100
10
1
0.1
Concentration (pg/ml)
24 hr
48 hr
72 hr
Time
A549
F15/LAM4/KZN
Beijing
Unique
F11
F28
H37Rv

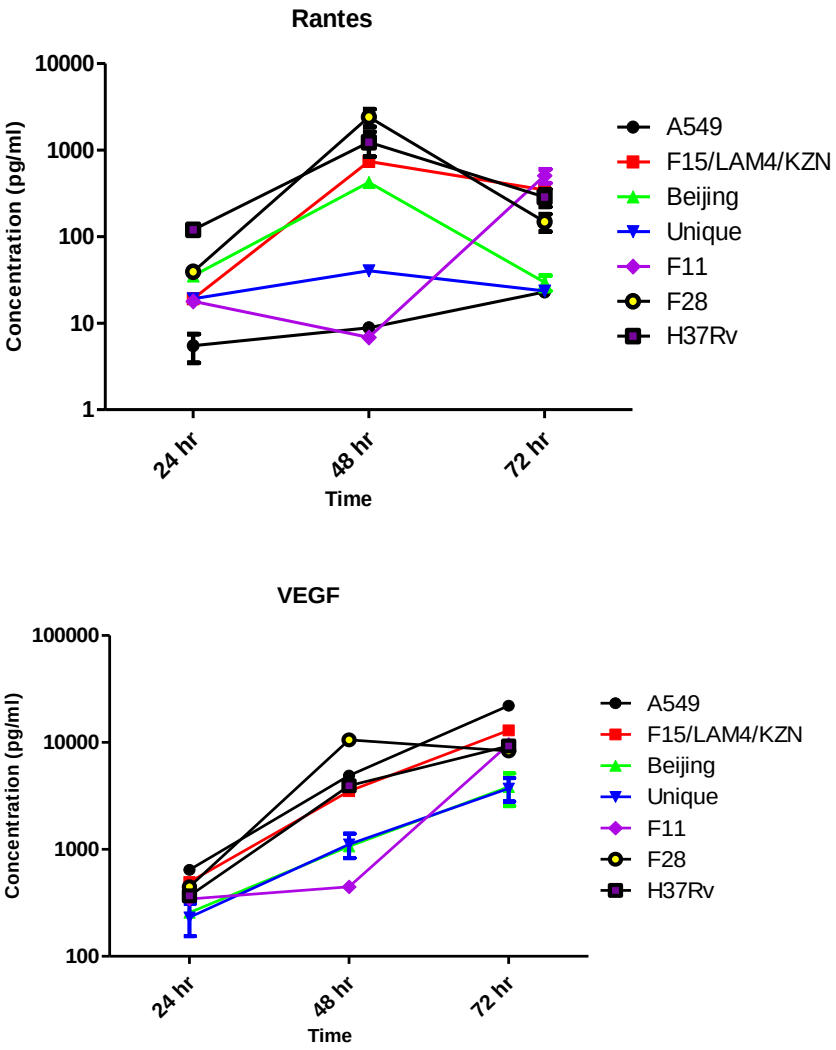


Fig. 5. Kinetic curves of chemokine production by epithelial cells infected with the F15/LAM4/KZN, Beijing, Unique, F11, F28 and H37Rv strains. A549 cells were infected with clinical strains of *M. tuberculosis* and laboratory strain H37Rv at an MOI~10:1. Supernatant was collected at 24, 48 and 72 hr post-infection. Cytokines and chemokines were measured by multiplexing 27-analytes using the Bio-Plex Manager 6.1. The data is presented as ±SEM of two independent experiments. Unpaired student t-test was used for statistical analysis ($P<0.05$ significant).

Clinical strains exhibited differential intracellular growth in pulmonary epithelial cells (Fig. 6), with the highest by the Beijing strain at 24 hr, F11 strain at 48 hr and F28 strain at 72 hr. These intracellular bacillary loads were significantly higher compared to other clinical strains. At 24 hr, significant differences were observed in the intracellular CFU/ml among the strains with the following exceptions: F11 compared to F28 and H37Rv; and F28 compared to the Unique strains, respectively. At 48 hr, all strain exhibited significant differences in the intracellular growth except for the F15/LAM4/KZN and Beijing strain, as well as between Beijing and Unique strain. Lastly, significant differences were observed for all strains at 72 hr except for the following: Beijing vs H37Rv and H37Rv vs F11, respectively (Fig. 6, Supplementary Table 4 and 5).

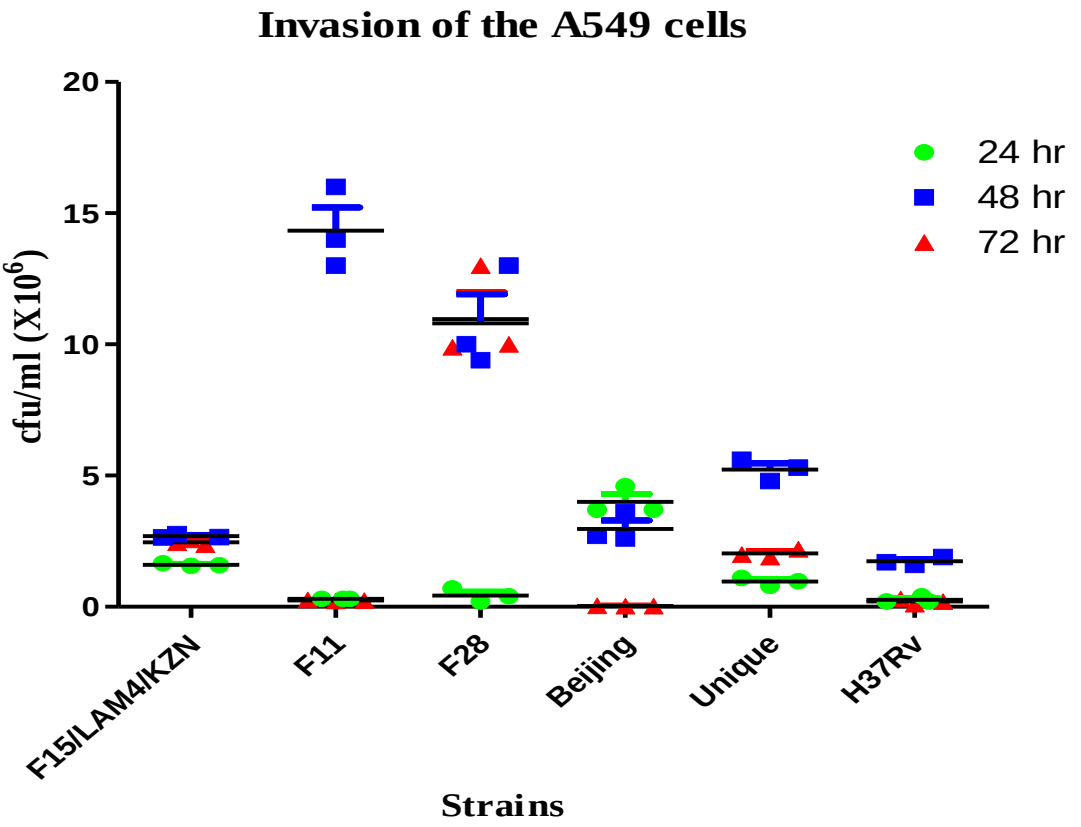


Fig. 6. Invasion of A549 cell by clinical strains of *M. tuberculosis* at 24, 48 and 72 hr after infection. Data is presented as ±SEM of three independent experiments plated in triplicate.

The A549 pulmonary epithelial cells were infected with clinical (F15/LAM4/KZN, F11, F28, Beijing, Unique) and a laboratory (H37Rv) strain of *M. tuberculosis* at an MOI~10:1. Intracellular growth of these strains varied within epithelial cells at different time intervals with highest growth observed for the Beijing strain at 24 hr, F11 strain at 48 and F28 strain at 72 hr. Paired t-test was performed among the strains and $P < 0.05$ was considered significant.

Multi-regression analysis

Different factors such as sampling intervals, variations in intracellular cfu/ml and strain identity were tested for their ability to influence cytokine/chemokine production by plotting analytes correlation matrix (Fig. 7) and computing significant influences of the variables of interest. The data was first normalized prior to regression and ANOVA analysis. It was observed that the sum of squares for the model used was greater than the residual sum of squares suggesting that factors were explained by the model that was fitted. From the three variables tested (cfu/ml, sampling intervals and strain identity), significant ($P < 0.05$) influences were caused by sampling intervals and not other factors. Using three factors that were tested, positive correlation was observed between most analytes as depicted in Fig. 7.

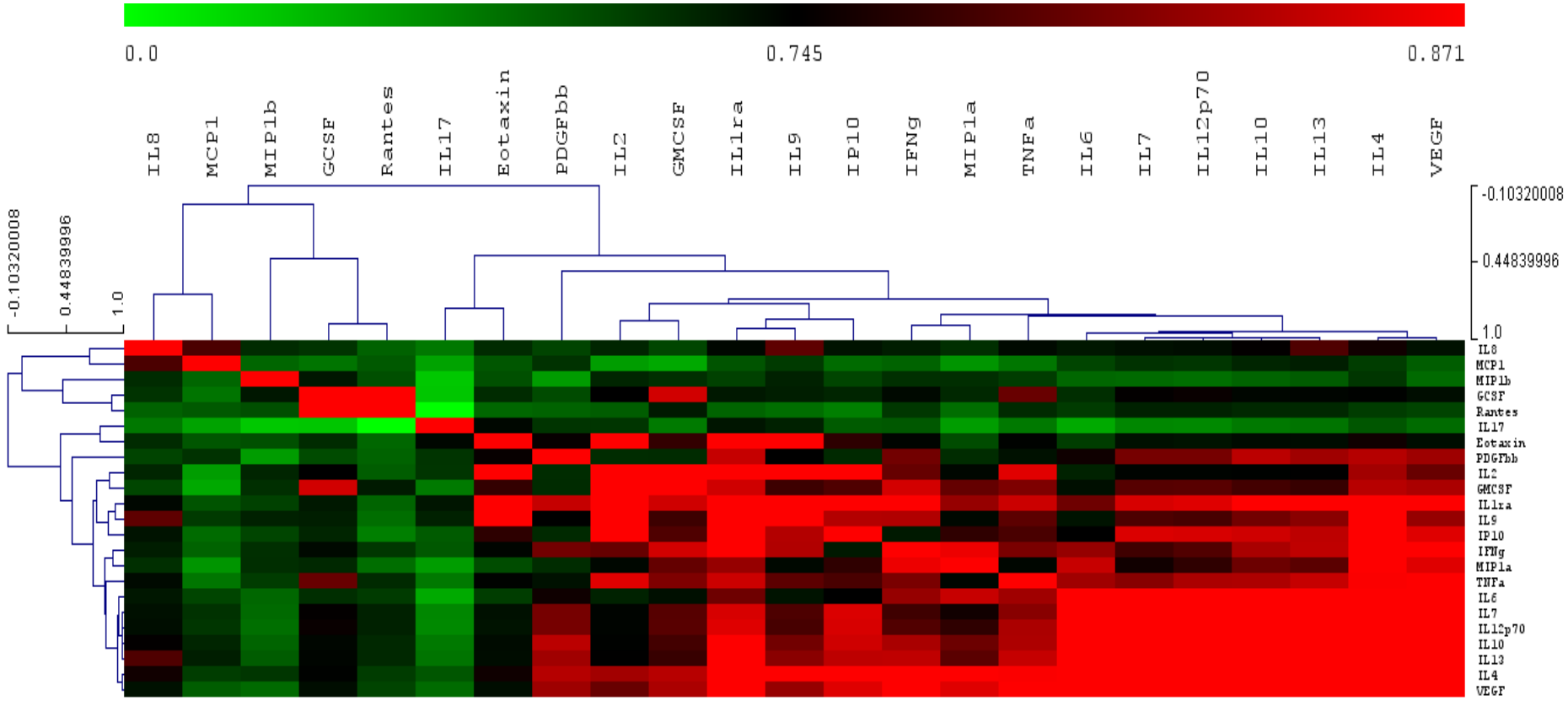


Fig. 7: Multi-regression analysis (Pearson) showing correlations between cytokine/chemokine production at all-time intervals. Strain identity, sampling intervals and intracellular Cfu/ml were tested in the ability to influence production of different analytes at all times tested using Genestat (v2.0) and SPSS (v16.0). Only sampling intervals significantly ($P < 0.05$) affected the production of different analytes compared to other factors tested.

Discussion and conclusion

Recent studies have provided increasingly compelling evidence, resulting in a paradigm shift, of the role of pulmonary epithelial cells from that of functioning largely as a protective barrier, to being intrinsically involved in the host immune response to TB infection (Lee *et al.*, 2009; Lin *et al.*, 1998; Sato *et al.*, 2002; Sharma *et al.*, 2007; Whitsett and Alenghat, 2015). In addition to the well described immune cells such as macrophages and dendritic cells, epithelial cells, infected by different *M. tuberculosis* genotypes, have also been reported to induce *in vitro* and *in vivo* production of cytokines/chemokines (Lin *et al.*, 1998). Their role in the adaptive immune response and granuloma formation was well described by Chuquimia *et al*. (2012).

Most studies however, reported on a limited number of cytokines/chemokines induced in either epithelial cells (Chuquimia *et al.*, 2012; Lee *et al.*, 2009; Lin *et al.*, 1998; Méndez-Samperio *et al.*, 2006; Sharma *et al.*, 2007; Wickremasinghe *et al.*, 1999; Zhang *et al.*, 1997) or macrophages/monocytes by clinical strains (Carmona *et al.*, 2013; Chakraborty *et al.*, 2013; Faksri *et al.*, 2014; Rhoades *et al.*, 1995), or non-clinical strains such as laboratory H37Ra, H37Rv and *M. bovis* (BCG) strain (Ameixa and Friedland, 2001; Andersson *et al.*, 2012; Chuquimia *et al.*, 2012; Keane *et al.*, 2000). Thus, the extent of the cytokine/chemokine response elicited by epithelial cells infected with characterized clinically relevant strains remained unexplored.

We have shown that pulmonary alveolar epithelial cells induce the production of a wide repertoire of cytokines and chemokines, peaking at 48 or 72 hr, in response to *in vitro* infection with *M. tuberculosis*. Moreover, this response differs significantly among a

laboratory and 5 clinical strains. The latter represent 4 strains belonging to well characterized, highly successful *M. tuberculosis* genotypes in several provinces of South Africa, including KwaZulu-Natal, and the Western and Eastern Cape (Chihota *et al.*, 2012; Muller *et al.*, 2013; Pillay and Sturm, 2007), as well as a Unique genotype. Strain families may be stratified by their level of virulence inferred by the magnitude of transmission within a population. Data from the KwaZulu-Natal Province in South Africa showed an over-representation of the drug resistant F15/LAM4/KZN followed by the F28, F11 and Beijing strains respectively between 2004-2005 (Chihota *et al.*, 2012). Hypervirulent or highly transmissible strains such as the Beijing strain, have been shown to induce low protective host cytokine responses (Manca *et al.*, 1999; Manca *et al.*, 2001; Ranjbar *et al.*, 2009; Tanveer *et al.*, 2009), that may explain the success of these strains epidemiologically (Chen *et al.*, 2014).

The production of anti-inflammatory cytokines (IL-1ra, IL-4 IL-9, IL-10, IL-13), pro-inflammatory cytokines (IL-2, IL-6, IL-7, IL-8, IL-12p70, IL-17, TNF-α, IFN-γ) as well as chemokines (Eotaxin, G-CSF, GM-CSF, IP-10, MCP-1, MIP-1a, MIP-1b, PDGF-bb, Rantes VEGF) was differentially induced at different times post-infection. The analytes IL-7; 10; 12p70, 13, 17 and VEGF were constitutively expressed by A549 cells.

Early production of the anti-inflammatory cytokines IL-10 and IL-22 in *M. bovis* (BCG) infected primary human bronchial epithelial cells was associated with a time dependent increase in the pro-inflammatory cytokine IL-6, but total inhibition of TNF-α and IFN-γ (Lutay *et al.*, 2014). In our study, IL-10 and IL-13 were produced at lower levels up to 72 hr. IL-22 was not included in the panel of analytes tested. A strong, early anti-inflammatory response was induced by the F28 strain as evidenced by peak concentrations of IL-1ra, IL-4 and IL-9 at 48 hr, but this decreased by 72 hr. The production of these cytokines increased

from 24 to 72 hr by epithelial cells induced by the F15/LAM4/KZN strain. This response pattern was similar for the other 4 strains, but at lower concentrations. The highest concentration was induced by the F15/LAM4/KZN strain compared to the others at the last time point tested. Since anti-inflammatory cytokines were produced at much lower concentration compared to pro-inflammatory cytokines and chemokines, pulmonary epithelial cells might not possess a strong anti-inflammatory response in the lungs during infection by these strains at an earlier time of infection. These anti-inflammatory cytokines from pulmonary epithelial cells are less likely to inhibit the protective Th1 response during early infection by clinical strains tested in the study.

IL-6, produced by many cell types (Akdis *et al.*, 2011) and capable of both pro- and anti-inflammatory activity (VanHeyningen *et al.*, 1997) is essential to induce IFN-γ during early lung infection (Saunders *et al.*, 2000). It has also been implicated in inhibition of macrophage response to IFN-γ, resulting in unsuccessful control of *M. tuberculosis* (Nagabhushanam *et al.*, 2003). Lee *et al.* (2009) reported on increased IL-6 production by epithelial cells that peaked at 48 hr and was reduced at 96 hr (Lee *et al.*, 2009). Our study also showed that epithelial cells infected by *M. tuberculosis* strains of different families induce substantial amounts of IL-6 at 48 and/or 72 hr, with the F15/LAM4/KZN and F28 inducing the highest production. This pattern was also observed for the induction of IFN-γ production. In contrast, the F11 strain induced high IL-6, but low IFN-γ. Notably, low concentrations of both cytokines were induced by the Beijing strain, similar to the Unique strain. This observation for the Beijing strain, as a hyper-virulent genotype (Caws *et al.*, 2008; Glynn *et al.*, 2002) is hardly surprising. Similar findings have been reported in monocyte/macrophage and animal models (Manca *et al.*, 1999; Manca *et al.*, 2001; Tanveer *et al.*, 2009).

It is likely that the low IFN-γ induction in epithelial cells by the Beijing strain acts in concert with the low response of macrophages, resulting in an inability to control *M. tuberculosis* in the early stages of infection (Reiling *et al.*, 2013), thus contributing to its capacity for extensive transmission. However, the low IFN-γ induction by the F11 and Unique strains remains unexplained. Unlike these 3 strains, the capacity to induce the release of high concentrations of IFN-γ may indicate the greater effectiveness of the STAT-1 pathway activated by the F15/LAM4/KZN and F28 strains, as reported for the laboratory strain, H37Rv (Sharma *et al.*, 2007). Furthermore, our findings were similar to that of Sharma *et al.* (2007), whereby induction of IFN-γ in epithelial cells progressed with the duration of infection and was maximal at 72 hr. However, higher concentrations of IFN-γ were detected in our study. This was observed for all strains with the exception of the F28 strain that peaked at 48hr and decreased by 72hr. The spread of the F15/LAM4/KZN and F28 strains in our KwaZulu-Natal population may be explained by delayed detection of drug resistance and initiation of appropriate treatment, rather than hypervirulence, whilst our data may suggest increased virulence of the F11 and the Unique strain due to induction of low IFN-γ compared to other clinical strains, despite the apparent lack of transmission of the latter. An alternate explanation may be that the Beijing strain family, unlike the F15/LAM4/KZN family, has had the opportunity through transmission in high risk settings, to co-evolve in humans substantially over time into "modern" strains, capable of subverting the TH1 response (Chen *et al.*, 2014; Rakotosamimanana *et al.*, 2010).

IL-7 plays a role in the development of B and T-cells, expansion of T-cells that is important for the control of *M. tuberculosis* (Maeurer *et al.*, 2000). The release of IL-7, despite being constitutively induced by uninfected epithelial cells, was significantly reduced at 48 and 72 hr

by the Beijing and Unique strains, unlike the other 3 clinical strains, which may further contribute to the success of the Beijing strain.

The repertoire of protective functions of TNF-α includes granuloma formation and maintenance, and induction of specific chemokines (Algood *et al.*, 2004), macrophage activation in conjunction with IFN-γ (Flesch and Kaufmann, 1990) and apoptosis control in infected cells (Keane *et al.*, 2000). TNF-α production by *M. tuberculosis* infected pulmonary epithelial cells was previously associated with the innate immune control and containment of TB infection (Lee *et al.*, 2009). As with the other cytokines, low amounts of TNF-α were induced by the Beijing and Unique strains at all time-points after infection in our study. In contrast, the F11, F28 and F15/LAM4/KZN strains induced high levels of TNF-α that peaked at either 48 or 72 hr after infection.

Low production of TNF-α could be detrimental to the host (Miller and Ernst, 2009) and is one of the survival strategies employed by *M. tuberculosis* (Balcewicz-Sablinska *et al.*, 1998; Keane *et al.*, 2000), including the highly pathogenic Beijing strain (Faksri *et al.*, 2014). The similarity in the behavior between the Beijing and Unique strains supports our hypothesis that the latter may share certain virulent factors to induce comparable behavior to the former strain. Different virulence factors harbored by clinical strains may explain complex and differential host response observed in epithelial cells in the production of protective cytokines such as TNF-α and IFN-γ.

IL-8 has anti-mycobacterial properties with the potential of inhibiting *M. tuberculosis* replication at the site of infection (O'Kane *et al.*, 2007). The production of IL-8 by epithelial cells infected with *M. avium*, *M. tuberculosis* H37Ra, H37Rv and uncharacterized clinical

strains has been previously documented (Lee *et al.*, 2009; Lin *et al.*, 1998; Wickremasinghe *et al.*, 1999). The production of the chemokine IL-8, elicited by both clinical and laboratory strains of *M. tuberculosis* but not avirulent *M. avium* and killed *M. tuberculosis,* was attributed not to virulence, but the ability to grow intracellularly (Lin *et al.*, 1998). In our study, similar concentrations of IL-8 were induced by all clinical strains at 48/72 hr, with the exception of the significantly increased and decreased amounts by the F15/LAM4/KZN and Beijing strains, respectively, at 72 hr post-infection. IL-8 secretion links the innate and adaptive immune response by serving as a chemo-attractant for leukocytes, mostly neutrophils, as well as other granulocytes (Andersson *et al.*, 2012). Increased neutrophil migration was observed in the *M. bovis* (BCG) infected epithelial cell model that induced production of IL-8 (Andersson *et al.*, 2012). The high levels of IL-8 induced by the F15/LAM4/KZN and F28 suggest that they may be more efficient at attracting large numbers of leukocytes *in vivo* compared to the Beijing and Unique clinical strains.

The recruitment of T-cells, monocytes and macrophages to the site of infection is crucial as they play a major role in granuloma formation and effective immune response. G-CSF and GM-CSF stimulate production of macrophages and granulocytes and their migration to the site of infection for granuloma formation (Nambiar *et al.*, 2010; Szeliga *et al.*, 2008; Thomas *et al.*, 2002). Nambiar *et al*. (2010) showed that mice infected with the *M. bovis* (BCG) strain induced moderate GM-CSF production necessary to prevent immunopathology of the lung tissue (Nambiar *et al.*, 2010). However, over-production of this chemokine has been associated with severe immunopathology (Lang *et al.*, 1987). In our study, a time dependent increase in both chemokines was observed for the F15/LAM4/KZN strain which peaked with high concentrations at 72hr. In contrast, the F28 peaked at 48 hr and rapidly declined to negligible amounts by 72hr. Very low concentrations were induced by the Beijing and

Unique strains at all intervals, except for the former at 48 hr. The differences in the quantity and time of production of these chemokines might be related to the time required for granuloma formation by the host. The low quantities of G-CSF and GM-CSF by the Beijing and Unique strains suggest delays in this process or ineffectual granuloma production, resulting in the lack of containment of the bacilli. Paradoxically, the high amounts of both G-CSF and GM-CSF by the F15/LAM4/KZN and F28 strains may be responsible for extensive lung damage, leading to increased infectivity and dissemination of the pathogen in the lungs.

Monocytes and alveolar macrophages are the main producers of MCP-1 and play an important role in granuloma formation (Silva Miranda *et al.*, 2012). *In vivo* studies have shown that increased production of MCP-1 were accompanied by a corresponding increase in the numbers of monocytes in the pleural fluid of the infected patients (Antony *et al.*, 1993) as well as in the BAL of TB infected individuals (Kurashima *et al.*, 1997). Although demonstrating an inability to effectively recruit monocytes to *M. tuberculosis* infected lungs, clearance of the pathogen by MCP-1 deficient mice remained unaffected (Lu *et al.*, 1998). *M. tuberculosis* infected epithelial cells can also produce MCP-1. Lin *et al.* (1998) showed a time and inoculum dose dependent increase in MCP-1 concentrations up to 6 days post infection. As observed by Lin *et al*. (1998), in our study, epithelial cells also spontaneously produced MCP-1. Levels of this chemokine increased up to 48 hr after infection and decreased at 72 hr in epithelial cells infected by all strains with the exception of the F11 strain which peaked at 72 hr. Increased amounts were induced by all clinical strains, with the highest by the F15/LAM4/KZN and F11 strains. Similar amounts were induced by the Beijing and Unique strains at both 48 and 72 hr, supporting the finding by Lin *et al*. (1998) that MCP-1 production is independent of strain virulence. The differences in MCP-1 production in epithelial cells most likely reflect strain specific phenotypes and may influence

the time taken for the involvement of other immune cells to further produce other protective cytokines.

As a result of its multiple receptor binding capacity (Proudfoot *et al.*, 2000), Rantes has the potential to activate numerous signaling pathways leading to a wide spectrum of cellular responses (Wong *et al.*, 2001). Skwor (2005) elucidated the regulatory role of Rantes in the cytokine/chemokine network. Whilst expression of the pro-inflammatory cytokines IL-8, TNF-α and IL-1β and chemokine MCP-1 was induced by recombinant Rantes in guinea pig macrophages, LPS exposure of Rantes pre-treated cells led to decreased expression of these genes at either 2 and/or 6 hr (Skwor, 2005). Similarly, TNF-α protein secretion by Rantes pre-treated macrophages was decreased following LPS exposure after 6 hr. Inconsistent findings with respect to IL-1β protein secretion have been reported (Locati *et al.*, 2002; Schindler *et al.*, 1990). In the present study, maximum production of Rantes was induced at 48 hr in epithelial cells by all clinical strains except the F11 strain at 72 hr. The Beijing and the Unique strains produced the lowest amount of Rantes at any time point. Failure to produce Rantes could be detrimental to the host as was observed in individuals with polymorphisms in the Rantes gene that rendered them more susceptible to TB infection compared to healthy controls (Ben-Selma *et al.*, 2011). Rantes production by epithelial cells could be important in activating the surrounding T-cells to favour a TH1 phenotype that could lead to bacterial clearance. This was observed with T-cells activated by dendritic cells that produced Rantes resulting in a TH1 phenotype of T-cells (Salam *et al.*, 2008). Rantes production by epithelial cells was stimulated by IL-1β production by monocytes (Wickremasinghe *et al.*, 2004). In contrast, in our study, *M. tuberculosis* stimulated epithelial cells were able to produce Rantes, despite the lack of induction of IL-1β production. This lack may be due to production of IL-1ra (data not shown) that inhibits production of IL-1

(Akdis *et al.*, 2011). Unlike our study, Lin *et al*. (1998) demonstrated that A549 cells were incapable of Rantes expression following infection with *M. tuberculosis*.

Similar to other cytokines/chemokines detected in this study, a host-specific response was observed by epithelial cells infected by different strains to produce IP-10. The highest concentrations were produced by the F15/LAM4/KZN and F11 strains at 72 hr and F28 strain at 48 hr. The Beijing and Unique strains again induced the lowest production of this chemokine. As with Rantes, IP-10 has been shown to activate T-cells to favour a TH1 phenotype and intracellular mycobacterial clearance (Salam *et al.*, 2008). In addition, both Rantes and IP-10 have been reported to be important for chemotaxis of other immune cells (Alam *et al.*, 1993) to the site of infection.

Production of IL-2, -12p70, -17, Eotaxin, β-chemokines (MIP-1α and MIP-1β), PDGF-bb and VEGF was also strain specific as observed with other analytes detected in this study, with peak production at either 48 or 72 hr after infection by each strain. Lowest production of all these analytes at 48 hr was induced by the F11 strain but increased at 72 hr, while Beijing and Unique strains induced lower concentration compared to other clinical strains. Our study demonstrated for the first time, that pulmonary epithelial cells produce these chemokines (Eotaxin, IL-2, PDGF-bb, MIP-1α and MIP-1β) in response to infection by clinical strains of *M. tuberculosis* in variable concentrations and peaking at different times. We hypothesize that the functional significance of epithelial MIP-1α and MIP-1β may play a supportive role in macrophage inhibition of *M. tuberculosis* replication at the site of infection. IL-17 is one of the important pro-inflammatory cytokine that act together with TNF and IL-1 and destroy cellular components of the pathogens (Chiricozzi *et al.*, 2010; Miossec *et al.*, 2009). IL-2 is important in regulating white blood cells that are required to render immunity (Liao *et al.*,

2011), while IL-12 is required for differentiation of T-cells to TH1 cells (Hsieh *et al.*, 1993). Production of these analytes detected in pulmonary epithelial cells indicates a crucial role of pulmonary epithelial cells to contribute to both local and adaptive immunity in a strain specific manner.

The diverse patterns of cytokine production shown by hierarchical clustering and PCA indicate distinct strain-specific patterns induced in pulmonary epithelial cells at an earlier time of infection. These differences can be attributed to differences in virulence factors that are yet to be identified in these clinical strains. Among the obvious differences in these patterns were cytokines induced by the F15/LAM4/KZN (48 and 72 hr) and F28 (48 hr) strains within the PCA plots. Both these clinical strains induce specific patterns that differed from those of other clinical strains. Generally, peak concentrations for most analytes were induced by the F28 strain at 48 hr and F15/LAM4/KZN strain at 72 hr post-infection. This might cause differences in cytokines/chemokines patterns as shown by the PCA plot. High concentrations of cytokines/chemokines by both these strains at an earlier time of infection might indicate an early response by the host compared to other clinical strains. Since pulmonary alveolar epithelial cells are present at the site of infection, these differences in cytokine levels induced by diverse clinical strains can influence down-stream changes and outcome of infection. The dominant South African F15/LAM4/KZN, F11 and F28 strains induced higher concentrations compared to the global prevalent Beijing strain and non-clustering Unique strain. This demonstrates a protective response induced by the F15/LAM4/KZN and F28 strain compared to the Beijing and Unique strain at an earlier time of infection. Moreover, the strain-specific immunological markers observed in this study might have resulted from interaction of specific pathogen-associated molecular patterns with

innate receptors, such as toll-like receptors, expressed in pulmonary epithelial cells (Li *et al.*, 2012).

There was no correlation between the intracellular growth and cytokine production as shown by the multi-regression analysis. These findings suggest that intracellular bacillary load did not influence production of most cytokines/chemokines tested at all-time intervals and that differential cytokine production might be due to diverse virulence factors rather than differences in the intracellular growth of clinical strains in pulmonary epithelial cells. Sampling intervals was the only significant factor to influence analytes production shown by multi-regression analysis. This might be due to peak production of all cytokines at 48 or 72 hr by the clinical strains, therefore supporting positive correlation between time of incubation and higher cytokine production.

Virulent strains, particularly of the Beijing family, evoked lower protective cytokine responses compared to less virulent ones in *in vivo* and *in vitro* studies (Chen *et al.*, 2014; Manca *et al.*, 2004; Manca *et al.*, 1999; Manca *et al.*, 2001; Portevin *et al.*, 2011; Tanveer *et al.*, 2009). The current data, with lower cytokine/chemokine production induced by the Beijing compared to the F15/LAM4/KZN, F11 and F28 strains concurs with those findings, suggesting that the Beijing strain may be the most virulent among clinical strains globally. The similar, consistently lower levels observed for the Unique compared to the Beijing strain, suggest that while the former was not detected in more than 1 patient in the study population, it nevertheless, may potentially share certain virulent factors as the latter, resulting in low cytokine induction by this strain. Virulent strains belonging to the F15/LAM4/KZN and Beijing family were reported to induce higher cytotoxicity of epithelial cells compared to the Unique strains and laboratory strains (Ashiru and Sturm, 2015). This might also explain

lower cytokine/chemokine production by Beijing and Unique-infected epithelial cells, however this does not explain higher cytokines/chemokines induced by the F15/LAM4/KZN strain. Further studies are warranted to test different hypothesis generated from our findings. The higher cytokine/chemokine responses of the F15/LAM4/KZN and other 2 clinical strains suggest that their transmissibility may be related to the failure of the TB control program as a result of profound delays in the detection and initiation of timeous and appropriate treatment/management of drug resistant cases rather than hyper-virulence. The strain specific response of these 3 genotypes probably reflects diversity in specific virulence traits that should be elucidated in future studies.

Several limitations may be observed in the study. The use of an *in vitro* system means that the findings emanating from this cannot be directly correlated to what happens *in vivo* in humans. Due to financial constraints, cytokine/chemokine analysis was performed in duplicate and not triplicate. Epithelial cells were not examined at sampling times for confluence and adherence. Another limitation was that it was not possible to rank these strains for their level of virulence in order to understand the different cytokine signatures that were obtained in this study despite molecular epidemiology studies being available. No data has been reported on the virulence characteristics of these strains apart from the Beijing strain, and only recently, limited information on the F15/LAM4/KZN strain (Smith *et al.*, 2014) without details on *in vitro* and i*n vivo* pathogenesis studies. Despite these shortcomings, valuable, novel information on the host response to epithelial cell infection has been generated.

In conclusion, our study demonstrates a strain-specific induction of a wide repertoire of cytokine/chemokine production by pulmonary alveolar epithelial cells at different times post-infection by virulent strains of *M. tuberculosis*. We therefore hypothesize that the diversity observed in the cytokine/chemokine production may reflect the presence of determinants that in turn may confer varying degrees of virulence on these strains and might explain the

complex interaction between the host and pathogen and outcomes of infection. Furthermore, the F15/LAM4/KZN strain is unlikely to be a hypervirulent strain due to induction of high concentrations of protective cytokines in epithelial cells, and its association with the development of the XDR-TB epidemic in the Tugela Ferry region, South Africa, was probably related to programmatic shortcomings such as delays in diagnosis, control and treatment programs for tuberculosis.

We further confirm and extend the findings of other studies that, epithelial cells via their extensive repertoire of cytokine/chemokine production, play a significant role in the host immune response, most likely by recruitment of other immune cells and in granuloma formation *in vivo*. Future pathogenesis studies on the *in vitro* host response to *M. tuberculosis* infection should include the use of epithelial cells due to their abundance in the human lung alveolar space compared to macrophages, as well as inclusion of different virulent *M. tuberculosis* strains.

CHAPTER 3: *Mycobacterium tuberculosis* CLINICAL STRAINS EXHIBIT DIFFERENTIAL AND STRAIN-SPECIFIC SIGNATURES IN PULMONARY EPITHELIAL CELLS

Abstract

Novel approaches to biomarker identification are urgently required in the design and development of effective new therapeutic strategies that take cognisance of host response, especially to genetically diverse, dominant strains of *M. tuberculosis* in specific geographic regions. Given the importance of pulmonary epithelial cells in host immune response to *M. tuberculosis* infection, few studies have focussed on global transcriptomics in this infection model with different strains of *M. tuberculosis.*

Therefore, this study was undertaken to elucidate differential gene expression and strain-specific molecular signatures in pulmonary epithelial cells during infection by clinical *M. tuberculosis* strains of varying pathogenicity. Transcriptomics was used to investigate changes in gene expression by directly sequencing RNA transcripts from pulmonary epithelial cells infected with strains belonging to four different genotypes and a Unique strain of *M. tuberculosis,* associated with drug resistance in KwaZulu-Natal. The Illumina HiSeq 2000 platform generated 50 bp reads that were mapped to the human genome (hg19) using Tophat (2.0.10). Differential expression between the uninfected and infected cells, as well as among the different strains, was quantified using Cufflinks (2.1.0) with a significant Q-value and a fold-change cut-off of 2. Gene expression varied among the strains with the total number of genes up-regulated and down-regulated shown in parentheses: F15/LAM4/KZN (1187), Beijing (1252), F11 (1639), F28 (870), Unique (886) and H37Rv (1179). A subset of 292 genes was commonly induced by all strains. Out of the 292 genes, 52 were down-

regulated and 240 were up-regulated. Down-regulation was associated with signal transduction, membrane associated functions, endopeptidase activity and steroid biosynthesis while up-regulation was associated with proteins found in the extracellular space, immune response genes, plasma membrane and cell receptors. The number of strain-specific genes activated by each strain were as follows: F15/LAM4/KZN (138), Beijing (52), F11 (255), F28 (55), Unique (185) and H37Rv (125). Strain specific molecular signatures associated with functional pathways were observed only in the F15/LAM4/KZN, Unique and H37Rv strains. These findings demonstrate that in addition to other cellular functions, pulmonary epithelial cells infected by *M. tuberculosis,* are involved in both innate and adaptive immune responses. Strain-specific molecular signatures identified in this study have the potential to be exploited for novel biomarker applications that could impact on host directed treatment strategies including immunotherapy.

Introduction

Despite the availability of effective drugs and advances in the development of rapid diagnostic techniques (Hillemann *et al.*, 2009; Ling *et al.*, 2008; Ulrich *et al.*, 2006), global burdens of TB, have not decreased significantly, with prevalence, incidence and mortality rates of approximately 2 billion, 9 million, and 1.5 million per year, respectively (WHO, 2014). These high burdens resulted largely from the emergence of drug resistant, including MDR and XDR strains (Abebe and Bjune, 2006; Klopper *et al.*, 2013; Shah *et al.*, 2007; Singh *et al.*, 2007; Velayati *et al.*, 2009) of *M. tuberculosis*, which are virtually untreatable (Klopper *et al.,* 2013). High HIV-co-infection rates (WHO, 2014), especially in poorly resourced countries, as well as the slow replication rate of this pathogen and the associated diagnostic delays and resultant delayed commencement of treatment have also contributed

significantly to high TB incidence rates (WHO, 2014). Moreover, the only effective available vaccine, BCG, is incapable of protecting individuals beyond childhood (Abebe and Bjune, 2006), and recent strategies to develop new vaccines have been either unsuccessful (Martin *et al.*, 2006; Waters *et al.*, 2009) or still under clinical investigation (Kaufmann, 2014). Upon testing of the latest and promising MVA85A TB vaccine, low tolerance with modest immunogenic and insignificant protection against *M. tuberculosis* in infant clinical trials was observed (Tameris *et al.*, 2013). Thus, pathogenesis studies that will enable the identification of novel pathogen and host specific biomarkers are urgently required for the development of rapid point of care diagnostic tools, and efficacious vaccines and therapeutics, especially against MDR and XDR-TB.

Genotyping has identified numerous strain families (de Viedma *et al.*, 2005; Glynn *et al.*, 2002; Streicher *et al.*, 2012; Valway *et al.*, 1998) that may elicit specific host responses based upon their unique virulence attributes (Koo *et al.*, 2012; Manca *et al.*, 2004; Manca *et al.*, 1999; Manca *et al.*, 2001; Subbian *et al.*, 2013). Different *M. tuberculosis* strains induce strain-specific host responses to infections in macrophages at different times post-infection (Koo *et al.*, 2012). Higher levels of certain cytokine/chemokine mRNA expression were observed in pulmonary epithelial cells infected with the less virulent *M. avium* compared to *M. tuberculosis* (Sato *et al.*, 2002). These studies elucidated the possible diversity of antigenic properties found in different mycobacterial species (Sato *et al.*, 2002), and expression patterns among *M. tuberculosis* strains (Dheenadhayalan *et al.*, 2006). A phenolic glycolipid was shown to be produced by hyper-virulent *M. tuberculosis* Beijing strains, resulting in lower host inflammatory responses and suppressed innate immune response (Reed *et al.*, 2004). Furthermore, genetic and phenotypic diversity among *M. tuberculosis* strains may be responsible for the underlying mechanism that results in differential host

response which can have implications on disease outcome (Kato-Maeda *et al.*, 2001; Malik and Godfrey-Faussett, 2005; Nicol and Wilkinson, 2008) and prevalence of that strain within the population. Thus, strain diversity is an important characteristic in the design and development of intervention strategies.

The host response to *M. tuberculosis* infection has been extensively studied by screening changes in gene expression using microarray analysis in mice (Jang *et al.*, 2008) and macrophages (Giacomini *et al.*, 2001; Koo *et al.*, 2012; Volpe *et al.*, 2006) as well as quantifying mRNA in epithelial cells using quantitative real time PCR (qRT-PCR) (Lee *et al.*, 2009; Lin *et al.*, 1998; Sato *et al.*, 2002). Transcriptomic studies in macrophages and dendritic cells using microarray analysis revealed changes in numerous immune related genes at different times of infection by *M. tuberculosis* (Tailleux *et al.*, 2008; Volpe *et al.*, 2006). Studies on gene expression in epithelial cells (Lee *et al.*, 2009; Lin *et al.*, 1998; Sato *et al.*, 2002; Wickremasinghe *et al.*, 1999; Wickremasinghe *et al.*, 2004) have focused only on a limited number of genes, whilst understanding of host-pathogen interactions using whole genome transcriptomics remains largely unexplored especially in epithelial cells infected with different clinical strains of *M. tuberculosis*. The importance of epithelial cells in pathogenesis has already been documented (Chuquimia *et al.*, 2013; Chuquimia *et al.*, 2012; Lee *et al.*, 2009; Lin *et al.*, 1998; Sato *et al.*, 2002; Sharma *et al.*, 2007), since they may be the first point of contact with the pathogen because of their abundance in the lung relative to macrophages (Dobbs *et al.*, 2009; Williams, 2003).

In this study, transcriptomics was used to investigate gene expression changes by using RNA-Seq of pulmonary epithelial cells infected with strains belonging to a Unique genotype, as well as four different genotype families of *M. tuberculosis*. We hypothesized that gene

expression and strain-specific molecular signatures would be differentially induced in pulmonary epithelial cells infected with the different clinical strains of *M. tuberculosis*.

Materials and methods

Details of selection of bacterial strains and infection of epithelial cells methods are provided in Chapter 2 and summarized in this chapter.

Bacterial isolates

Clinical strains of *M. tuberculosis* F15/LAM4/KZN, Beijing , F11 , F28 and a Unique strain used in the study were isolated from sputum specimens of patients in KwaZulu-Natal and characterized in Medical Microbiology and Infection Control, University of KwaZulu-Natal. The laboratory strain *M. tuberculosis* H37Rv was included as a virulent control. *M. tuberculosis* isolates were cultured in 10 ml Middlebrook 7H9 broth supplemented with 50% glycerol, 20% Tween-80 and 10% OADC at 37 ºC aerobically to a mid-log phase. Glycerol stocks of an actively growing culture at mid-log phase were stored at -70 ºC to avoid multiple passages and loss of virulence. For each infection assay, cultures were revived in 7H9 broth and grown to an OD_{600nm} of 0.8-1, where an OD_{600nm} of 1 = ~$1X10^8$ Colony forming units (cfu)/mL (Larsen *et al.*, 2007).

Infection of epithelial cells

Stock cultures of A549 type II pulmonary epithelial cells (ATCC CCL 185) were grown in 25 cm^2 TCFs containing EMEM (Lonza, South Africa) supplemented with 10% heat inactivated FCS (Lonza, South Africa). A pilot study was performed prior to experimental procedure to

determine optimal MOI, level of infection at each time intervals to be tested and confluent cells after infection. After optimizing the methodology in the pilot study, confluent cells were infected in two separate biological replicates with each strain of *M. tuberculosis* at an MOI of ~10:1 in 25 cm^2 TCFs and incubated at 37^oC in 5% CO_2 for 48 hr. Infected pulmonary epithelial cells were washed with PBS three time to remove extracellular adherent cells prior to cell lysis. Thereafter, cells were lysed with tritonX-100, washed twice with PBS, serially diluted and plated in triplicate on 7H11 Middlebrook agar plates. The cfu/ml was calculated after incubation at 37^oC in 5% CO_2 for 3-4 weeks.

RNA isolation from epithelial cells

RNA was isolated from uninfected as well as infected A549 epithelial cells using the RNeasy kit (Qiagen, South Africa) according to the manufacturer's instructions. Briefly, cells were lysed with RLT buffer together with 10µl β-mercaptoethanol to inhibit RNase activity and transferred to a column. This was washed with 700 µl of RW1 buffer followed by washing twice with 500 µl of RPE buffer. RNA was eluted twice with 40 µl of RNase-free water, quantified using a Nanodrop 2000c Spectrophotometer (ThermoScientific, South Africa) and stored in 10µl aliquots at -70ºC to prevent freeze-thaw cycles and retain RNA quality. The integrity of the RNA was assessed by RNA curves in the Spectrophotometer and RNA gel electrophoresis by estimating the sizes of 18S and 28S eukaryotic RNA subunits (Appendix B, Supplementary Fig. 2).

Library preparation and RNA sequencing

The RNA-Seq library for Illumina HiSeq 2000 sequencing for two biological replicates was prepared using the Illumina true-Seq RNA sample kit following the manufacturer's

procedure. Briefly, 200 ng of total RNA in a volume of 50 µl was mixed with 50 µl RNA purification magnetic beads. The fragmented mRNA was converted to double stranded cDNA that was repaired, tailed, and ligated with indexed adaptors. The adaptor linked cDNA library was then PCR amplified. The PCR products were purified using Agencourt RNAClean XP magnetic beads and electrophoresed on an Agilent high sensitivity DNA chip for quality control. Each of four samples used per lane for 50 bp sequencing were pooled in equimolar concentration and sequenced for single reads of 50 cycles.

Mapping and transcript assembly

Mapping, transcripts assembly and differential gene expressed was carried out within the South African National Bioinformatics Institute server, operating within the linux command line. Each job was executed using scripts described in the publicly available "Tuxedo Suite" from Trapnell *et al*. (2012) The details of reads processing and analysis were as follows: The sequencing reads were processed and mapped to the UCSC (*Homo sapiens*) reference genome (hg19) using Bowtie operating under TopHat (v. 2.0.9) as a mapping manager with the following command line: tophat -p 4 -o tophat_samplename -G references/genes.gtf --transcriptome-index references/hg19.mRNA references/Bowtie2Index/genome data/samplename.fastq. The alignment statistics for all reads was greater than 95% for all samples. All short reads were then assembled using Cufflinks (v. 2.0.2) that utilized the method of upper quartile normalization to increase the accuracy of differential analysis for different genes using the following command: cufflinks -p 4 –o fileoutput Condition_1_Replicate_1/accepted_hits.bam. Cuffdiff was used to calculate differential expression between the uninfected and infected epithelial cells and calculate a significant (<0.05) Q-values as well as fold changes between these two conditions (Trapnell *et al.*, 2012)

using the following command line: cuffdiff -o A549vsBeijing -L A549,Beijing -p 4 -N --compatible-hits-norm -b references/Bowtie2Index/genome.fa -M references/humanmask.gtf -u references/genes.gtf tophat_sample1/accepted_hits.bam,tophat_sample2/accepted_hits.bam tophat_sample13/accepted_hits.bam, tophat_sample14/accepted_hits.bam. CummeRbund (v. 2.0) was used for pairwise comparison and to visualize the Cuffdiff output generating scatter plots as well as volcano plots that shows differential expression and significantly regulated genes induced by the different strains in epithelial cells (Goff *et al.*, 2012).

Enrichment and functional analysis

The Molecular Signature Database (MSigDB) (http://www.broadinstitute.org/gsea/index.jsp) was used to enrich and functionally classify differentially expressed genes with a significant Q-value and a fold change of greater than two induced by different clinical strains of *M. tuberculosis*. Sets of genes that are co-regulated to induce a particular biological function or pathway were identified by MSigDB. Statistical cut-off FDR for the analysis was set at $P < 0.05$, in which C5 curated was selected to elucidate biological functions associated with differentially expressed genes, while C2 curated was selected to understand Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathways associated with mRNA genes of interest. Heat maps were generated to indicate the level of fold changes between uninfected and infected epithelial cells. Clustering of gene expression profiles among *M. tuberculosis* strains was performed by MeV (4.0.9) (Saeed *et al.*, 2003).

Identification of strain-specific molecular signatures

The total number of differentially expressed genes with a significant Q-value and a fold change cutoff of 2 was compared among clinical strains using the Bioinformatics &

Evolutionary Genomics database (http://bioinformatics.psb.ugent.be/webtools/Venn/). Both up-regulated and down-regulated genes were compared at a combination of six datasets, and the output for each strain combination and strain specific gene signatures was obtained. Expression levels of each comparison of enriched genes (MSigDB) were created using MeV (4.0.9) and compared for all strains, different strain comparisons and strain specific genes, respectively.

Quantitative real time PCR

In order to validate and confirm RNA-Seq results, six cytokine and chemokine genes were randomly selected for qRT-PCR analysis. Primers (Invitrogen, Life Technologies, South Africa) for these genes are shown in Table 1. The PCR conditions for each run were modified from original references and used accordingly. Denaturing temperature for all selected genes was 95 ºC, while annealing and extension temperatures were between 55-65 ºC and 55 to 72 ºC, respectively. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression where ΔCt = Ct (gene of interest) – Ct (normalizer_GAPDH). The fold change was calculated as ΔCt (sample)/ΔCt (calibrator_uninfected A549) and graphs were plotted using Graph pad prism (v. 5).

Table 1: Cytokine/chemokine primers used for quantifying mRNA by real time PCR

Gene	Forward	Reverse	Ref
IL6	ATGTAGCCGCCCCACACAGA	CATCCATCTTTTTCAGCCAT	(Lin *et al.*, 1998)
IL8	ATGACTTCCAAGCTGGCCGTG	TTATGAATTCTCAGCCCTCTTCAAAAACT TCTC	(Lin *et al.*, 1998)
MCP-1	CAAACTGAAGCTCGCACTCTCGCC	ATTCTTGGGTTGTGGAGTGAGTGTTCA	(Lin *et al.*, 1998)
GM-CSF	TGGCTGCAGAGCCTGCTGCTC	TCACTCCTGGACTGGCTCCC	(Sato *et al.*, 2002)
TNF-α	TCTCGAACCCCGAGTGACAA	TATCTCTCAGCTCCACGCCA	(Sato *et al.*, 2002)
IFN-γ	ATGAAATATACAAGTTATATCTTGGCTTT T	GATGCTCTTCGACCTTGAAACAGCAT	(Sato *et al.*, 2002)
GAPDH	GAGTCAACGGATTTGGTCGT	AAATGAGCCCCAGCCTTCT	(Rivas-Santiago *et al.*, 2005)

Results

Differentially expressed genes with a significant Q-value and a fold change of ≥2 varied among the strains. There were common genes induced by all clinical and laboratory strains used while other genes were only shared by different strain combinations. Strain-specific molecular signatures were also obtained and these signatures affected different Kegg and Reactome pathways in pulmonary epithelial cells.

Differential gene expression in epithelial cells infected with clinical strains of *M. tuberculosis*

Differential genes expression obtained from Cuffdiff varied among the clinical and laboratory strains of *M. tuberculosis* compared to the uninfected epithelial cells. The number of differentially expressed genes induced in epithelial cells by the different strains are shown in parenthesis: F15/LAM4/KZN (1187), F11 (1639), F28 (870), Beijing (1252), Unique (886) and H37Rv (1179). Scatter plots of pairwise comparison of differentially expressed genes induced by all strains relative to the uninfected epithelial cells showed variation in some gene expression patterns, whilst others were common among the strains. According to the patterns of gene expression, the strains could be categorized into 2 groups with similar profiles, viz, (i) F28, Unique, H37Rv strains and (ii) F15/LAM4/KZN, F11, Beijing strains (Fig. 1). Volcano plots showed significantly up- (red) and down (green)-regulated genes compared to all genes within the transcriptomics data of infected pulmonary epithelial cells compared to the uninfected A549 cells (Fig. 2).

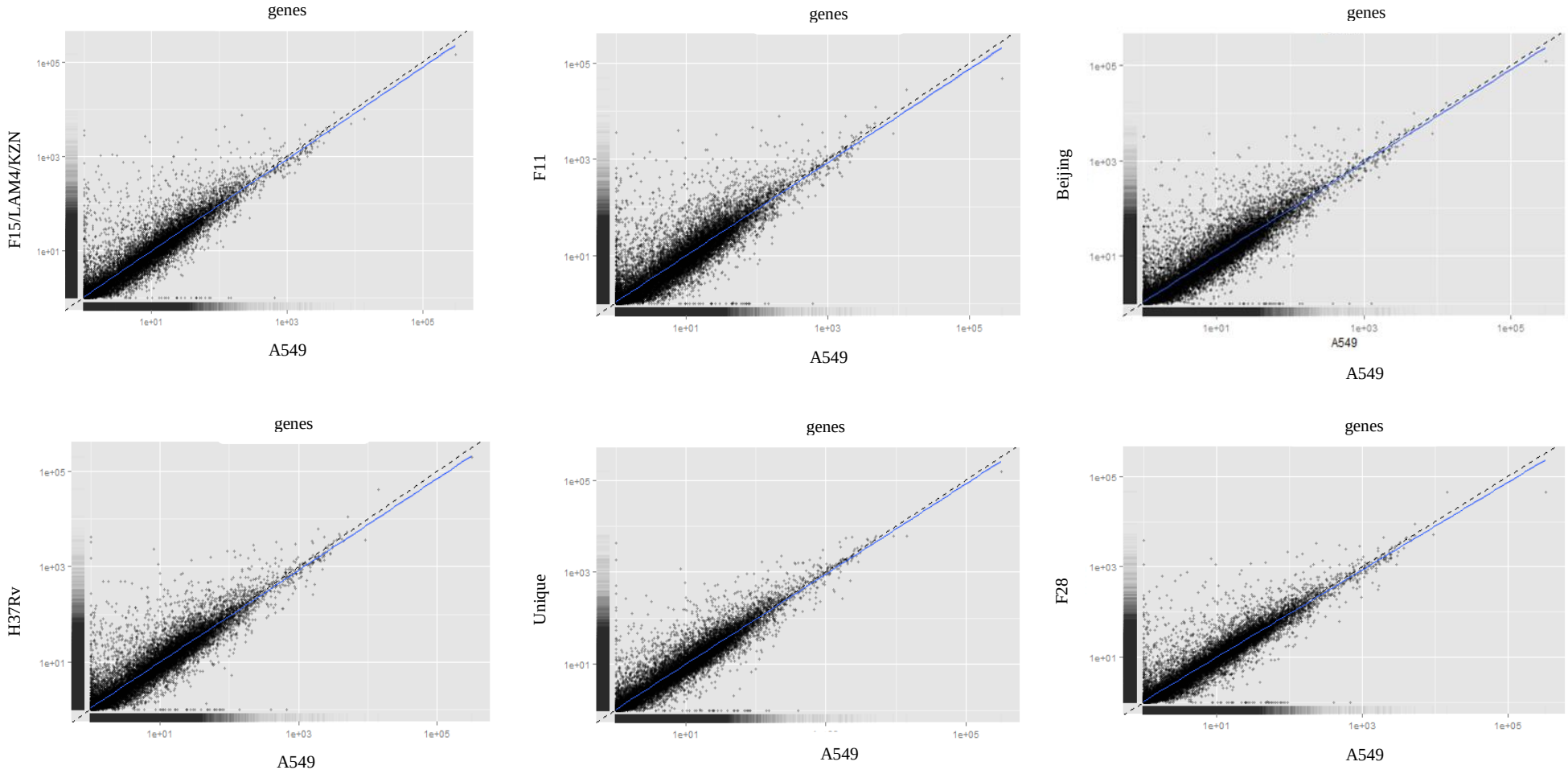
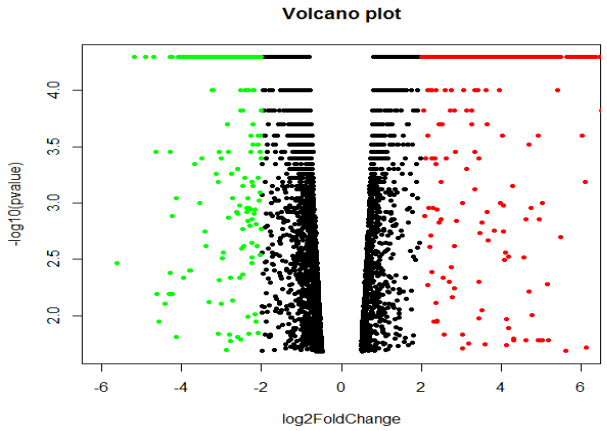
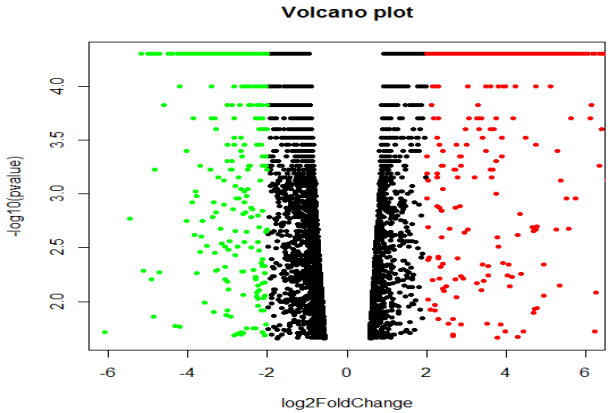


Fig. 1. Scatter plots showing pairwise comparison of differentially expressed genes in epithelial cells induced by different clinical and laboratory strains of *M. tuberculosis* in infected relative to un-infected A549 pulmonary epithelial cells. Differentially expressed genes varied among the strains: F15/LAM4/KZN, F11, F28, H37Rv, Unique and Beijing. Scatter plots were created by using R: Cummberbund.

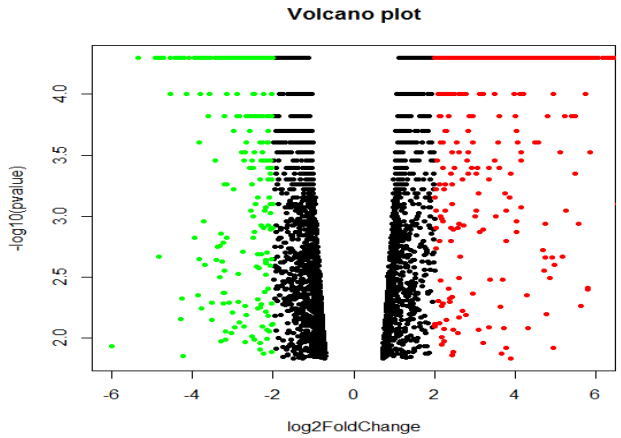
M. tuberculosis F15/LAM4/KZN strain



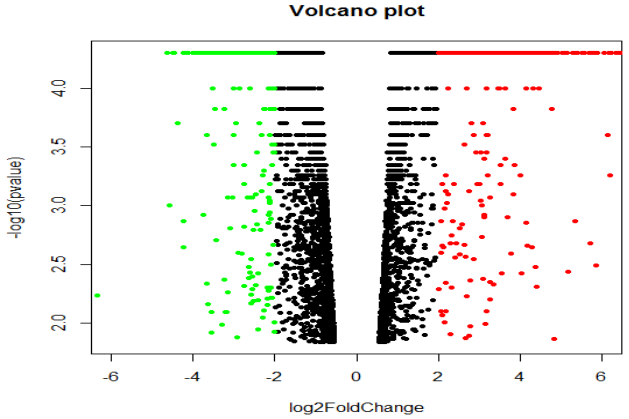
M. tuberculosis F11 strain



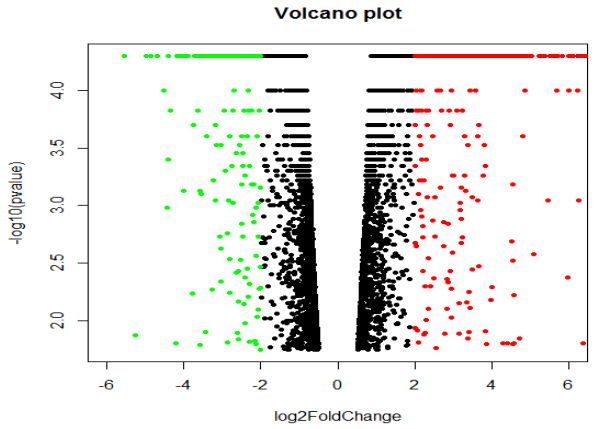
M. tuberculosis Beijing strain



M. tuberculosis F28 strain



M. tuberculosis Unique strain



M. tuberculosis H37Rv strain

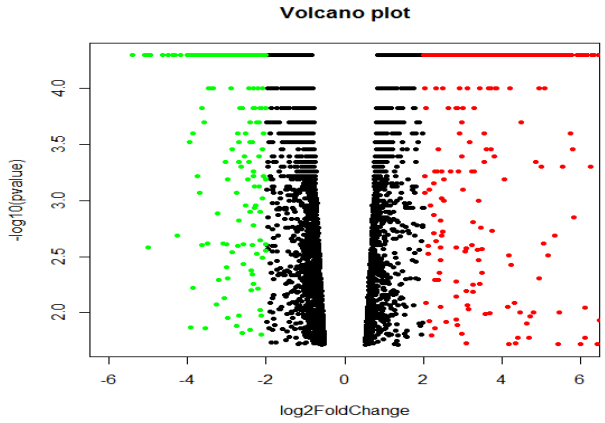


Fig. 2. Volcano plots showing significantly up- (red) and down (green)-regulated genes against all genes (black) in the transcriptomic data of infected pulmonary epithelial cells compared to uninfected A549 controls. Significantly regulated genes varied among clinical strains. Volcano plots were created by using the statistical software, R.

The top 10 most up-regulated and down-regulated genes are shown in Table 2. Among these, *OASL, P13* and *RSAD2* were commonly up-regulated by all strains. *CSF3, IF127, PI3, OAS2* and *CLEC4E* were up-regulated by all clustering strains and H37Rv but not the Unique strain. *IF16* was induced only by the Beijing and Unique strains in the top 10 up-regulated genes. Among the top 10 down-regulated genes, similar to the up-regulated ones, several genes were uniquely induced by the different strains, whilst none was commonly induced by all. Eight and 3 different genes were down-regulated in combination by 2 and 3 strains, respectively. Of these, *DYSF* and *CNTD2* were induced by the Beijing and the Unique strains within top 10 down-regulated genes (Table 2).

Table 2: The total number and the 10 most up- and down-regulated genes induced by different *M. tuberculosis* strains in A549 pulmonary epithelial cells

	F15/LAM4/KZN	F11	F28	Beijing	Unique	H37Rv
Up-regulated	**773**	**997**	**542**	**853**	**624**	**778**
	CSF3	***RSAD2***	*CSF3*	*CSF3*	*CA9*	*CSF3*
	RSAD2	*CSF3*	*CLEC4E*	***PI3***	*AHSG*	***PI3***
	OAS2	*TNFAIP6*	***RSAD2***	*IFI27*	*IGFBP5*	***RSAD2***
	OASL	***OASL***	*IFI27*	***OASL***	*CMPK2*	*CLEC4E*
	TNF	*CLEC4E*	***PI3***	***RSAD2***	***OASL***	***OASL***
	IFI27	*IFI27*	*OAS2*	*CLEC4E*	*IFITM1*	*MMP10*
	TNFAIP6	***PI3***	***OASL***	*OAS2*	*IFIT1*	*MMP1*
	CLEC4E	*OAS2*	*CMPK2*	*IFI6*	*IFI6*	*IFI27*
	IL23A	*IFIT2*	*IFI44L*	*CMPK2*	***RSAD2***	*OAS2*
	PI3	*NOD2*	*NOD2*	*CFB*	***PI3***	*CMPK2*
Down-regulated	**414**	**642**	**328**	**399**	**262**	**401**
	CERS1	*MYPN*	*SSTR5-AS1*	*MYPN*	*SLAMF7*	*HLF*
	SSTR5-AS1	*VEPH1*	*EGR3*	*VEPH1*	*AQPEP*	*SSTR5-AS1*
	KIF17	*ST6GALNAC3*	*SSTR5*	*DYSF*	*DYSF*	*F7*
	RHBDL1	*DDC*	*CNTN1*	*HRK*	*FAM129A*	*DDC*
	HOXA13	*MAGI2-AS3*	*CERS1*	*RAB39B*	*KLHDC7B*	*SSTR5*
	COL26A1	*PABPC1L*	*ESRP1*	*CDKL2*	*IL16*	*DOK7*
	CILP2	*LOC728392*	*MATN2*	*CNTD2*	*KIAA1045*	*NR0B1*
	KCNIP3	*GPNMB*	*SYTL1*	*KIF17*	*ADAMTS16*	*MYPN*
	RTN4RL1	*TRIM67*	*SCEL*	*MPP1*	*CNTD2*	*MATN2*
	RCOR2	*RNFT2*	*APOE*	*ST6GALNAC3*	*ESRP1*	*KRT20*

Expression pattern of common genes induced by all *M. tuberculosis* strains in pulmonary epithelial cells

Differentially expressed genes between uninfected and infected pulmonary epithelial cells were compared with RNA-Seq Tuxedo tools. The Bioinformatics & Evolutionary Genomics database (http://bioinformatics.psb.ugent.be/webtools/Venn/) was used to identify genes that were induced by all strains in pulmonary epithelial cells as well as genes shared by different strain comparisons. A total of 292 genes were induced by all strains and the expression profile of 152 of these is illustrated in Fig. 2. HLA genes (*HLA-B*, *HLA-C*, *HLA-F* and *HLA-H*) were significantly up-regulated together with surface markers (*CD38*, *CD68* and *CD274*) and other receptors (*TLR5* and *NOD2*). Additionally, these clinical strains induced an increase in MMP (*MMP1* and *MMP10*) genes. Moreover, expression of *MMP7* was induced only by the Beijing and F11 strains, while down-regulation of *MMP14* was induced by these 2 strains and the Unique strain. Down-regulation of the *MMP2* gene was induced only by the F28 strain compared to other strains (data not shown). Among the up-regulated genes, increased expression of cytokines/chemokine (*IL1A*, *IL1B*, *IL8*, *IL23A*, *CCL2*, *CCL5*, *CCL20*, *CXCL1*, *CXCL5*) together with other transcriptional factors (*STAT1*, *STAT2*, *IRF7*) was observed with differences in the level of expression among clinical strains (Fig. 3).

Fig. 3 shows hierarchical clustering of the 100 most up-regulated and all (52) down-regulated genes in epithelial cells among the strains. Down- and up- regulated genes cluster together with a few exceptions (*MMP10*, *CA9*, *LBP*, *GDA*, *SLC1A2*, *GPR1*). The lowest fold changes of these commonly shared genes was induced by the Unique strain followed by the laboratory H37Rv strain compared to the much higher changes by the clinical strains. Among the clustering clinical strains, the higher fold changes were induced by the F15/LAM4/KZN,

Beijing and F11 strains, compared to the F28 strain (Fig. 3; Appendix B, Supplementary Table 1).

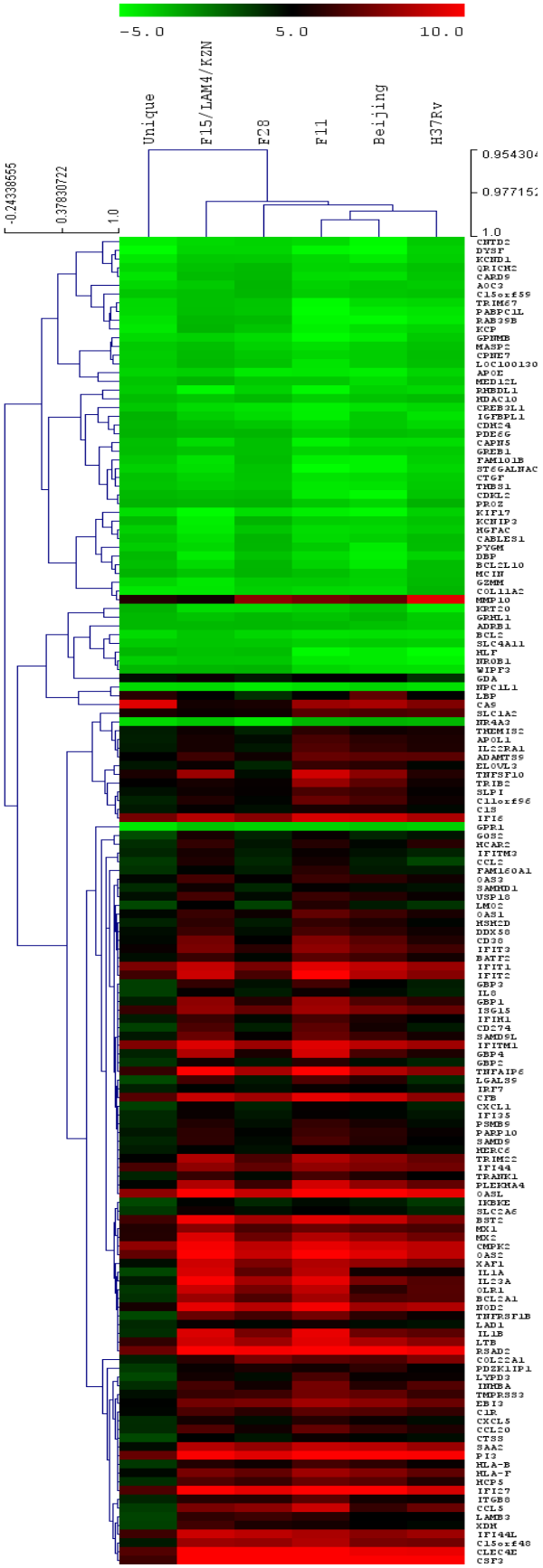


Fig. 3. Fold changes showing gene expression profiles of top 100 (out of 240) up-regulated, and all (52) down-regulated genes in A549 pulmonary epithelial cells at 48 hr post-infection. Hierarchal clustering indicates that highly virulent *M. tuberculosis* strains cluster together and induce a similar behaviour in epithelial cells compared to less virulent Unique strain. The Tuxedo RNA-Seq pipeline was used to identify differentially expressed genes between uninfected and infected epithelial cells by F15/LAM4/KZN, Beijing, F11, F28, Unique clinical strains and a laboratory H37Rv strain of *M. tuberculosis*. The fold changes were compared among the strains and a heat map was created using MeV. The F15/LAM4/KZN, F11 and Beijing strains induced higher fold changes compared to the F28, Unique and H37Rv strains.

Gene Ontology analysis

Gene ontology enrichment analysis (MSigDB: C5) was performed on 292 genes to clarify the biological, molecular and cellular functions associated with both up (n = 240) - and down-regulated (n = 52) genes. These functions ranged from signal transduction, membrane associated functions, endopeptidase activity, and steroid biosynthesis of down-regulated genes, to extracellular space, immune system processes, defense response, response to external stimuli, plasma membrane and receptor binding functions of the up-regulated genes (Table 3).

Table 3: Biological functions associated with up- and down-regulated genes in A549 pulmonary epithelial cells

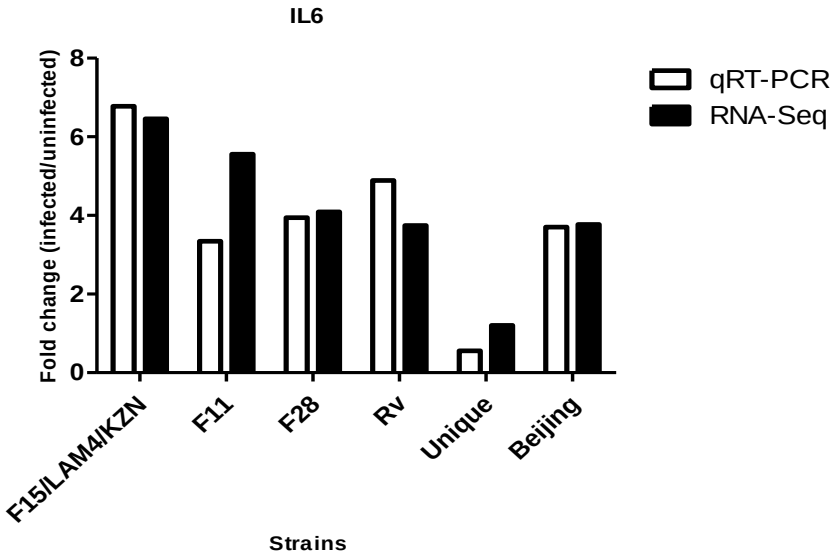
	Gene ontology functions	Genes
Down-regulated genes	Signal transduction	*APOE, CARD9, ADRB1, RHBDL1, GPR1, KCNIP3, CAPN5, BCL2L10, NR4A3, CDKL2*
	Membrane associated functions	*AOC3, BCL2, CDH24, SLC4A11, DYSF, CTGF, GPNMB, HDAC10*
	Endopeptidase activity	*HGFAC, MASP2*
	Steroid biosynthesis	*NPC1L1, NR0B1*

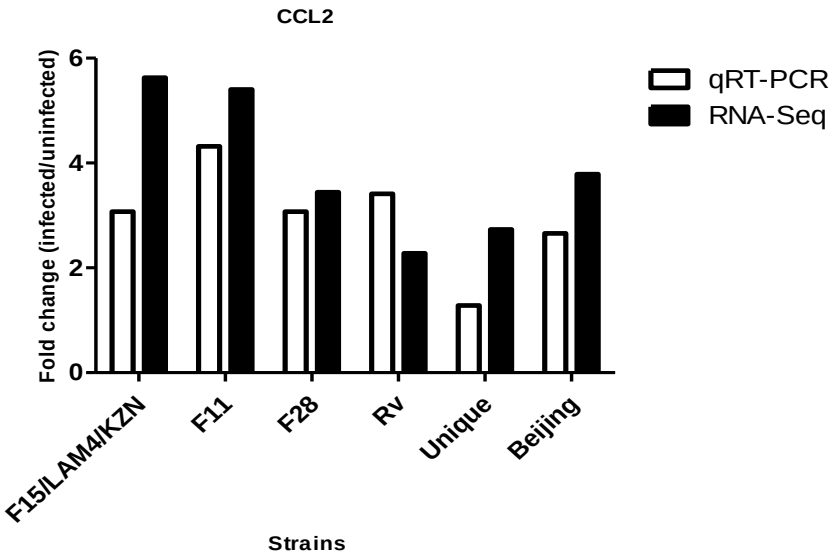
Up-regulated genes	Extracellular space	*CCL20, IL8, INHBA, EBI3, SECTM1, CCL2, CXCL1, IL1A, LBP, LGALS3BP, CSF3, IL1B, FGFBP1, GPX3, CP, PRSS3, MMP10, LOXL2, APOL1, CHI3L2, SERPINA1, FBLN5, ADAMTS9, PI3, CDA, SPINT1*
	Immune system processes	*CCL20, IL8, INHBA, EBI3, SECTM1, CCL2, CXCL1, CXCR4, CCL5, RSAD2, BST2, IFITM3, IFI6, CD274, TRIM22, CTSS, DPP4, GBP2, PF4*
	Defense response	*CCL20, IL8, INHBA, CXCL1, IL1A, LBP, LGALS3BP, CXCR4, CCL5, RSAD2, TNFAIP6, AHSG, MX1, HLA-B, MX2, HCP5*
	Response to external stimulus	*CCL20, IL8, INHBA, CCL2, CXCL1, IL1A, LBP, CXCR4, CCL5, PF4, TNFAIP6, AHSG, PLAUR, ITGB3, CXCL5, STC1*
	Plasma membrane	*EBI3, CXCR4, BST2, IFITM3, PLAUR, ITGB3, TNFSF10, IFITM1, ROBO1, ADAM12, DNER, CD38, OLR1, SLC16A3, PTPRN2, ANPEP, EMR1, KCNJ6, ITGA5, ITGB8, SLC22A11, HAS3, CEACAM5, LAPTM5, MUC1, CEACAM7, LYPD3, GJB2, PLSCR1*
	Receptor binding	*CCL20, IL8, INHBA, SECTM1, CCL2, CXCL1, CSF3, FBLN5, CCL5, PF4, CXCL5, STC1, IGF2, C3, APOL2, OASL*

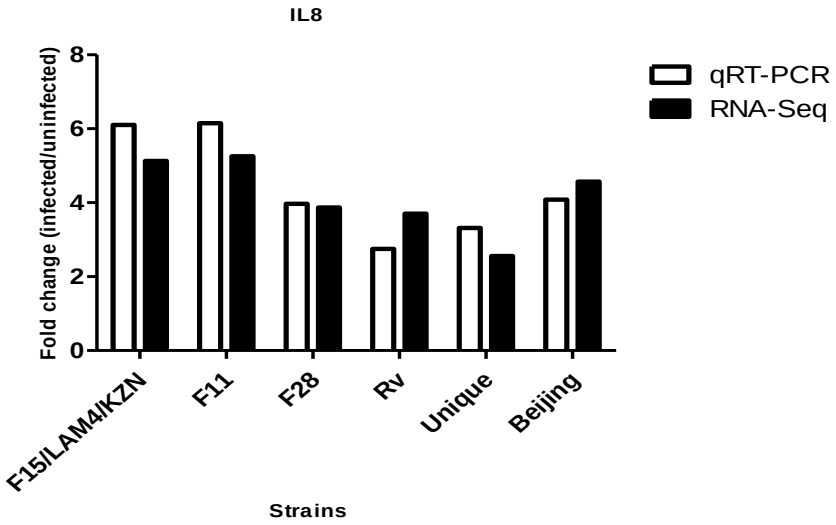
Validation by qRT-PCR

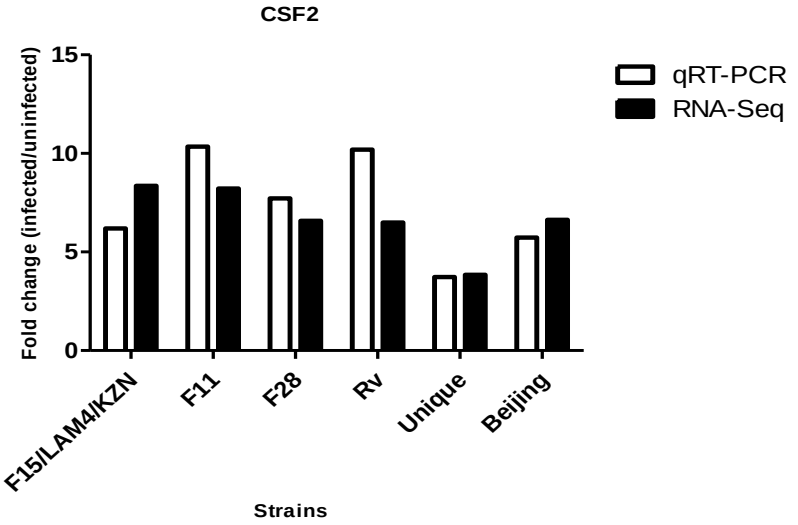
Validation of RNA-Seq transcriptomics data of 6 cytokine and chemokine genes showed increased expression of *IL6, IL8, CCL2, CSF2, TNF-α, IFN-γ* mRNA at 48 hr after infection by different strains of *M. tuberculosis* compared to the uninfected epithelial cells. Fold

changes detected by qRT-PCR ranged from 0.055 to 12.64. A fold change of below 0.4 was obtained for *IFN-γ* mRNA. The validation results were consistent with those of RNA-Seq, with the exception for the *IFN-γ* mRNA that was not detected by the latter. The differences in the fold changes between the 2 techniques ranged from 0.117 to 3.71, but these were not significant except for *IFN-γ* ($P = 0.0131$). There was a positive ($r^2 > 0.5$) correlation between the RNA-Seq and qRT-PCR for *IL6, IL8, CCL2, CSF2, TNF-α* validating the expression profiles detected by the RNA-Seq. Correlation could not be performed for *IFN- γ* because it was not detected by RNA-Seq, hence it had an expression of zero (Fig. 3).


IL6
Fold change (infected/uninfected)
0
2
4
6
8
qRT-PCR
RNA-Seq
F15/LAM4/KZN
F11
F28
Rv
Unique
Beijing
Strains


CCL2
Fold change (infected/uninfected)
0
2
4
6
qRT-PCR
RNA-Seq
F15/LAM4/KZN
F11
F28
Rv
Unique
Beijing
Strains


IL8
Fold change (infected/uninfected)
0
2
4
6
8
qRT-PCR
RNA-Seq
F15/LAM4/KZN
F11
F28
Rv
Unique
Beijing
Strains


CSF2
Fold change (infected/uninfected)
0
5
10
15
qRT-PCR
RNA-Seq
F15/LAM4/KZN
F11
F28
Rv
Unique
Beijing
Strains

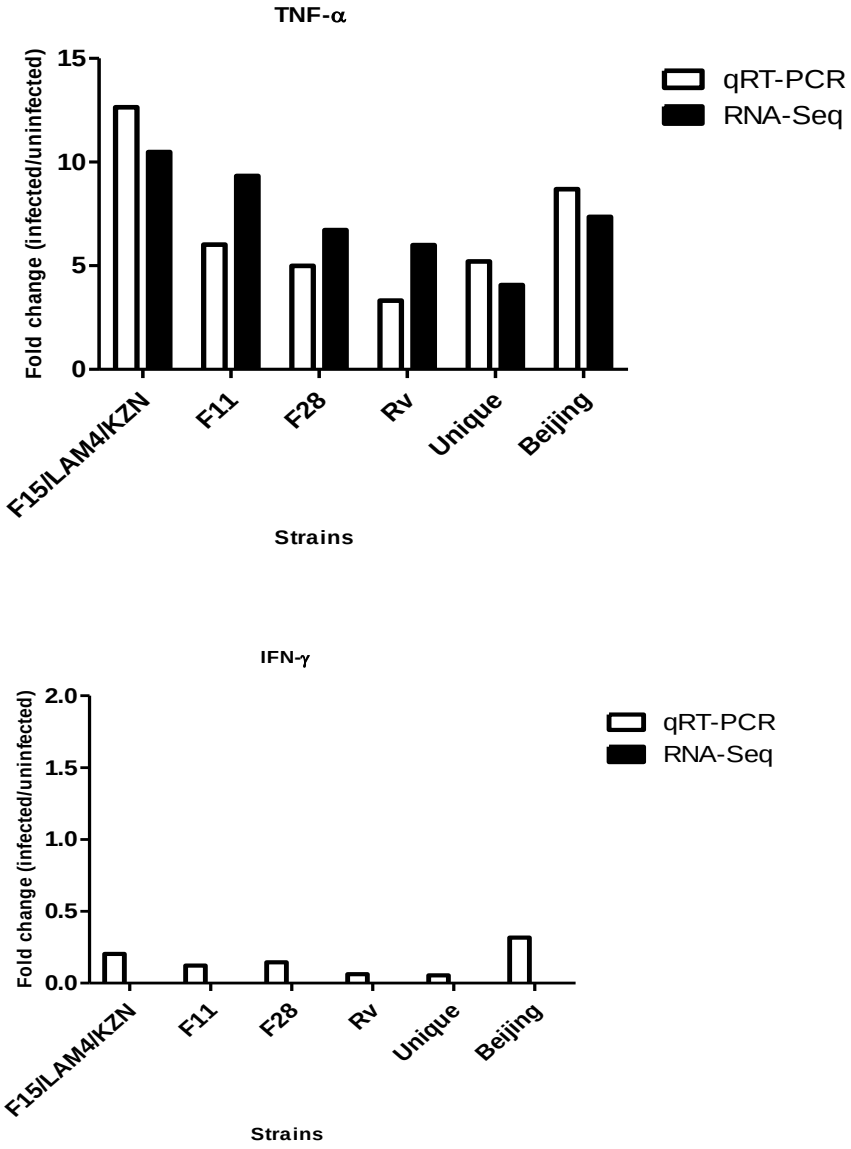


Fig. 4. The expression and validation of cytokine/chemokine (*IL6*, *IL8*, *CCL2*, *CSF2*, *TNF-α*, *IFN-γ*) mRNA levels was performed with quantitative real time PCR. RNA was extracted at 48 hr post-infection from *M. tuberculosis* infected pulmonary epithelial cells. The genes of interest were normalized by using the house keeping gene *GAPDH* and the expression data for two independent experiments are presented as fold changes between the infected and uninfected epithelial cells for both RNA-Seq and quantitative real time PCR. The expression levels shown by quantitative real time PCR were comparable with RNA-Seq with the exception of the *IFN-γ* gene that was detected by the real time PCR and was less than 0.4 fold changes in epithelial cells infected by all strains.

Kegg and Reactome pathways: Strain-specific signatures in pulmonary epithelial cells

In order to identify strain-specific molecular signatures induced by different strain comparisons and strain specific functions in epithelial cells, all differentially expressed genes (fold change ≥2) were compared between all strains using the publically available Bioinformatics & Evolutionary Genomics database (http://bioinformatics.psb.ugent.be/webtools/Venn/). The number of induced genes and associated pathways that were activated/down-regulated, varied with different strain comparisons as shown by Fig. 5 and Table 4. These pathways were identified by selecting KEGG and Reactome pathways in the MSigDB (C2 curated). Changes in genes that activate or down-regulate specific KEGG and Reactome pathways were observed for the different pathways as shown by (Table 4).

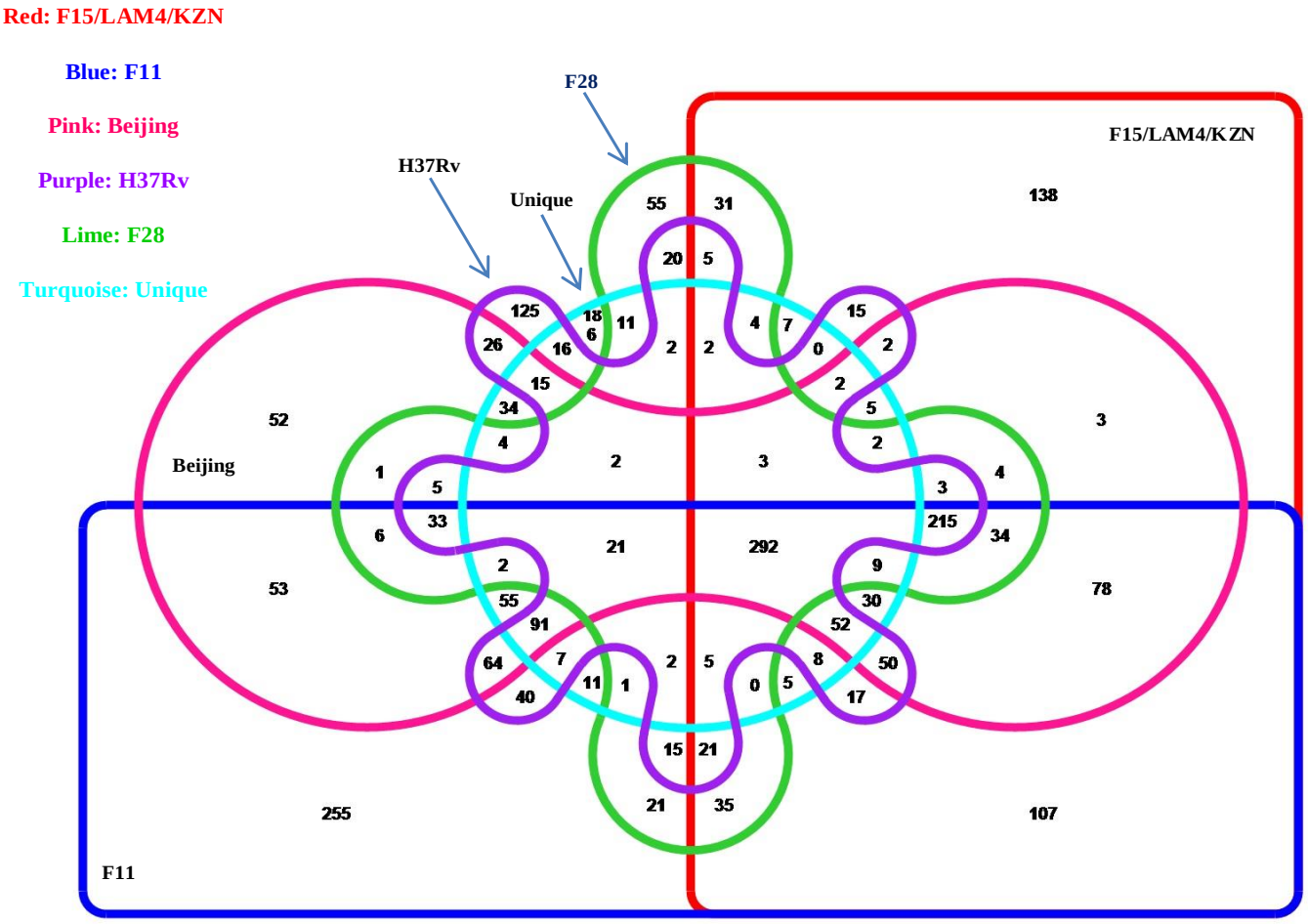


Fig. 5. A six-way Venn diagram showing molecular signatures shared by different strain combinations. Differentially expressed genes were compared among the strains to reveal strain-specific signatures in pulmonary epithelial cells at 48 hr post-infection. Intersections and the Venn diagram were created using the Vennture software. The number of strain-specific signatures was as follows: F11 (255), F15/LAM4/KZN (138), F28 (55), Beijing (52), Unique (186) and H37Rv (125).

All strains except the Unique strain activated cytokine signalling, IFN-γ signalling, JAK-STAT pathways, cytokine-cytokine signalling and signalling of interleukins, and down-regulated the axon guidance pathway. Metabolism of β-alanine and the cholesterol biosynthesis pathways were activated by F15/LAM4/KZN, Beijing, F11, Unique and H37Rv

strains only. These strains also down-regulated Terpenoid backbone biosynthesis and phenylalanine metabolism. The Beijing, F11, Unique and H37Rv strains activated carbohydrate and amino acid metabolism, glycolysis/gluconeogenesis, and transmembrane transport pathways, whilst down-regulating genes involved in amino acid synthesis and interconversion. Genes specific for F15/LAM4/KZN, Beijing and F11 activated apoptosis and RIG-I-like receptor signalling pathway. The TLR 4 signalling, Integrin cell surface interactions, Plasma membrane MyD88:Mal cascade and Toll Receptor Cascades pathways were activated by the F15/LAM4/KZN, F11 and F28 strain comparison. The F15/LAM4/KZN and F11 up-regulated genes involved in the adaptive immune system, Toll-like and B cell receptor signalling. No significant KEGG and Reactome pathways were activated/down-regulated by the molecular signatures specific for the gene comparisons of Beijing, F11 and F28 strains. Some of the genes involved in the same pathway were shared in different strain comparisons. This was observed with the JAK-STAT signalling and cytokine-cytokine receptor interaction pathways induced by the genes shared by all but Unique strain, while on the other hand, the F15/LAM4/KZN strain specifically activated the same pathway. Similarly, the up-regulated genes shared by all but the F28 strain were involved in cholesterol biosynthesis, while Beijing and F11 specific genes were also involved in the same pathway.

The highest number of strain-specific molecular signatures in *M. tuberculosis* infected pulmonary epithelial cells were present in the F11 (255), followed by the Unique (187), F15/LAM4/KZN (138), H37Rv (125), F28 (55), and Beijing (52) strains, respectively. Each strain induced strain specific genes in pulmonary epithelial cells (Table 4), however only the F15/LAM4/KZN, Unique and H37Rv gene signatures induced overlapping KEGG and Reactome pathways. The F15/LAM4/KZN strain activated the cytokine-cytokine receptor interaction and the JAK-STAT pathway while also down-regulating other genes involved in

the same cytokine-cytokine receptor pathway. The Unique strain specifically down-regulated genes that are involved in developmental biology, exon guidance, L1CAM interactions, NCAM signalling for neurite out-growth and netrin-1 signalling. Furthermore, the Unique strain up-regulated genes involved in sugar metabolism and variety of other pathways as shown in Table 4. Genes involved in chronic myeloid leukemia, TGF-beta signalling pathway and cell cycle were down-regulated in H37Rv infected epithelial cells (Table 4, Appendix B, Supplementary Table 2 and 3).

Table 4: Strain-specific signatures and their respective KEGG and Reactome pathways affected by different strain combinations

Strain combinations	**Total number of gene signatures**	**Pathways: Kegg and Reactome**
F15/LAM4/KZN Beijing F11 F28 H37Rv	215	**Down-regulated** Axon guidance **Up-regulated** Cytokine Signalling in immune system, Immune System, Interferon/ interferon gamma signalling, Cytokine-cytokine receptor interaction, Pathways in cancer, Signalling by interleukins, JAK-STAT signalling pathway, Endosomal/Vacuolar pathway
Beijing F11 H37Rv Unique	91	**Down-regulated** Amino acid synthesis and interconversion (transamination) **Up-regulated** Metabolism of amino acids and derivatives, Metabolism of carbohydrates, Glucose metabolism, Transmembrane transport of small molecules, Transport of glucose and other sugars, bile salts and organic acids, metal ions and amine compounds, Glycolysis, Glycine, serine and threonine metabolism, SLC-mediated transmembrane transport, Glycolysis / Gluconeogenesis
F15/LAM4/KZN Beijing F11 Unique	30	**Up-regulated** Regulation of Complement cascade
F15/LAM4/KZN Beijing F11	78	**Up-regulated** Apoptosis, Genes involved in extrinsic pathway for apoptosis, Genes involved in apoptosis
F15/LAM4/KZN F11 F28	35	**Up-regulated** Integrin cell surface interactions, Plasma membrane MyD88:Mal cascade, Activated, TLR4 signalling, Toll receptor cascades
Beijing F11	53	**Up-regulated** Cholesterol biosynthesis
F11 F28	21	**Down-regulated** Mitotic G2-G2/M phases

F11 H37Rv	40	**Down-regulated** Butanoate metabolism
F15/LAM4/KZN F11	107	**Up-regulated** Immune system, Leishmania infection, Cytokine signalling in immune system Adaptive immune system, Toll-like receptor signalling pathway, Signaling by the B cell receptor (BCR), Genes involved in interferon signalling, Cytokine-cytokine receptor interaction, Antigen activates B Cell receptor leading to generation of second messengers
H37Rv Unique	16	**Up-regulated** Glucuronidation, Ascorbate and aldarate metabolism, Pentose and glucuronate interconversions, Porphyrin and chlorophyll metabolism, Drug metabolism - other enzymes, Starch and sucrose metabolism, Steroid hormone biosynthesis, Retinol metabolism, Metabolism of xenobiotics by cytochrome P450, Genes involved in Phase II conjugation
Unique	186	**Down-regulated** Developmental Biology, Exon guidance, L1CAM interactions, NCAM signalling for neurite out-growth, Netrin-1 signalling **Up-regulated** Common Pathway, Starch and sucrose metabolism, Pentose and glucuronate interconversions, Complement and coagulation cascades, Formation of Fibrin Clot (Clotting Cascade), Metabolism of xenobiotics by cytochrome P450, Drug metabolism - cytochrome P450, Porphyrin and chlorophyll metabolismRetinol metabolism
H37Rv	125	**Down-regulated** Chronic myeloid leukemia

Discussion and conclusion

Molecular epidemiological studies have identified genetically diverse strains of *M. tuberculosis* that vary in virulence (Coll *et al.*, 2014; Coscolla and Gagneux, 2010; Fleischmann *et al.*, 2002). This diversity has been reported to impact on clinical outcome as exhibited by the capacity of several of these strains for increased transmission rates (Brites and Gagneux, 2012; Mihret *et al.*, 2012), rapid progression to active disease (Nahid *et al.*, 2010), and differential host immune response (Koo *et al.*, 2012; Manca *et al.*, 1999; Subbian *et al.*, 2013), including subversion of the host response that may be associated with increased virulence (Hoal-van Helden *et al.*, 2001; Nicol and Wilkinson, 2008). In an effort to identify strain specific molecular signatures that may be associated with virulence, we investigated the global transcriptomic profile by RNA-Seq of the epithelial cells infected with the F15/LAM4/KZN strain that has been transmitted largely in its drug resistant form (Pillay and Sturm, 2007), and responsible for an XDR-TB outbreak with high mortality rates in the

province of KwaZulu-Natal in the mid 2000's (Gandhi *et al.*, 2006). We compared this to the transcriptomes of epithelial cells infected with the Beijing, F11, F28 and Unique strains that are also widely transmitted in this province as well as the laboratory strain H37Rv infected cells. The qRT-PCR assay validated the RNA-Seq data with the exception of *IFN-γ*, suggesting the reliability of the latter technique (Fig 3).

Epithelial cells in immune response

Host infection by *M. tuberculosis* results in a variety of changes that favour the growth and replication of the bacilli within the lungs. Epithelial cells, in addition to their protective functions, simultaneously play a significant role in both the innate and adaptive immune response during infection by *M. tuberculosis* via induction of the cytokines/chemokines (Chuquimia *et al.*, 2012; Lee *et al.*, 2009; Lin *et al.*, 1998; Sato *et al.*, 2002; Sharma *et al.*, 2007; Stadnyk, 1994). The current study revealed differential changes in the transcripts of numerous genes of epithelial cells infected with the different clinical strains, similar to studies in other infection models such as monocytes and macrophages (Koo *et al.*, 2012; Manca *et al.*, 2004) as well as in mice (Manca *et al.*, 1999). Strain specific responses were also observed.

The differential gene expression levels and strain specific responses may be due to the varying virulence determinants of these clinical strains. In our study, the Unique (non-clustering) strain exhibited the lowest fold change in the expression of the 100 most up- and all 52 down-regulated genes in epithelial cells compared to the clustering strains. This may suggest that lower gene expression, including immune related genes may be associated with less virulent, non-clustering *M. tuberculosis* strains in epithelial cells. However, this does not

correlate with our findings that the Unique strain behaved similar to the Beijing strain with respect to the low cytokine/chemokine production in our previous work (Chapter 2). A limitation in our study is that further investigations were not performed to ascertain the virulence of the Unique strain. The low cytokine/chemokine induced by the Unique strain in our previous work may be associated with low mRNA changes induced by this strain. However, similar low cytokine/chemokine production but higher gene expression levels by the Beijing strain suggests the involvement of other complex mechanisms that require further investigation.

The up-regulation of the *CSF3, IF127, PI3, OAS2* and *CLEC4E* genes amongst the top-10 up-regulated genes in only clustering strains and H37Rv but not the Unique strain suggest that these may represent host factors that may be associated with increased transmissibility of the former strains. Functions associated with these up-regulated genes range from stimulating stem cells to produce granulocytes (Nambiar *et al.*, 2010; Szeliga *et al.*, 2008; Thomas *et al.*, 2002), promoting cell death (Rosebeck and Leaman, 2008), antimicrobial peptides (Molhuizen *et al.*, 1994; Neto *et al.*, 2014), controlling cellular function (Besse *et al.*, 1998) and recognition of cell wall components of Mycobacteria (Schoenen *et al.*, 2010).

Since epithelial cells play a crucial role in initiating an inflammatory response in innate immune response (Chuquimia Flores, 2011; Chuquimia *et al.*, 2012), our results suggest that less virulent or lower transmissible strains of *M. tuberculosis* may tend to induce lower inflammatory response through involvement of cytokines and chemokines. Our findings differ from that reported by Lin *et al*. (1998) and Sato *et al*. (2002) where *M. tuberculosis* was compared with the less virulent *M. avium*. The higher expression levels of *TNF-α* and

CSF2 by the latter organism were explained by the use of different receptors by the latter to facilitate adhesion to and entry into host cells (Lin *et al.*, 1998; Sato *et al.*, 2002).

Our study indicated that up-regulated genes in epithelial cells are involved in immune and defence response, activated crucial immune associated surface markers, cell receptors and transcriptional factors during infection. The role of epithelial cells in production of chemo-attractant proteins and cytokines as a defence mechanism has been well studied (Lee *et al.*, 2009; Lin *et al.*, 1998; Sato *et al.*, 2002; Sharma *et al.*, 2007; Wickremasinghe *et al.*, 1999). In this study, increased expression of cytokines (*IL1A*, *IL1B*, *IL8*, *IL23A*) and chemokines (*CCL2*, *CCL5*, *CCL20*, *CXCL1*, *CXCL5*) to inhibit bacterial replication at the site of infection was induced by all strains. Additionally, activation of *STAT1* (Sharma *et al.*, 2007), *STAT2* (Stockinger and Decker, 2009), *IRF7* (Honda *et al.*, 2005) transcriptional factors results in an increased protective response in epithelial cells by the production of pro-inflammatory cytokines including IFN-γ, that is crucial for the anti-tuberculosis clearance at the site of infection (Sharma *et al.*, 2007). The observed changes in pulmonary epithelial cells support mounting evidence of the involvement of epithelial cells in both innate and adaptive immune response during *M. tuberculosis* infection. Lower fold changes of immune associated genes induced by the Unique strain compared to other clinical strains, is suggestive of a less vigorous immune response of epithelial cells during infection by this strain.

Antigen presentation

The role of epithelial cells as antigen presenting cells (APCs) has been well studied in type II pulmonary epithelial cells (Cunningham *et al.*, 1997; Cunningham *et al.*, 1994; Debbabi *et al.*, 2005; Fehrenbach, 2001; Harbeck *et al.*, 1988; Lo *et al.*, 2008). The increased fold changes of the HLA genes (*HLA-B*, *HLA-C*, *HLA-F* and *HLA-H*) and some of the surface

markers (*CD38*, *CD68* and *CD274*) induced by all clinical and laboratory strains in our study lend further support to their findings that alveolar pulmonary epithelial cells most likely play a significant role in antigen presentation via the MHC class I molecules (Mack *et al.*, 2013). Antigen presentation by epithelial cells was further characterized by the increased expression of the *TLR5* and *NOD2* receptors that are known to play a role in antigen recognition and presentation (Cooney *et al.*, 2010; Takeda and Akira, 2005). Epithelial cells may not be classified as professional APCs (Kumari and Saxena, 2011), however, their contribution to T-cell trafficking through antigen presentation indicates their important immunological functions during TB infection (Gereke *et al.*, 2009) by linking both the innate and adaptive immune response. Inhibition of the antigen presentation phenotype during infection by *M. tuberculosis* has been identified as an intracellular persistence strategy of this pathogen (Flores-Batista *et al.*, 2007; Harding and Boom, 2010). This was not the case with our current findings, although we observed differences in expression levels among the clinical strains.

Granuloma formation

Matrix metalloproteinases (MMPs) belong to a family of enzymes that play a significant role in various cellular activities (Parks and Shapiro, 2001), promotion of early granuloma formation during *M. tuberculosis* infection, as well as dissemination of the pathogen through extracellular matrix destruction (Izzo *et al.*, 2004; Parks and Shapiro, 2001). In our study, all strains induced increased expression of the *MMP1* and *MMP10* genes in epithelial cells, with the highest expression by the H37Rv strain compared to clinical strains. There were variations in the expression of other MMP genes (data not shown) shared by different comparisons of clinical strains. The diversity in expression of these granuloma associated genes induced in pulmonary epithelial cells may influence the time needed for the granuloma formation during infection by different clinical strains of *M. tuberculosis* based on their

virulence. *MMP1* was the most highly up-regulated of all MMP genes tested, whilst both MMP1 and MMP3 protein levels were both increased in primary human monocytes of patients with TB, with the former being strongly associated with immunopathology of the lungs in patients (Elkington *et al.*, 2011). However, the genotype of the infecting strain was not determined, and therefore no associations with differential expression and strain virulence could be drawn. The association between strain virulence and MMP gene expression needs to be explored in future studies by investigating times taken by different strains to induce formation of the granuloma in mice models as well as understanding MMP genes in other cell types.

Molecular signatures among strain combinations

In addition to common genes activated or down-regulated in epithelial cells infected by different strains of *M. tuberculosis*, we further attempted to identify strain specific molecular signatures by examining the pathways that are affected by each strain. Several pathways were activated or down-regulated in epithelial cells either by the F15/LAM4/KZN, Unique or H37Rv alone or by different strain comparisons (Table 4). All strains up-regulated genes of the immune system except the Unique strain, reflecting a failure to provoke a more vigorous host immune response at 48 hr compared to others. Activation of the immune associated pathways by clustering strains, but not the Unique strain, may be attributed to increased expression of the JAK-STAT pathway that has been known to contribute to effective host immune response (Stockinger and Decker, 2009). The same JAK-STAT pathway was up-regulated by the F15/LAM4/KZN specific genes that may perform different functions within the same pathway.

Genes involved in apoptosis

Several studies have shown that whilst virulent *M. tuberculosis* is capable of inducing apoptosis in host cells, this process can also be inhibited via the regulation of 3 genes: *nuoG*, *SecA2* and *pknE* (Chaussabel *et al.*, 2003; Danelishvili *et al.*, 2003; Velmurugan *et al.*, 2007). Thus, avirulent mycobacteria induce a more vigorous apoptotic response than virulent mycobacteria (Briken and Miller, 2008; Keane *et al.*, 2000). Schaible *et al*. (2003) showed that *M. tuberculosis* infected macrophages undergo apoptosis to overcome the loss of antigen presenting capacity by facilitating antigen cross presentation to T-cells (Schaible *et al.*, 2003). Contrary to macrophages, H37Rv-infected alveolar epithelial cells inhibited apoptosis mediated by the down-regulation of the pro-apoptotic genes *bad* and *bax*, and up-regulation of the anti-apoptotic genes *bcl-2* and *Rb* (Danelishvili *et al.*, 2014). In addition, the apoptosis associated caspase (*casp1, casp3* and *casp10*) genes were also repressed by the up-regulation of *bcl-2* in A549 epithelial cells (Danelishvili *et al.*, 2003).

In the present study, activation of the genes (*CASP10*, *TRADD*, *CFLAR*, *BID*, *CASP7*, *TMEM173*) involved in the apoptosis pathway was surprisingly induced in epithelial cells by only the F15/LAM4/KZN, Beijing and F11 strains, but not the other virulent F28, Unique and H37Rv strains. Despite the down-regulation of the *BCL2* genes by all strains, only F15/LAM4/KZN, Beijing and F11 significantly affected the apoptosis pathway with the up-regulated *CASP10* gene. Up-regulated apoptotic genes may facilitate rapid, earlier progression to disease in patients infected with the former clinical strains due to apoptosis of the epithelial cells. Alveolar macrophages infected with *M. tuberculosis* underwent apoptosis mediated by TNF-α (Keane *et al.*, 1997). These authors also showed that lower macrophage apoptosis induced by virulent *M. tuberculosis* occurred via inhibition of TNF-α by soluble TNFR2 (sTNFR2) that was stimulated by IL-10. In a subsequent study, they reported a lack of correlation between levels of TNF-α; sTNFR2 or IL-10 and virulent or avirulent strains

(Keane *et al.*, 2000). Similarly, TNF-α and IL-10 production in epithelial cells infected with different virulent strains (Chapter 2) showed no correlation with our contradictory transcription profiles, suggestive of diverse virulence determinants among the virulent strains tested in the present study.

Genes involved in toll-like receptor signalling

In the mouse model, TLR 4 signalling was associated with a late chronic protective response, *M. tuberculosis* control at the infection site, dissemination from the lungs, inhibition of macrophages and pro-inflammatory cytokines (Abel *et al.*, 2002; Branger *et al.*, 2004). In the current study, TLR 4 signalling and toll receptor cascades pathways were specifically up-regulated in epithelial cells by the F15/LAM4/KZN, F11 and F28 strains, indicating a potential protective role for these pathways in late stages of infection by these strains. These findings lend support to those of Carmona *et al*. (2013), in that different strains of *M. tuberculosis* are preferentially recognised by different TLRs which may impact on the immune response through the differential production of cytokines/chemokines (Carmona *et al.*, 2013).

Genes involved in cholesterol metabolism

Host lipids, including cholesterol, play a significant role in TB pathogenesis, as high levels of these have been implicated in granuloma development during infection (Kim *et al.*, 2010) as well as during persistence of *M. tuberculosis* within the host (Ouellet *et al.*, 2011). In our study, the *HMGCS1* and *FDPS* genes involved in cholesterol biosynthesis were up-regulated in all strains except the F28. Additionally, genes specific for the Beijing and F11 strains up-regulated the cholesterol biosynthesis pathway (*CYP51A1*, *HSD17B7*, *HMGCR*) and metabolism of lipids and lipoprotein (*CYP51A1*, *HSD17B7*, *HMGCR*, *CYP24A1*, *CHKA*).

These findings suggest that the F28 strain, unlike the others, may not be able to sequestrate host cholesterol as a source of carbon and energy (Ouellet *et al.*, 2011), and uses alternate sources such as sugars and amino acids to survive and replicate within epithelial cells as seen with other intracellular pathogens (Eisenreich *et al.*, 2010). The additional cholesterol biosynthesis genes may confer an advantage to the Beijing and F11 strains in their ability to mobilize host cholesterol, and contribute to increased virulence compared to the other 3 clinical strains.

Strain-specific molecular signatures

Significant up-regulation/down-regulation of specific pathways resulted in molecular signatures that were specific for the F15/LAM4/KZN, Unique and H37Rv strains. Cytokine-cytokine receptor and JAK-STAT pathways were specifically regulated by the F15/LAM4/KZN strain, which seems capable of significantly provoking an effective host immune response at an earlier time in epithelial cells. Despite this capacity, the successful spread of the F15/LAM4/KZN strain might be fuelled by XDR and compromised host immunity (Gandhi *et al.*, 2006; Pillay and Sturm, 2007) rather than hypervirulent attributes.

The Unique strain specifically down-regulated several pathways, including a netrin-1 pathway that is known to inhibit leukocyte migration in both *in vitro* and *in vivo* models (Ly *et al.*, 2005). This could lead to increased migration of leukocytes to the lungs resulting in improved bacterial clearance, and may explain the low prevalence of this strain within the population. Up-regulation of the starch and sucrose metabolism might be beneficial to the Unique strain as these may be used as carbon and energy sources in addition to host cholesterol. Metabolic changes and control of major metabolic pathways such as sugars are tightly controlled by external signals (Eisenreich *et al.*, 2013). These may include certain

proteins and other molecules produced by the Unique strain to activate these pathways within epithelial cells. Decreased sugar (mainly glucose and glycogen) metabolism within mice (Shin *et al.*, 2011) and guinea pigs (Somashekar *et al.*, 2012) was induced by *M. tuberculosis* infection, in contrast to our findings, where increased expression of different sugar pathways including pentose and glucuronate sugars was induced by the Unique strain in epithelial cells. These differences might be attributed to different infection models and different strains of *M. tuberculosis* used in the different studies. Metabolomics studies to elucidate sugar profiling have not yet been documented in pulmonary epithelial cells infected with different *M. tuberculosis* clinical strains. However, such information would serve to broaden our knowledge of the different nutritional requirements of these clinical strains to facilitate further understanding of the complex-host pathogen interactions.

Infection with the laboratory adapted H37Rv strain might not necessarily result in responses similar to those elicited by other clinical strains as was shown in our study by several down-regulated pathways that were unaffected by other clinical strains. Mononuclear phagocytes have been known to be the main source of TGF-β in the lungs (Aung *et al.*, 2005), however, our study revealed that epithelial cells infected with the laboratory strain may reduce the production of TGF-β through down-regulation of its pathway. TGF-β has anti-inflammatory properties and its inhibition was shown to improve lymphocyte response and *M. tuberculosis* containment by macrophages (Hirsch *et al.*, 1997). Highly virulent and transmissible clinical strains did not down-regulate the TGF-β pathway, which highlights differences in using laboratory and clinical strains isolated in patients. This data emphasizes the importance of including clinical strains when profiling host response.

Molecular signatures that were specific for the Beijing, F11 and F28 strain combinations did not affect changes in KEGG and Reactome pathways, however, they may be involved in other host biological functions that can be further explored in future studies. *In vivo* studies utilising global transcriptome analysis of human blood samples have provided evidence that molecular signatures show promise as targets for the development of TB diagnostics (Lesho *et al.*, 2011), treatment monitoring (Cliff *et al.*, 2012), vaccine and drug therapies (Berry *et al.*, 2010). However, the influence of strain identity on host immune response in pulmonary epithelial cells has not been considered. Our data shows clearly, that specific host genes and signalling pathways are induced in epithelial cells infected with virulent strains of diverse genetic background.

The main technical limitation in this study is that epithelial cells were not examined at sampling times for confluence and adherence. Another limitation is the lack of prior knowledge on virulence determinants in our clinical strains which could have explained differential expression patterns in pulmonary epithelial cells. Another limitation is the lack of literature on metabolic changes in pulmonary epithelial cells during infection with a wide range of clinical strains belonging to either different strain families or lineages. Such knowledge may have enabled a deeper understanding of the interaction of certain host factors that are crucial during *M. tuberculosis* infection, as well as an understanding on some of the changes observed for the different genes that were expressed.

In conclusion, the current study adds to the growing body of evidence that epithelial cells play a significant role in both the innate and adaptive response, and that genetically different virulent strains stimulate a strain specific immune host responses with the potential to impact downstream changes, granuloma formation and clinical outcome. The shared 292 and strain-

specific molecular signatures identified in this study can potentially be exploited in the design of efficacious vaccines and adjunctive immunotherapies. These findings warrant further exploration and confirmation in future *in vivo* studies on mice and patients infected with virulent strains that dominate in TB endemic regions.

CHAPTER 4: CANONICAL PATHWAYS, NETWORKS AND TRANSCRIPTIONAL FACTOR REGULATION BY CLINICAL STRAINS OF *Mycobacterium tuberculosis* IN PULMONARY ALVEOLAR EPITHELIAL CELLS

Abstract

The high prevalence and spread of *M. tuberculosis* globally has identified the need for further investigation of host associated molecular mechanisms that may affect the outcome of infections by different strains. The current study investigated epithelial cell pathways, networks and transcriptional factors that are induced upon infection with clinical *M. tuberculosis* strains belonging to 4 genotype families and a Unique strain. Epithelial cells were infected at MOI~10:1 and RNA samples were collected at 48 hr post-infection. The RNA-Seq Tuxedo pipeline (Bioinformatics) was used to obtain differentially expressed genes. Changes in affected pathways, networks and transcriptional factors were identified using Ingenuity Pathway Analysis (IPA).

The top canonical pathways differed among the strains with the interferon signalling and hepatic Fibrosis/hepatic stellate cell activation pathways being among the top 5 pathways in all the strains. Immune related pathway enrichment was similar in the Beijing and Unique strains, whilst the F15/LAM4/KZN showed closer relatedness to the F11 strain, and the F28 strain clustered closely to the H37Rv strain. The induction of top scoring networks varied among the strains. The functions associated with these gene networks involved antimicrobial response, developmental disorder, organismal injury, infectious disease and cellular development. Among the transcriptional factors, only *EHL*, *IRF7*, *PML*, *STAT1*, *STAT2* and *VDR* were induced by all clinical strains while the induction of other factors, were strain, viz., F15/LAM4/KZN (*SOX2*), Beijing (*TBL1X*), F11 (*ANKRD42*, *BRCA1*, *GLI3*, *GLIS2*, *IRF2*,

MEIS1, *NKX2-5*, *RELA*, *RFX5*, *TFCP2L1*), F28 (*SOX8*), Unique (*BHLHE41*, *EGR1*, *FOSL1*, *IRF4*, *KLF2*) and H37Rv (*EGR2*, *EN2*, *MLXIPL*, *MYC*, *OLIG1*, *PPARGC1B*). The heterogeneity in the molecular mechanisms elucidated in infected epithelial cells in the current study may account for the widespread dissemination of certain strains such as the Beijing strain, while other strains are easily eradicated within the population. This may be mediated through low protective immune associated pathways induced by the Beijing strain compared to the F15/LAM4/KZN, F11 and F28 genotypes. However, similarities in the behaviour induced by the Beijing and Unique strains require further investigation. Activation of the different pathways, networks and transcriptional factors revealed in the current study may explain the diversity in the inflammatory response, and may be an underlying mechanism that results in differential host response that determines the outcome of infection by different clinical strains of *M. tuberculosis*.

Introduction

The high global TB burden (WHO, 2014), particularly in resource constrained countries, has been fuelled by the high HIV prevalence (Churchyard *et al.*, 2014; Elizabeth *et al.*, 2003), and the emergence of MDR, XDR and TDR *M. tuberculosis* strains (Marahatta, 2010; Pillay and Sturm, 2007; Streicher *et al.*, 2012; Udwadia, 2012; Udwadia *et al.*, 2012). The lack of effective anti-TB drugs to treat these highly resistant strains, resulting in patients remaining infectious for extensive periods, has greatly facilitated escalating TB transmission rates. This problem is further heightened by the low protective power of the current BCG vaccine to prevent infection beyond childhood (Abebe and Bjune, 2006).

Novel biomarkers are therefore, urgently needed as targets for drug, vaccine and immunotherapeutic agents. Much research effort has been directed towards the pathogen (Caminero *et al.*, 2010; Dutta *et al.*, 2014; Schoeman and du Preez, 2012; Swanepoel and Loots, 2014) and not much towards host targets (Hawn *et al.*, 2013; Maiga *et al.*, 2012; Roca and Ramakrishnan, 2013; Tobin *et al.*, 2012). Alternate targets such as host immune pathways and/or networks may offer novel options for therapeutic interventions, since conventional approaches have realised limited success thus far (Azuma *et al.*, 2013; Quy *et al.*, 2003; Sadacharam *et al.*, 2007).

Computational analysis identified numerous proteins involved in the complex interplay between *M. tuberculosis* and the host following infection (Rapanoel *et al.*, 2013). The clinical outcome of infection depends on the presence of different virulence factors by the infecting strain (Caws *et al.*, 2008; Manca *et al.*, 1999; Tsenova *et al.*, 2005), host genotype (Caws *et al.*, 2008), and host molecular pathway changes (Koo *et al.*, 2012). Host molecular mechanisms including pathways and transcriptional factor changes in macrophages (Koo *et al.*, 2012; Tailleux *et al.*, 2008), DCs (Tailleux *et al.*, 2008), and mouse models (Kang *et al.*, 2011) during *M. tuberculosis* infection have been well described. High immune related pathway enrichment in *M. tuberculosis* H37Rv infected mice were observed for the different immune cell signalling pathways and involvement of host receptors in recognition of bacteria and viruses (Kang *et al.*, 2011). *In vitro* studies performed on macrophages and DCs revealed common, as well as diverse host immune associated pathways and metabolic functions that were associated with *M. tuberculosis* infection (Tailleux *et al.*, 2008). Simultaneous interrogation of the *M. tuberculosis* H37Rv transcriptome identified, in addition, host cell specific pathogen transcriptional signatures in the same study (Tailleux *et al.*, 2008). An immune activation network that included crucial transcriptional factors (*STAT3* and *STAT5A*) was significantly up-regulated by the CDC1551 strain, while the Beijing strain activated the

lipid metabolism network in macrophages (Koo *et al.*, 2012). The importance of strain virulence on pathway changes was also observed in macrophages infected with an avirulent H37Ra strain that induced a more potent apoptosis compared to the virulent H37Rv strain (Behar *et al.*, 2011; Hart *et al.*, 1989). This occurred despite comparable amounts of TNF produced by both strains that greatly influence the extrinsic apoptosis pathway (Behar *et al.*, 2011).

A study modelling macrophage response during *M. tuberculosis* infection revealed that cytokine, MAPK and calcium signalling featured among the top regulated of numerous signalling pathways involving diverse, activated proteins (Sambarey *et al.*, 2013). Whole blood transcriptional studies showed that IFNI signalling holds promise in the development of vaccines and therapeutics, whilst a 393-transcript signature could play a role in diagnosis of active tuberculosis (Berry *et al.*, 2010). However, the contribution of IFNI signalling to the outcome of TB infection remains to be elucidated in future studies (Blankley *et al.*, 2014).

A wealth of knowledge has been generated in *in vivo* and *in vitro* studies demonstrating diverse signalling pathways and transcriptional signatures related to host metabolism and immune associated response, and with significant potential as biomarkers in novel diagnostics as well as host-directed therapy for anti-TB interventions. The heterogeneous host immune response to infection with strains of diverse genetic background, and in different infection models has been well documented (Manca *et al.*, 2004; Manca *et al.*, 1999; Manca *et al.*, 2001; Rakotosamimanana *et al.*, 2010; Subbian *et al.*, 2013).

Despite this increasing knowledge, few studies have focused on understanding cellular response to clinical strains of *M. tuberculosis* in pulmonary epithelial cells (Lin *et al.*, 1998) although there is compelling evidence showing the importance of these cells in TB pathogenesis (Chuquimia *et al.*, 2013; Chuquimia *et al.*, 2012). Moreover, transcriptomics

studies to understand pathways, networks and transcriptional factor regulation induced by *M. tuberculosis*, particularly of diverse genotypes, within these cells, have not been elucidated to date.

Recently, we have shown that dominant strains in South Africa, induce differential cytokine production and gene expression in epithelial cells (Chapter 2 and 3). In this study, we used the IPA software to quantitatively analyse the enrichment of canonical pathways and networks as well as transcriptional factor regulation induced by different clinical strains on pulmonary epithelial cells.

Materials and methods

Bacterial isolates and infection of pulmonary epithelial cells

Infection of the A549 pulmonary epithelial cells with one laboratory and 5 clinical strains was performed as described in Chapters 2 and 3. *M. tuberculosis* strains belonging to the F15/LAM4/KZN, Beijing, F11, F28 families, and a Unique genotype, were previously isolated from patients in the KwaZulu-Natal region of South Africa. Briefly, the F15/LAM4/KZN, Beijing, F11, F28 and Unique strains were grown in Middlebrook 7H9 broth [supplemented with 50% glycerol, 20% Tween-80 and 10% OADC] until the log phase was reached. Glycerol stocks of these cultures were stored at -70 ºC. Prior to infection, these stocks were revived and grown until mid-log phase. A549 pulmonary epithelial cells (ATCC CCL 185) were infected with each strain at an MOI of ~10:1 in 25 cm^2tissue culture flasks and incubated at 37 ºC in 5% CO_2 for 48 hr.

RNA extraction, sequencing and mapping, and transcript assembly were performed as described in Chapter 3. Briefly, RNA samples from infected and uninfected pulmonary

epithelial cells were extracted using the RNeasy kit (Qiagen, South Africa). RNA concentrations were quantified using the nanodrop (ThermoScientific, South Africa) and stored at -70ºC. The integrity of the RNA was assessed using the Agilent 2100 Bioanalyzer (Johns Hopkins Deep Sequencing & Microarray Core Facility, USA). The TruSeq RNA Sample Preparation Kit v2 was used to prepare the RNA sequencing library, while the Illumina HiSeq platform was used to sequence 50 bp for each sample. The sequencing reads were mapped into the UCSC (Homo sapiens) reference genome (Hg19) using TopHat (2.0.9), aligned with cufflinks (2.0.2), and differential gene expression between infected and uninfected cells were calculated using cuffdiff (Trapnell *et al.*, 2012; Trapnell *et al.*, 2010).

Ingenuity Pathway Analysis

Differentially expressed genes (Cutoff ≥2) were used to investigate significantly affected canonical pathways, gene networks and transcriptional factors induced in pulmonary epithelial cells infected with different clinical strains of *M. tuberculosis*. Changes in canonical pathways were investigated with IPA (7.0) (Ingenuity Systems, USA), and an enrichment score for each pathway was displayed as a heat map using MeV (4.0.9). The networks induced by each of the clinical and laboratory strains were compared by IPA. Genes differentially expressed between infected and uninfected pulmonary epithelial cells, and IPA hypothetical interacting genes found in the IPA knowledge base system, were used to generate networks based on their connectivity with a maximal network size of 35 genes. Graphical representation of networks with their scores was computed by IPA. The nodes within the networks represent genes and lines indicate biological relationships (direct or indirect) with other genes based on published literature within the IPA software. The network score is based on hypergeometric distribution and is calculated with the right tailed Fisher's Exact Test. Moreover, the score was used to rank networks according to the input dataset.

This did not necessarily reflect the significance of that network but rather an approximation of the original focus gene list. IPA listed the top molecular and cellular functions as well as physiological system development and function of co-regulated differentially expressed genes (Appendix C, Supplementary Table 1).

Results

Different canonical pathways, networks and transcriptional factors that were activated or down-regulated in pulmonary epithelial cells infected with clinical strains of *M. tuberculosis* were identified using IPA. IPA also showed network interrelationships among clinical strains and their enrichment scores, based on the involvement of different genes from the input dataset. Differential regulation of transcriptional factors was observed with some of these being shared among the strains while others were strain specific.

Top 10 canonical pathways

The top 10 canonical pathways varied among the strains (Table 1). The interferon signalling and Hepatic Fibrosis/Hepatic Stellate Cell Activation pathways were among the top 5 canonical pathways induced by all the strains, while Antigen Presentation was among the top 10 in all but the Unique strain. The role of pattern recognition receptors for bacteria and viruses was induced by the F15/LAM4/KZN, F11, F28 and the H37Rv strains only. The super pathway of Cholesterol Biosynthesis was induced by only the Beijing and Unique strains, while only the former induced the Cholesterol Biosynthesis I pathway. All strains except the F15/LAM4/KZN and H37Rv induced the lipid sensing liver X alpha

and beta/retinoid X receptor (LXR/RXR) pathway activation. Communication between innate and adaptive cells was induced by the Unique and the H37Rv strains only, whilst granulocyte adhesion and diapediesis was induced by all except the F15/LAM4/KZN strain. The farnesoid X receptor/ retinoid X receptor (FXR/RXR) activation pathway was induced by only the Unique strain.

Table 1: The top 10 canonical pathways induced by each strain of *M. tuberculosis* in pulmonary epithelial cells at 48 hr post-infection

Canonical pathways for the F15/LAM4/KZN **strain**	**Genes**		
	Up-regulated	**Down-regulated**	**-log(p-value)**
Interferon Signalling	17/36 (47%)	1/36 (3%)	1.30E+01
Dendritic Cell Maturation	33/179 (18%)	5/179 (3%)	1.20E+01
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	27/127 (21%)	4/127 (3%)	1.16E+01
TREM1 Signalling	21/75 (28%)	2/75 (3%)	1.10E+01
Hepatic Fibrosis / Hepatic Stellate Cell Activation	33/197 (17%)	5/197 (3%)	1.07E+01
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	39/298 (13%)	8/298 (3%)	9.84E+00
Atherosclerosis Signalling	26/123 (21%)	2/123 (2%)	9.77E+00
Antigen Presentation Pathway	15/37 (41%)	0/37 (0%)	9.31E+00
Acute Phase Response Signalling	30/169 (18%)	2/169 (1%)	8.89E+00
Type I Diabetes Mellitus Signalling	23/110 (21%)	2/110 (2%)	8.78E+00

Canonical pathways for the Beijing strain	Genes		-log(p-value)
	Up-regulated	Down-regulated	
Hepatic Fibrosis / Hepatic Stellate Cell Activation	35/197 (18%)	5/197 (3%)	1.14E+01
LXR/RXR Activation	23/121 (19%)	4/121 (3%)	8.84E+00
Superpathway of Cholesterol Biosynthesis	13/29 (45%)	0/29 (0%)	8.58E+00
Interferon Signalling	13/36 (36%)	1/36 (3%)	8.21E+00
Atherosclerosis Signalling	24/123 (20%)	2/123 (2%)	8.01E+00
Granulocyte Adhesion and Diapedesis	29/177 (16%)	3/177 (2%)	7.93E+00
Acute Phase Response Signalling	27/169 (16%)	4/169 (2%)	7.85E+00
Antigen Presentation Pathway	13/37 (35%)	0/37 (0%)	7.05E+00
Agranulocyte Adhesion and Diapedesis	25/189 (13%)	4/189 (2%)	5.70E+00
Cholesterol Biosynthesis I	7/13 (54%)	0/13 (0%)	5.52E+00

Canonical pathways for the F11 strain	Genes		-log(p-value)
	Up-regulated	Down-regulated	
Interferon Signalling	20/36 (56%)	1/36 (3%)	1.49E+01
Hepatic Fibrosis / Hepatic Stellate Cell Activation	40/201 (20%)	7/201 (3%)	1.26E+01
Dendritic Cell Maturation	35/179 (20%)	3/179 (2%)	9.03E+00
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	44/304 (14%)	8/304 (3%)	8.51E+00
TREM1 Signalling	21/76 (28%)	1/76 (1%)	8.07E+00
Type I Diabetes Mellitus Signalling	25/111 (23%)	2/111 (2%)	7.93E+00
Antigen Presentation Pathway	15/37 (41%)	0/37 (0%)	7.92E+00
Granulocyte Adhesion and Diapedesis	32/179 (18%)	3/179 (2%)	7.41E+00
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	25/127 (20%)	3/127 (2%)	7.20E+00
LXR/RXR Activation	23/128 (18%)	5/128 (4%)	7.13E+00

Canonical pathways for the F28 **strain**	**Genes**		
	Up-regulated	**Down-regulated**	**-log(p-value)**
Acute Phase Response Signalling	22/169 (13%)	8/169 (5%)	1.09E+01
Hepatic Fibrosis / Hepatic Stellate Cell Activation	25/197 (13%)	7/197 (4%)	1.06E+01
LXR/RXR Activation	15/121 (12%)	9/121 (7%)	9.91E+00
Interferon Signalling	12/36 (33%)	1/36 (3%)	9.07E+00
Granulocyte Adhesion and Diapedesis	26/177 (15%)	2/177 (1%)	9.05E+00
Antigen Presentation Pathway	13/37 (35%)	0/37 (0%)	8.90E+00
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	20/127 (16%)	2/127 (2%)	7.99E+00
Dendritic Cell Maturation	23/179 (13%)	3/179 (2%)	7.65E+00
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	28/298 (9%)	6/298 (2%)	7.09E+00
TREM1 Signalling	14/75 (19%)	1/75 (1%)	6.48E+00

Canonical pathways for the Unique **strain**	**Genes**		
	Up-regulated	**Down-regulated**	**-log(p-value)**
LXR/RXR Activation	20/121 (17%)	4/121 (3%)	9.80E+00
Acute Phase Response Signalling	26/169 (15%)	2/169 (1%)	9.40E+00
Interferon Signalling	12/36 (33%)	1/36 (3%)	9.00E+00
Granulocyte Adhesion and Diapedesis	23/177 (13%)	1/177 (1%)	6.44E+00
Hepatic Fibrosis / Hepatic Stellate Cell Activation	21/197 (11%)	4/197 (2%)	6.13E+00
FXR/RXR Activation	18/127 (14%)	1/127 (1%)	5.88E+00
Super pathway of Cholesterol Biosynthesis	9/29 (31%)	0/29 (0%)	5.77E+00
Role of Tissue Factor in Cancer	12/110 (11%)	5/110 (5%)	5.53E+00
Atherosclerosis Signalling	15/123 (12%)	2/123 (2%)	4.86E+00
Communication between Innate and Adaptive Immune Cells	11/91 (12%)	3/91 (3%)	4.64E+00

Canonical pathways for the H37Rv strain	Genes		
	Up-regulated	Down-regulated	-log(p-value)
Hepatic Fibrosis / Hepatic Stellate Cell Activation	33/201 (16%)	6/201 (3%)	1.20E+01
Interferon Signalling	13/36 (36%)	1/36 (3%)	8.87E+00
Antigen Presentation Pathway	12/37 (32%)	1/37 (3%)	7.65E+00
Atherosclerosis Signalling	21/124 (17%)	2/124 (2%)	6.99E+00
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	20/127 (16%)	3/127 (2%)	6.79E+00
Dendritic Cell Maturation	25/179 (14%)	3/179 (2%)	6.71E+00
Granulocyte Adhesion and Diapedesis	27/179 (15%)	1/179 (1%)	6.71E+00
Communication between Innate and Adaptive Immune Cells	18/92 (20%)	1/92 (1%)	6.64E+00
TREM1 Signalling	16/76 (21%)	1/76 (1%)	6.53E+00
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	32/304 (11%)	6/304 (2%)	6.23E+00

Cholesterol biosynthesis and immune related canonical pathways

Clinical strains of *M. tuberculosis* enriched different immune related pathways shown by their P-values in the heat map in Fig. 1 and Supplementary Table 2. The Beijing strain induced the highest cholesterol biosynthesis via 3 different pathways (I, II and III), followed by the Unique and F11 strains while the other 2 clinical strains least affected these pathways. The F15/LAM4/KZN and F11 strains induced the highest immune related pathway enrichment in epithelial cells compared to other clinical strains. Very little difference was observed among the strains in their enrichment scores (P-values) for both the interferon and differential regulation of cytokine production in intestinal epithelial cells by IL-17A and IL-17F.

The less enriched pathways by all the strains were the inhibition of matrix metalloprotease, chemokine signalling, CCR5 signalling in macrophages, STAT3, IL-22 signalling, IL-1 signalling, CD40 signalling, IL10 signalling and macropinocytosis signalling pathways. A higher enrichment for the F15/LAM4/KZN and F11 strains was observed for the TLR, IL-12 signalling and production in macrophages, IL-6 signalling, iNOS signalling, apoptosis signalling, role of JAK1, JAK2 and TYK2 in interferon signalling and IL-15 signalling pathways. The highest P-value for the JAK/STAT pathway was induced by the F11 strain followed by the F15/LAM4/KZN strain, while the Beijing, Unique and H37Rv strains induced least enrichment of this pathway. The different *M. tuberculosis* strains differentially activated epithelial pattern recognition receptors. The F15/LAM4/KZN strain induced the highest, while the Unique strain induced the lowest P-values of the epithelial cell pattern recognition receptors. Hierarchical clustering for enrichment of immune associated pathways revealed similar patterns of pathway enrichment induced by Beijing and Unique strains, F15/LAM4/KZN and F11 and F28 and H37Rv strains, respectively (Fig. 1; Appendix C, Supplementary Table 2).

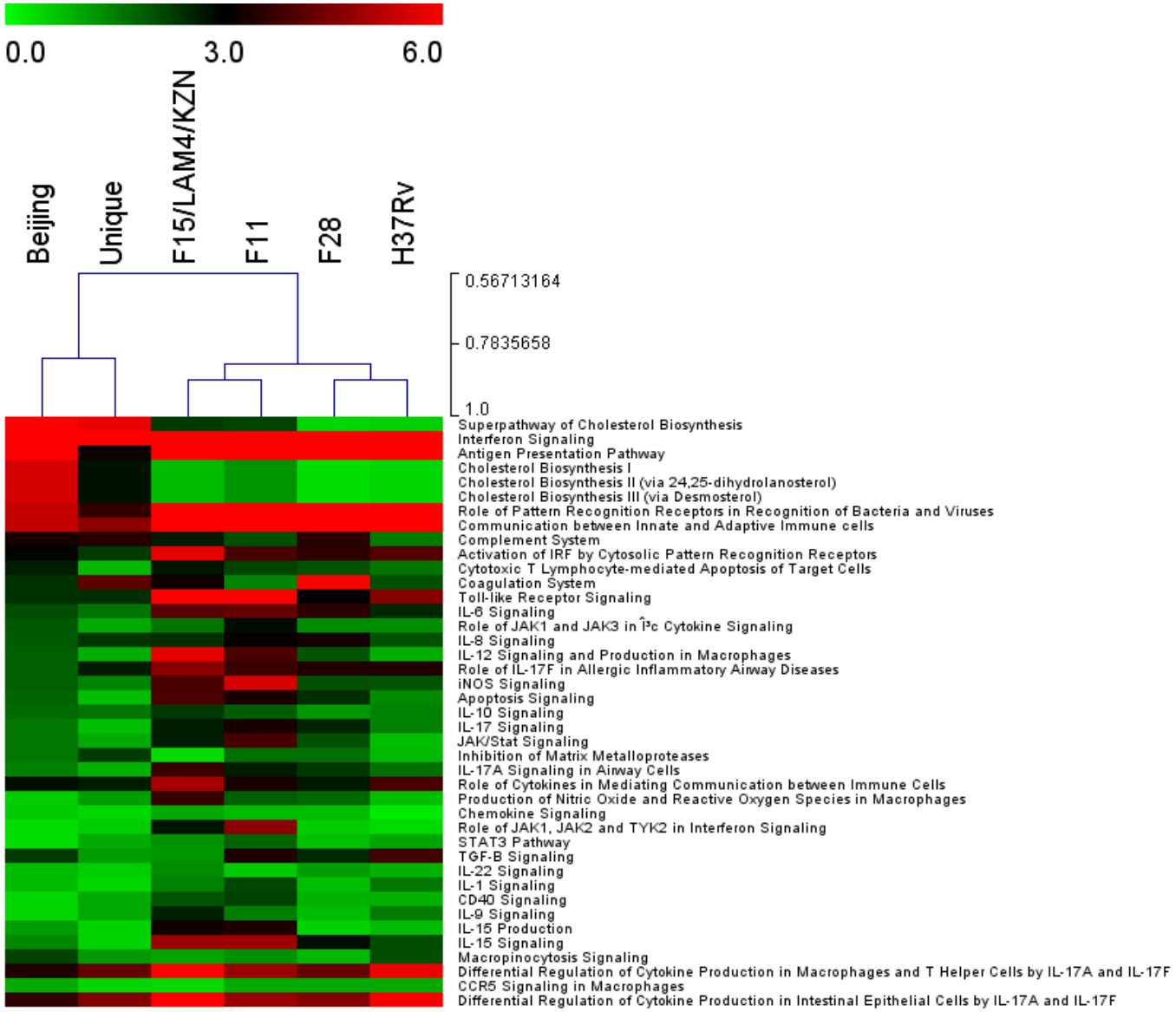


Fig. 1. Heat map showing the P-values of the cholesterol biosynthesis and immune associated canonical pathways in pulmonary epithelial cells infected with clinical strains of *M. tuberculosis* at 48 hr post-infection. The highest enrichment was induced by the F15/LAM4/KZN and F11 strains in the immune associated pathways while the Beijing and Unique strains induced the highest enrichment for the cholesterol biosynthesis pathways compared to other strains. Hierarchical clustering indicated similar enrichment profiles between the Beijing and Unique strains, the F15/LAM4/KZN and F11 strains and the F28 and H37Rv strains, respectively.

Toll-like receptor pathway

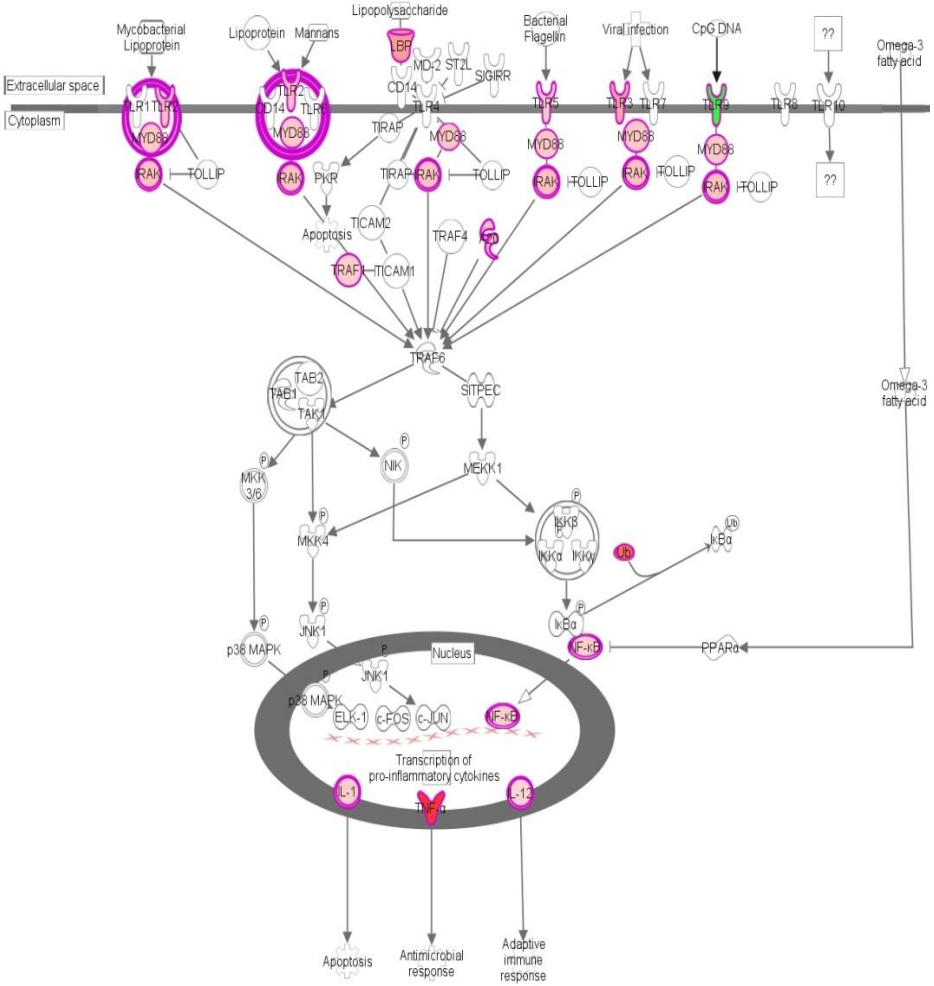
The TLR pathway plays a significant role in the innate immune response in recognition of different bacterial cell wall components and nucleic acids in pathogens (Li *et al.*, 2012). In the current study, *TLR5* and *IL-1* were up-regulated by all strains within this pathway. The activation of the other TLRs varied among strains (Fig. 2). The H37Rv strain down-regulated the expression of *TLR1* compared to the clinical strains, whilst the F15/LAM4/KZN, F11 and F28 strains up-regulated *TLR2*. All strains down-regulated the *TLR9* which was unaffected by the H37Rv strain. The Myeloid differentiation primary response gene (88) (*MYD88*) that facilitates signal transduction in the TLR pathway, was up-regulated by Beijing, F11, F15/LAM4/KZN and H37Rv strains. The Interleukin-1 receptor-associated kinase 1 (*IRAK*) gene was up-regulated by all except the Unique strains. Increased *IL-12* expression was induced by the F11 and F15/LAM4/KZN strains, while the latter and H37Rv strains induced an increase in *TNF-α* gene expression within the pathway. Signal transduction in this pathway resulted in activation of transcriptional factors that facilitate transcription of pro-inflammatory cytokines. The *C-Fos* transcriptional factor was up-regulated by only the Beijing, F11 and Unique strains while *NF-κB* was only activated by F15/LAM4/KZN and F11 strains (Fig. 2).

Interferon signalling pathway

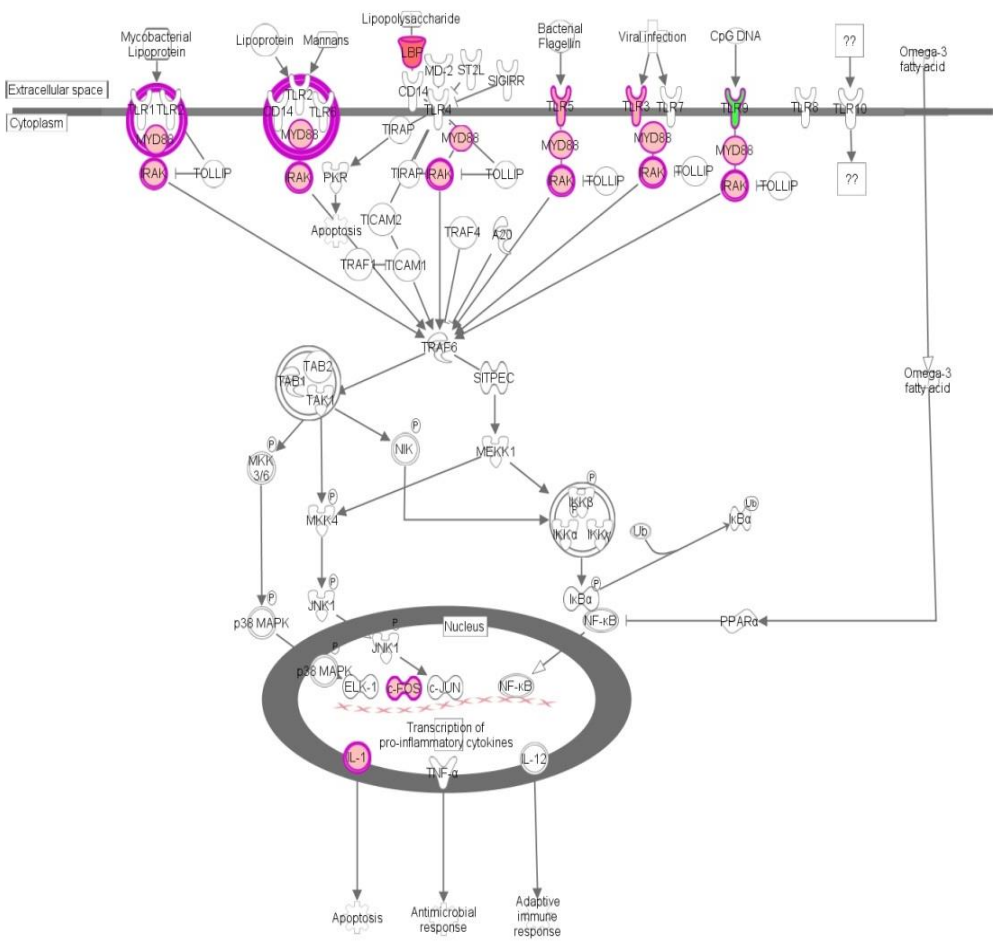
For all strains, interferon signalling was among the top five enriched pathways that resulted in the activation of essential transcriptional factors required for an effective immune response in epithelial cells. The F15/LAM4/KZN and F11 strains both activated *JAK2* and transmembrane receptors (*IFNγRα* and *IFNAR2*) together with the *IRF1* genes. All strains induced a down-regulation of the *BCL-2* while up-regulating a wide range of genes (*STAT1*,

STAT2, *TAP1*, *IFITM3*, *IFITM1*, *IFITM2*, *PSMB8*, *IFI35*, *IFIT1*, *OAS1*, *MX1* and *IFIT3*) within the pathway. No gene expression changes were observed for the following genes by all strains: *JAK1*, *TC-PTP*, *PIAS1*, *GIP2*, *GIP-3*, *TYK2*, *IFNR1*, *BAK*, *BAX*. The *SOCS1*, *IFNγRβ*, and *NF-κB p65* genes were activated only by the F11 strain and not other strains. Increased expression of *IRF9* was induced by all but Unique strain. No change for the *IFITM2* gene was observed for the F28 strain while other strains showed an increased expression of this gene within the pathway (Fig. 2).

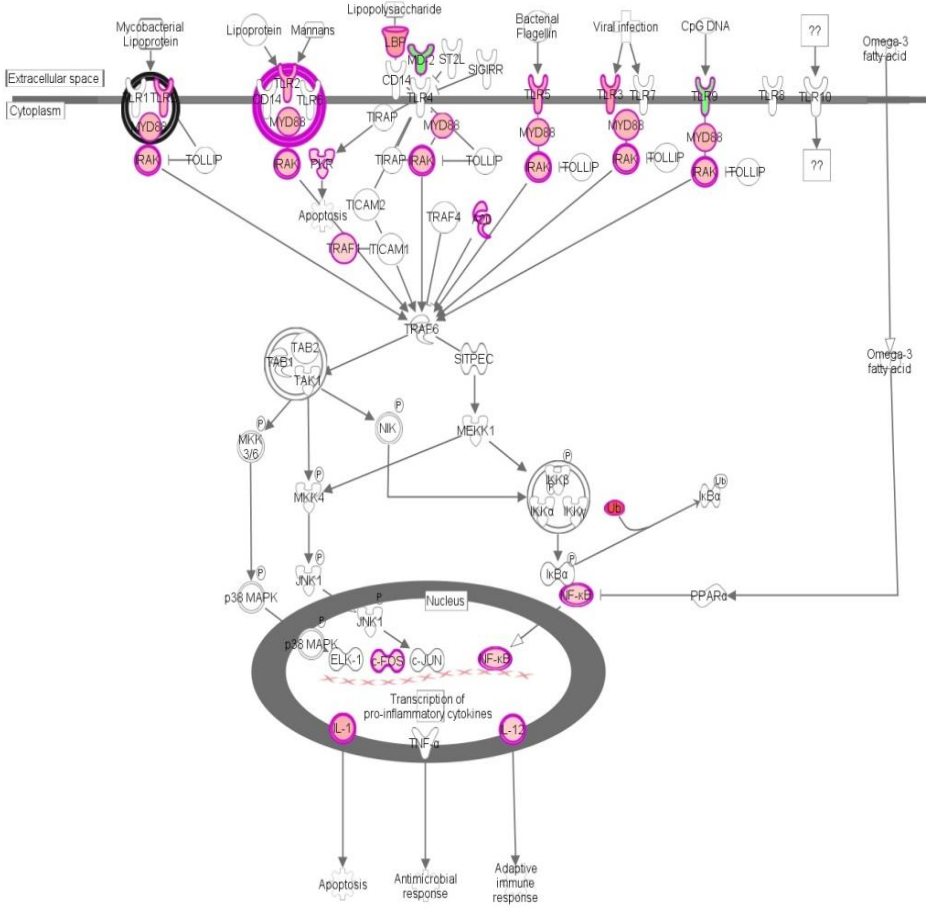
M. tuberculosis F15/LAM4/KZN strain



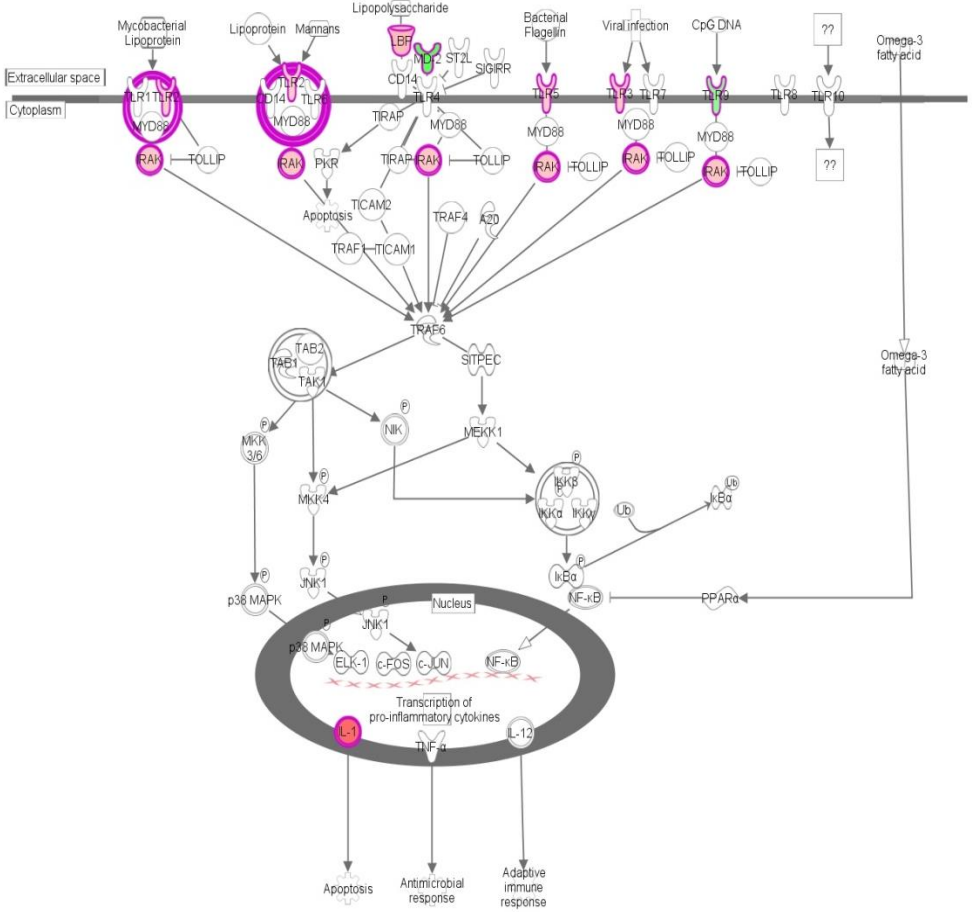
M. tuberculosis Beijing strain



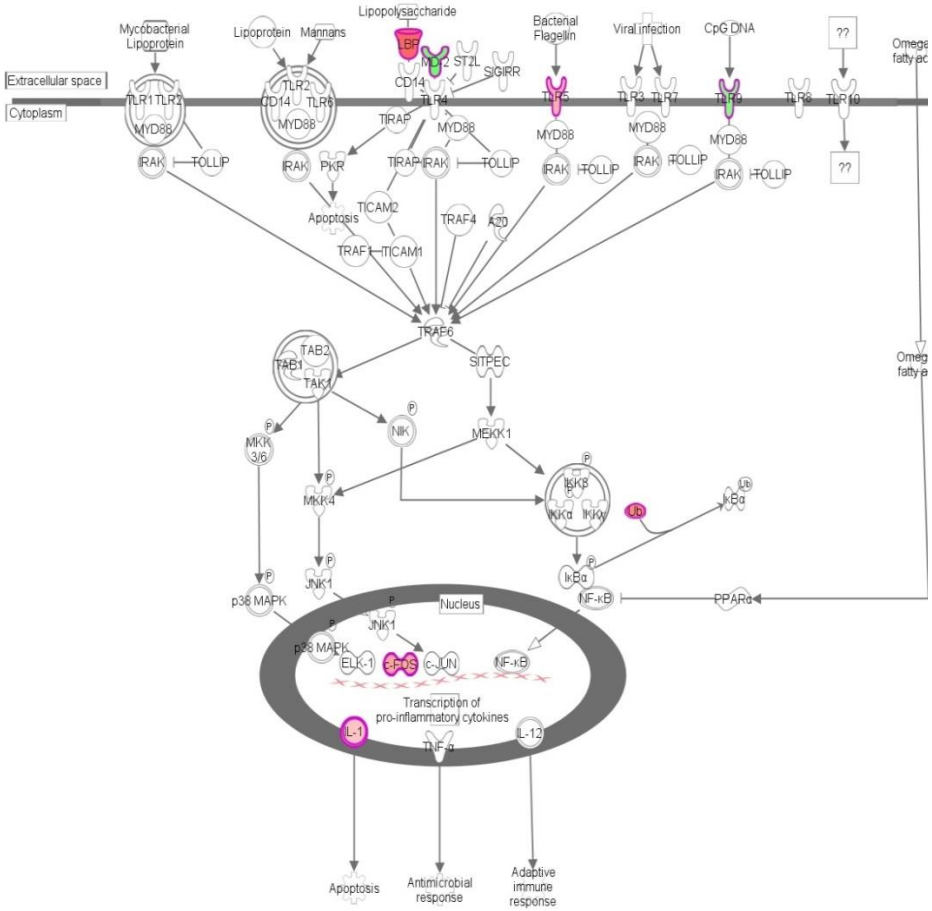
M. tuberculosis F11 strain



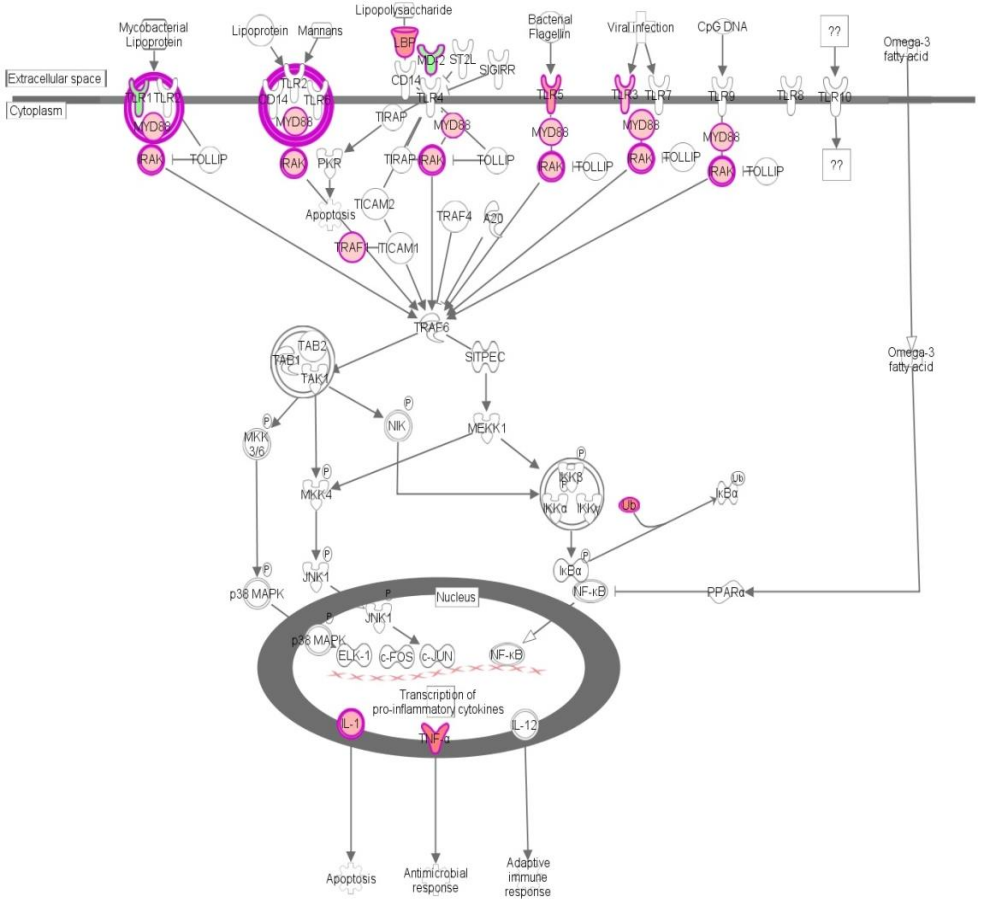
M. tuberculosis F28 strain



M. tuberculosis Unique strain

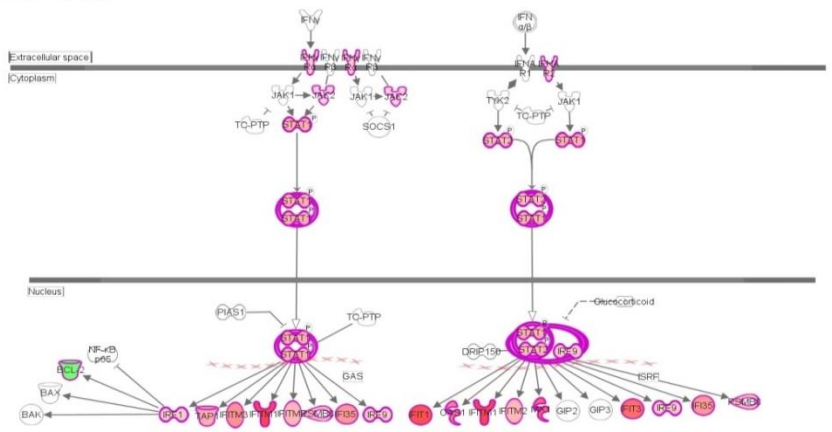


M. tuberculosis H37Rv strain

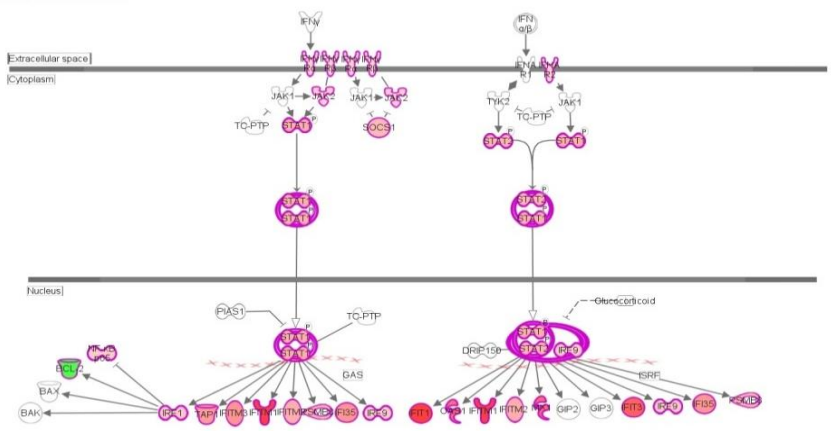


Interferon signalling

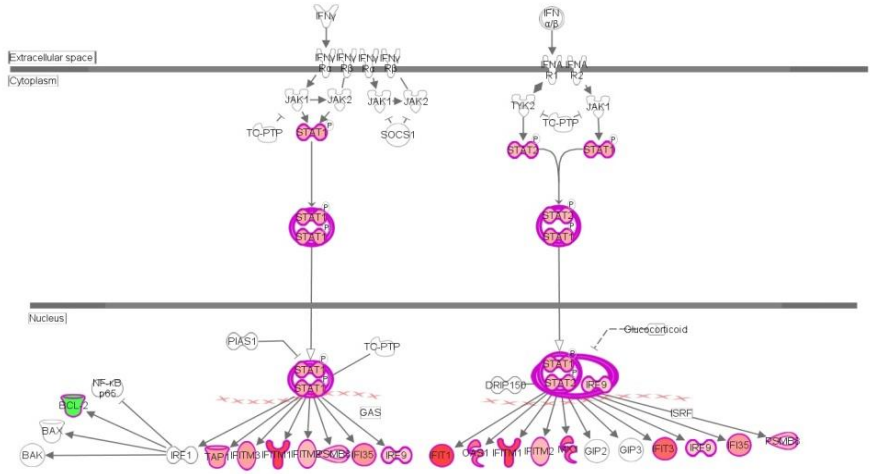
M. tuberculosis F15/LAM4/KZN strain



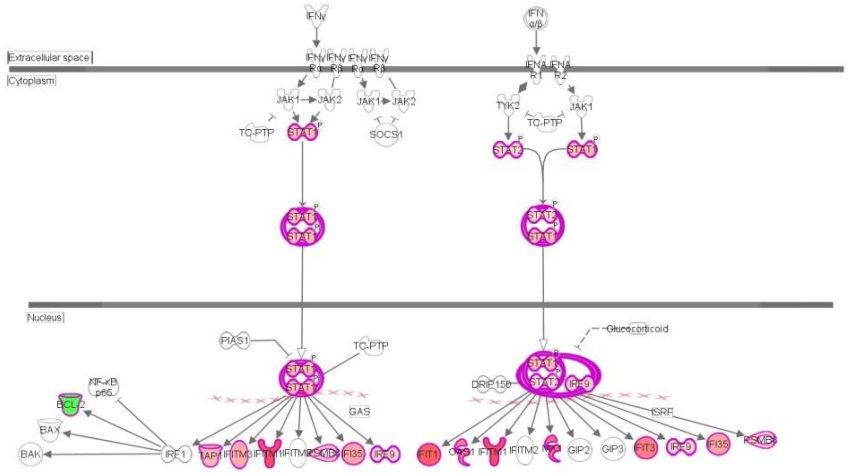
M. tuberculosis F11 strain



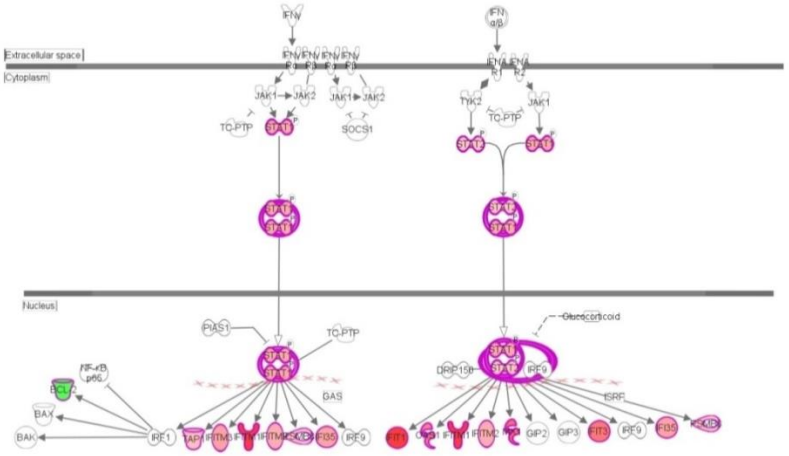
M. tuberculosis Beijing strain



M. tuberculosis F28 strain



M. tuberculosis Unique strain



M. tuberculosis H37Rv strain

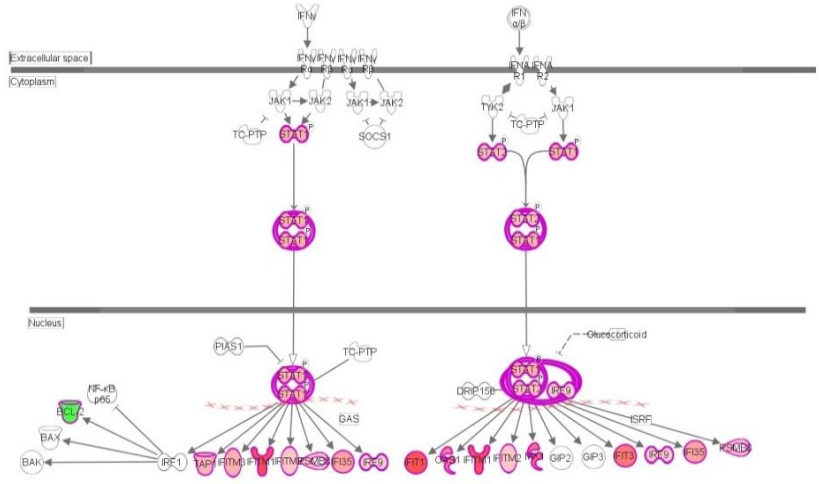
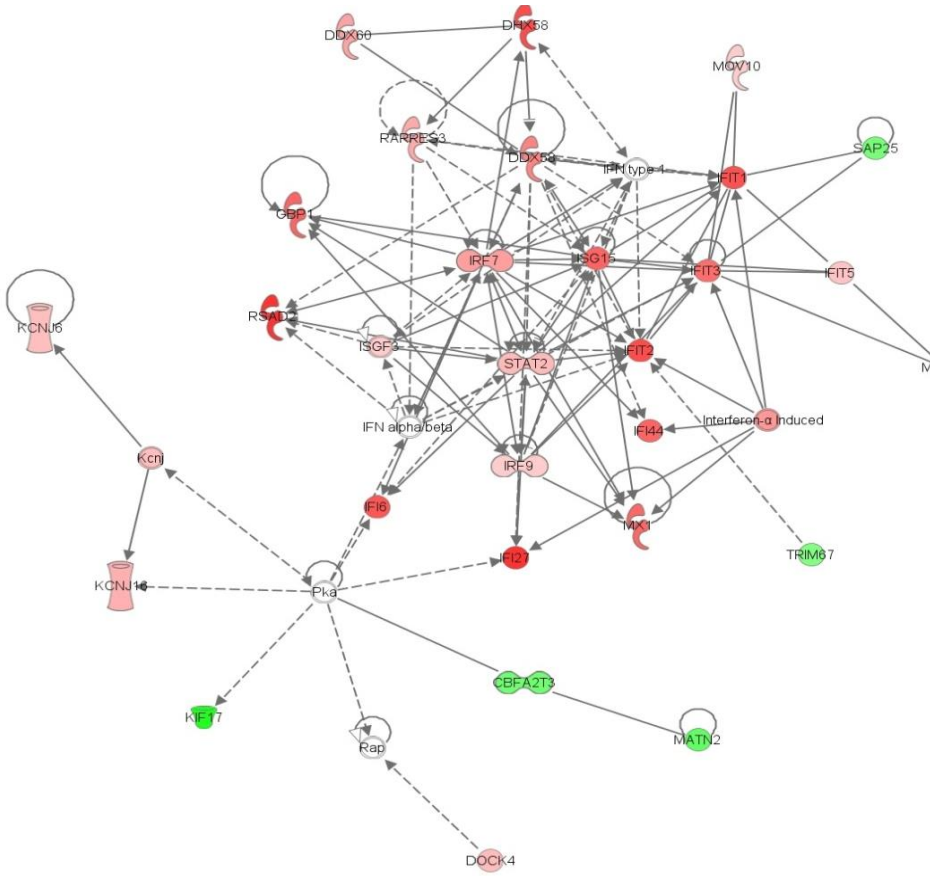


Fig. 2. IPA analysis of the toll like receptor and interferon signalling pathways induced by the Beijing, F11, F28, H37Rv, F15/LAM4/KZN and Unique strains in pulmonary alveolar epithelial cells. Interferon signalling and Hepatic Fibrosis/Hepatic Stellate Cell Activation pathways were among the top 5 canonical pathways induced by clinical strains. There were differences in some of the genes activated/down-regulated by each strain within these pathways in pulmonary epithelial cells. Red: Up-regulated genes, Green: Down-regulated genes.

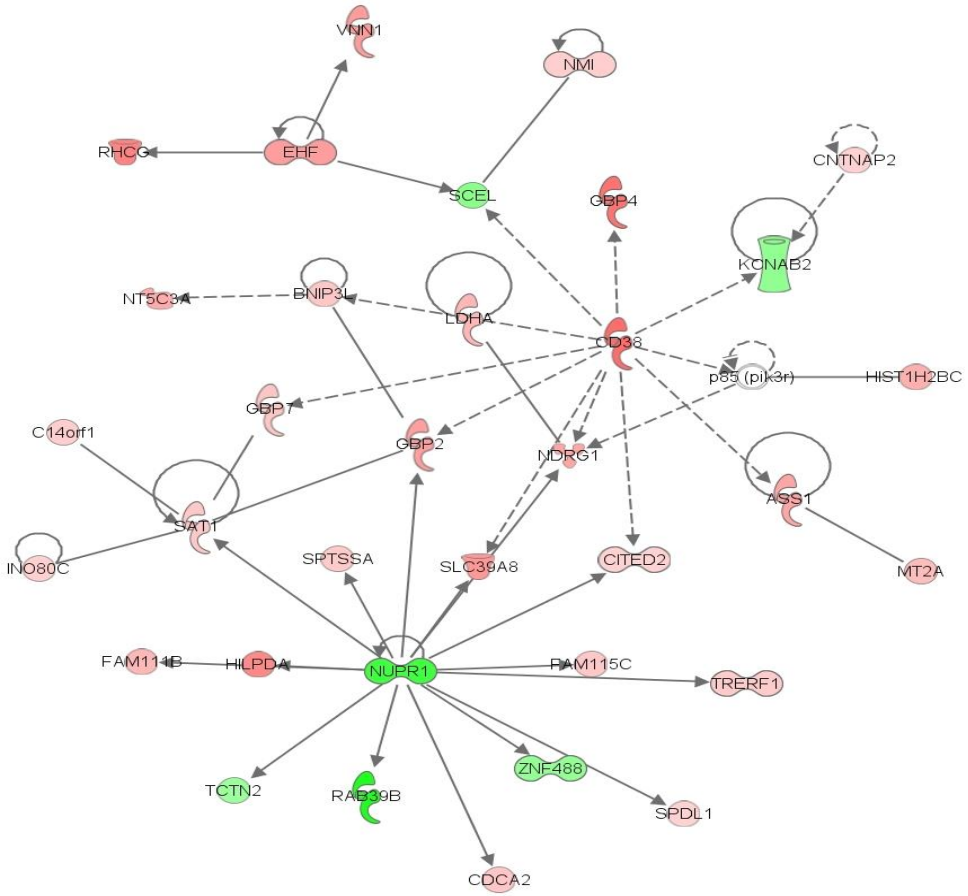
Top scoring gene networks in pulmonary epithelial cells induced by clinical strains of *M. tuberculosis*

Gene networks, associated with diverse functions within pulmonary epithelial cells were differentially regulated by each strain (Fig. 3). The top scoring networks, with their enrichment scores, and their associated molecular functions, induced in pulmonary epithelial cells differed among the strains. The highest scoring network (score=33) induced by the F15/LAM4/KZN strain, was associated with antimicrobial response. The highest enriched network for the Beijing strain was the developmental disorder network (score=47), while the organismal injury and abnormality network (score=34) was by the F11 strain. Lipid metabolism was the top network (score=40) affected by the genes induced by the F28 strain, while the infectious disease network (score=43) was induced by the Unique strain. Genes associated with cellular development (score=42) were induced by the H37Rv strain (Fig. 3).

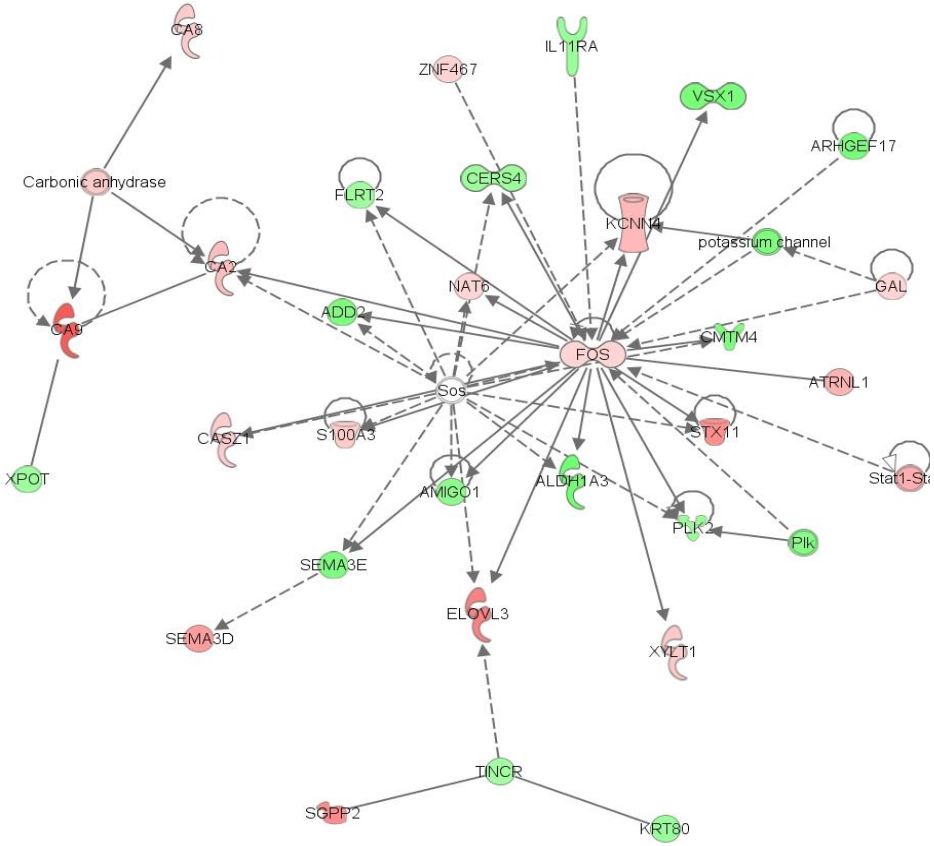
M. tuberculosis F15/LAM4/KZN strain



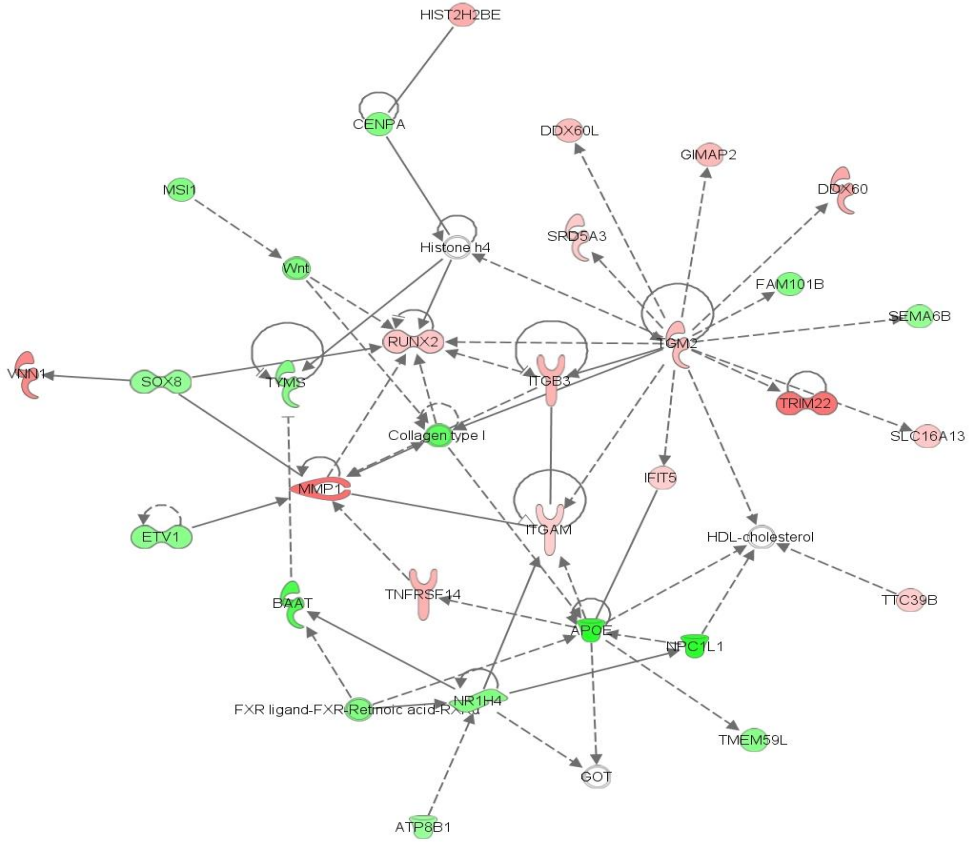
M. tuberculosis Beijing strain



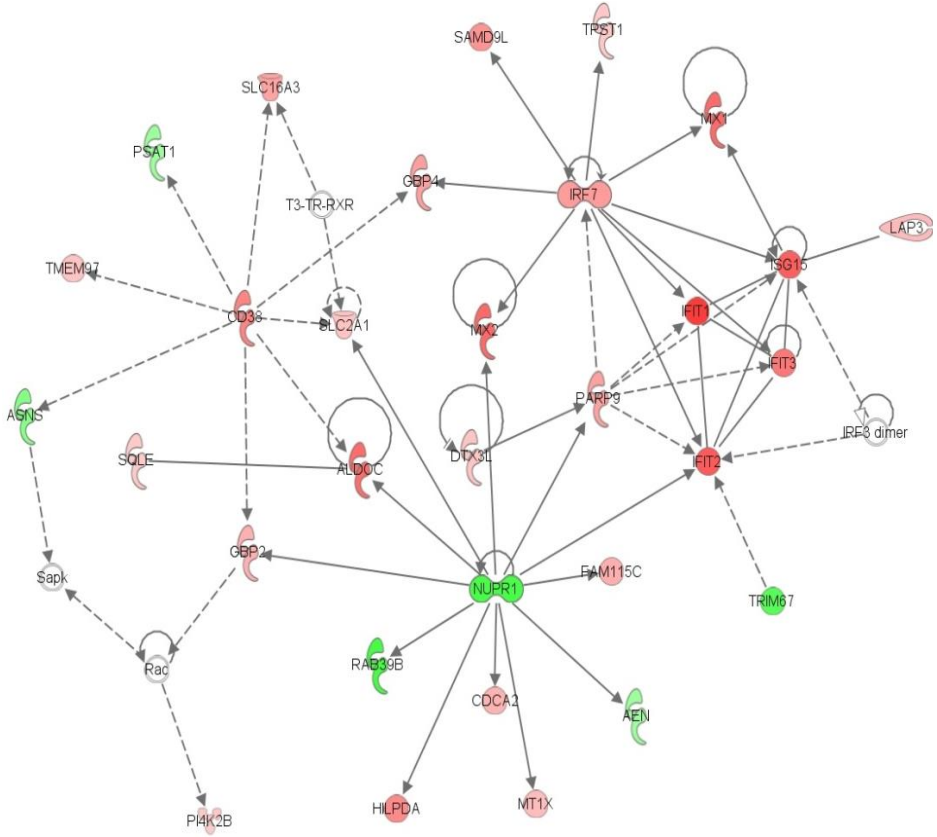
M. tuberculosis F11 strain



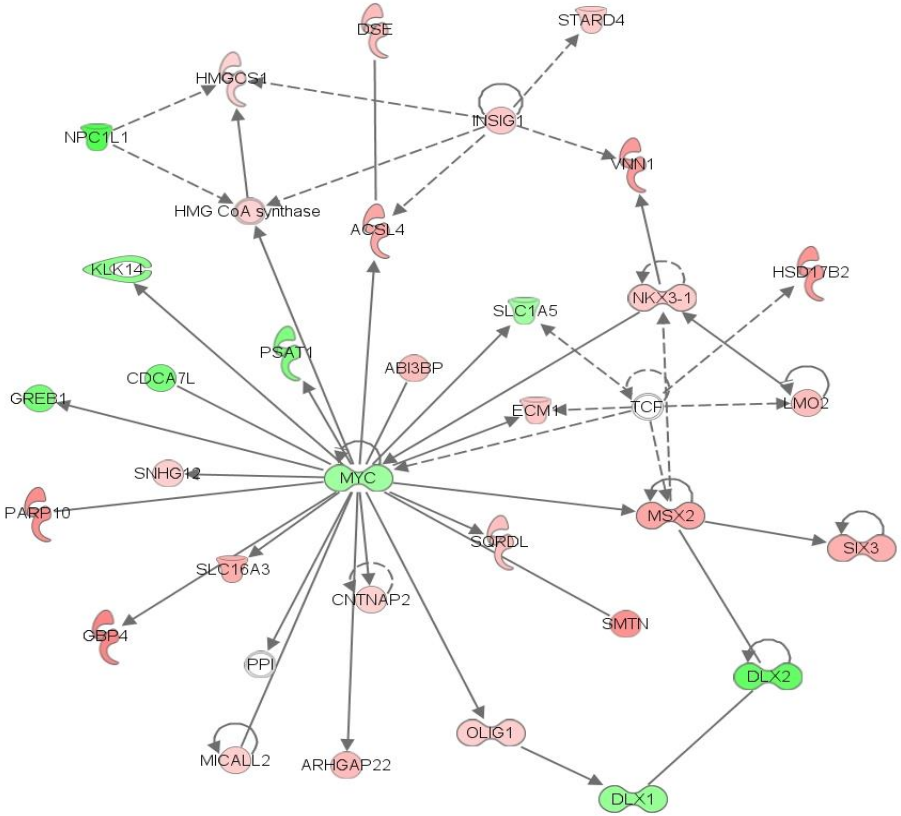
M. tuberculosis F28 strain



M. tuberculosis Unique strain



M. tuberculosis H37Rv strain



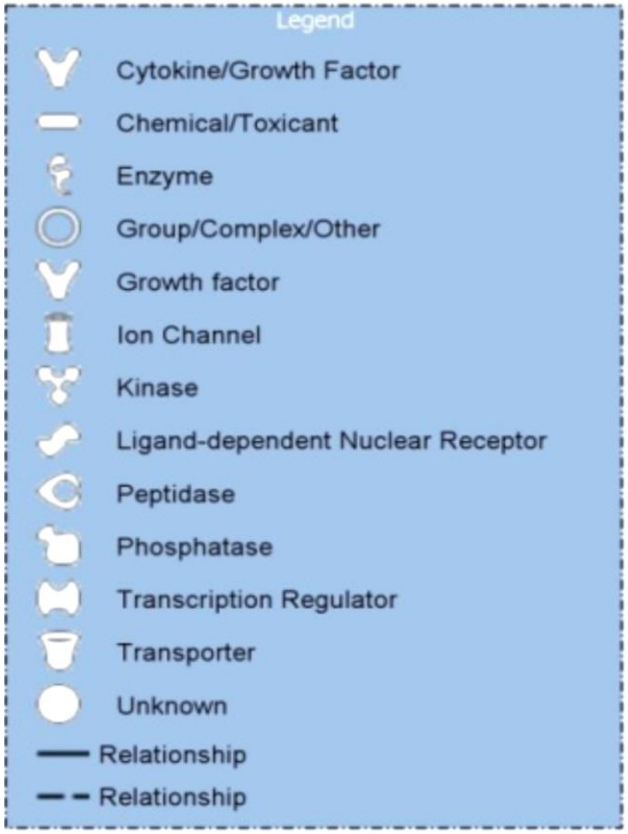


Fig. 3. Top IPA scoring networks induced by the F15/LAM4/KZN, Beijing, F11, F28, Unique and H37Rv strains of *M. tuberculosis* in pulmonary epithelial cells at 48 hr post-infection. The top scoring networks induced by different strains differed with their associated functions and enrichment scores as follows: F15/LAM4/KZN: Antimicrobial Response (33), Beijing: Developmental Disorder (47), F11: Organismal Injury and Abnormalities (34), F28: Lipid Metabolism (40), Unique: Infectious Disease (43), H37Rv: Cellular Development (42). Red: Up-regulated genes, Green: Down-regulated genes.

Differential regulation of transcriptional factors by clinical strains of *M. tuberculosis*

Transcriptional factors which are located on the upstream region of the genes greatly influence gene regulation and pathways within the genome. Some of the changes in pathways associated with infection by different strains might be explained by specific regulation of each transcriptional factor. In this study, clinical strains of *M. tuberculosis* differentially regulated the expression of diverse transcriptional factors which may be associated with complex down-stream molecular changes during infection (Fig. 4; Appendix C, Supplementary Table 3). *BATF2*, *EHL*, *HLF*, *HLX*, *IRF7*, *MSX2*, *PML*, *STAT1*, *STAT2*, *VDR* transcriptional factors were induced by all strains, albeit at different fold changes. These transcriptional factors were mainly involved in interferon and cytokine signalling pathways. Variations in transcriptional factor fold changes were observed for different strain combinations.

Up-regulation of *BRCA2*, *IFI16*, *IRF9*, *NLRC5* and *STAT5A* factors was significantly induced by all strains except the Unique strain. The functional categories of these factors include immune system processes and cell response to IFN-γ treatment. The F15/LAM4/KZN and F11 strain specific transcriptional factors included *CYLD*, *HOXB4*, *IRF1*, *IRF8*, *NFκB1* and *NFκBIZ* that are involved in interferon signalling pathway and other immune system processes. The *ATF3* and *RCAN1* transcriptional factors are involved in repressing gene expression including those involved in TLR pathway, while the *RCAN1* factor has been linked to disease neuropathology. Both *ATF3* and *RCAN1* were down regulated by the H37Rv and Unique strain while the *CEBPD* factor, associated with immune response, cell differentiation, metabolism and cell death, was up-regulated by these strains respectively (Fig. 4; Appendix C, Supplementary Table 3).

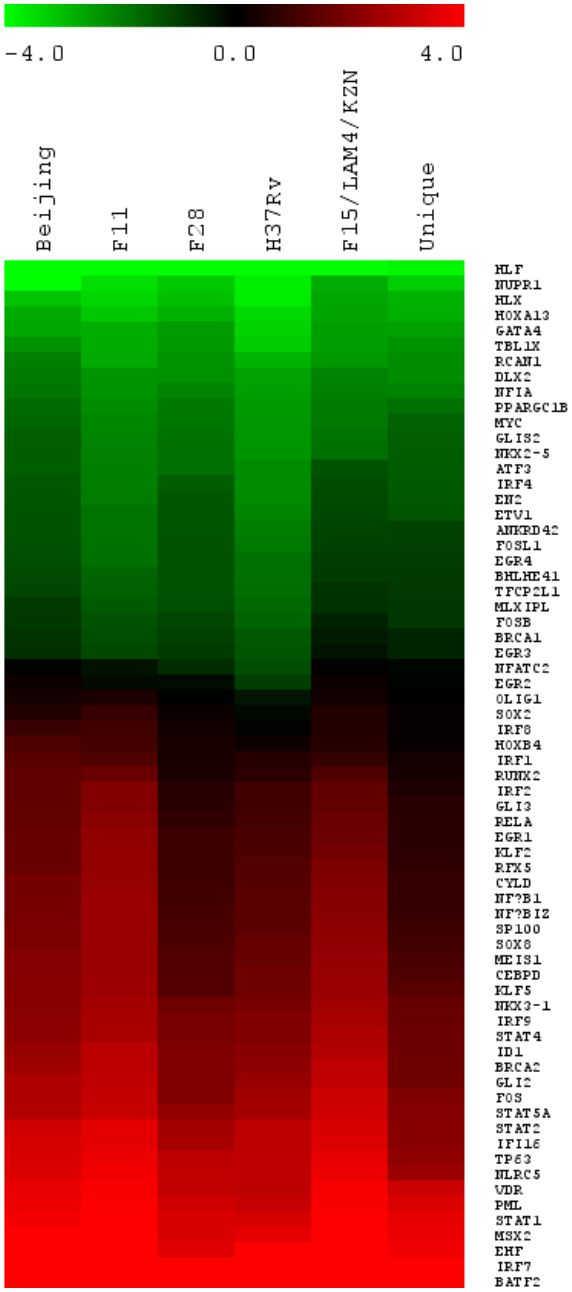


Fig. 4. Fold changes of transcriptional factors induced by clinical strains of *M. tuberculosis* in pulmonary epithelial cells at 48 hr post-infection. Up-stream regulators were identified by the IPA software based on published literature and fold changes of transcriptional factors induced by each strain are shown as a heat map. Genes are sorted according to their expression fold changes: Green-down-regulated, Red-up regulated.

The *TBL1X* factor was Beijing specific, and is involved in a variety of functions including signal transduction, gene regulation, RNA processing, vesicular trafficking and cytokine

skeleton assembly. The F11 specific transcriptional factors were *ANKRD42, BRCA1, GLI3, GLIS2, IRF2, MEIS1, NKX2-5, RELA, RFX5, TFCP2L1* and their functions were NF-κB heterodimer formation, transcriptional activator of histone H4 to nuclear translocation and activation, normal development, kidney development and neurogenesis. The *SOX8* factor was specific for the F28 strain and is associated with determination of the cell fate as well as regulating the embryonic development. The transcriptional factors *EGR2, EN2, MLXIPL, MYC, OLIG1* and *PPARGC1B* that code for proteins required for heterodimeric complex formation that activate carbohydrate response elements (ChoRE) motifs and these factors were specific for the H37Rv strain. The transcriptional factor *SOX2*, crucial for maintaining self-renewal, was F15/LAM4/KZN specific. Factors associated with the Unique strain were *BHLHE41, EGR1, FOSL1, IRF4* and *KLF2* which are involved in cell differentiation and mitogenesis, cell proliferation, differentiation, and transformation, lung development, embryonic erythropoiesis, epithelial integrity, T-cell viability and adipogenesis (Table 2).

Table 2: Strain specific transcriptional factors that are regulated by each strain in pulmonary epithelial cells

Beijing	**F11**	**F28**	**H37Rv**	**F15/LAM4/KZN**	**Unique**
TBL1X	*ANKRD42*	*SOX8*	*EGR2*	*SOX2*	*BHLHE41*
	BRCA1		*EN2*		*EGR1*
	GLI3		*MLXIPL*		*FOSL1*
	GLIS2		*MYC*		*IRF4*
	IRF2		*OLIG1*		*KLF2*
	MEIS1		*PPARGC1B*		
	NKX2-5				
	RELA				
	RFX5				
	TFCP2L1				

Discussion and conclusion

Molecular mechanisms associated with host response to infection by dominant strain families of *M. tuberculosis* in South Africa have been largely unexplored. To our knowledge, this is the first *in vitro* study using RNA-Seq to analyse changes induced in pathways, networks and transcriptional factors in pulmonary epithelial cells infected with clinical strains of *M. tuberculosis*. Epithelial cells, because of their increased presence in the alveolar space compared to macrophages (Chuquimia *et al.*, 2012; Dobbs *et al.*, 2009), may be the first to

encounter the pathogen, and there is increasing evidence of their involvement in host immune response (Lee *et al.*, 2009; Lin *et al.*, 1998; Sato *et al.*, 2002; Sharma *et al.*, 2007).

The identification of strain specific pathway activation might provide insights into potential novel targets that may be useful for the design of host directed immunotherapies or vaccine development that take into consideration the genetic heterogeneity of different strain families in different geographic settings. Virulent strains may activate certain pathways that will beneficiate them, whilst some strains may lack this ability, to the host advantage.

The present study demonstrated that infection of pulmonary epithelial cells induced changes in different canonical pathways, networks and transcriptional factors by different *M. tuberculosis* strains. Enrichment of the top 10 immune related canonical pathways was differentially induced by each strain in this study. The F15/LAM4/KZN and F11 strain activated the most immune related pathways that were highly enriched by these, but not the other 4 strains. These pathways included host cell recognition receptors, IL-12 signalling and production in macrophages, JAK/STAT signalling, role of JAK1, JAK2 and TYK2 in interferon signalling, IL-15 signalling and production pathways, suggestive of some similarity in the behaviour between these two strains. These pathway changes differed markedly compared to the other 3 clinical strains. The activation of protective immune associated pathways alludes to lower virulence of the F15/LAM4/KZN and F11 strains.

The interferon signalling pathway was among the top 5 activated pathway induced by all strains in our study. This may indicate that different types of interferons are induced in pulmonary epithelial cells during early infection with *M. tuberculosis*. This may explain the findings of Sharma *et al*. (2007) that *M. tuberculosis* infected epithelial cells are a source of IFN-γ (Sharma *et al.*, 2007), mediated by the activation of both types I and II interferon pathways. No differences were observed in the activation of the interferon pathways by the

clinical strains used in our study. Interferon pathways were shown to differentiate TB infection from other infectious diseases (Berry *et al.*, 2010). In contrast, another study showed that the interferon pathways were activated by both TB and melioidosis infections (Koh *et al.*, 2013) indicating a general host response to both these infections. These results suggest that the interferon signalling pathways are not differentially induced by different clinical strains of *M. tuberculosis*.

The cholesterol biosynthesis pathways were the most distinguishable canonical pathways among the clinical strains. These were highly enriched by the Beijing strain followed by the Unique strain compared to the others. These results suggest differences in host metabolism in response to different strains of *M. tuberculosis*, with host lipids preferentially utilized as carbon and energy sources during intracellular growth by the Beijing and Unique strains compared to others. The need to utilize host cholesterol using the ABC-like transport system (Pandey and Sassetti, 2008), as well as the ability of *M. tuberculosis* to grow and persist within cholesterol rich host cells has been well established (Kim *et al.*, 2010; Singh *et al.*, 2012). Cholesterol has been shown to be required for *M. tuberculosis* intracellular adaptation and host immune impairment within host cells (Gatfield and Pieters, 2000; Martens *et al.*, 2008). *M. tuberculosis* mutants defective in cholesterol utilization and degradation lacked the ability to grow in animal models of TB pathogenesis, indicating the importance of cholesterol in intracellular persistence and survival within the host (Chang *et al.*, 2009; Pandey and Sassetti, 2008; Yam *et al.*, 2009). High cholesterol synthesis induced by the Beijing and Unique strains may contribute to higher virulence and persistence within the host cells compared to the other strains. This characteristic may be associated with the increased virulence of the Beijing strains globally (Glynn *et al.*, 2002), however, this may not necessarily be linked to increased transmissibility of the Beijing strain as suggested by the less prevalent Unique strain.

Host protective pathways, such as the LXR/RXR pathway, are essential for effective response to *M. tuberculosis*. LXRα and β isoform knockout mice were more susceptible to infection with increased bacterial loads, severe lung pathology and decreased TH1 and TH17 functions in the lungs (Korf *et al.*, 2009), compared to the wild type mice following treatment with LXR agonists. In our study, this pathway was among the top 10 induced by the F11, F28 and Unique strains only. This is suggestive of a potential protective immune response induced by these strains within host epithelial cells. This may be the molecular mechanism responsible for the low prevalence of the Unique compared to the Beijing strains within the population, despite the ability of the former to induce an increase in cholesterol biosynthesis required for intracellular survival. It is possible that the prevalence and spread of F11 and F28 strains within the South African population (Chihota *et al.*, 2012; Victor *et al.*, 2004) may be due to alternative molecular mechanisms despite the induction of the LXR/RXR pathway by these strains.

The LXR activation pathway has been identified as a potential pathway to be explored for host directed therapy (Hawn *et al.*, 2013). Thus, the diversity exhibited in this pathway among clinical strains should be an important consideration in the design of such therapies. LXR influenced the activation of the RXR which forms a complex and modulates gene expression (Hawn *et al.*, 2013). In the current study, RXR activation was induced only by the Unique strain in the top 10 canonical pathways, indicating a potent gene expression heterodimer complex formed between the LXR and RXR to induce an increased protective response by this strain.

The diversity observed in the TLR pathway may not necessarily be due to the interaction of strain specific components of the different *M. tuberculosis* strains. The rationale for this thinking is that the RNA was sampled at 48 hr post-infection, while attachment and invasion can occur from 10 min-2 hr of exposure to *M. tuberculosis* (Garcia-Perez *et al.*, 2003) which

can induce differential gene expression of the TLRs. The least enrichment of TLR pathway was induced by the Unique strain compared to other clinical strains. Low enrichment of this pathway by the Beijing, F28 and Unique strains may be the molecular mechanism associated with the low production of pro-inflammatory cytokines induced by these strains compared to other clinical strains (Chapter 2), since stimulation of TLRs results in the production of pro-inflammatory cytokines (Mitchell *et al.*, 2010).

The *ATF3* transcriptional factor is known to negatively regulate expression of TLRs (Whitmore *et al.*, 2007). This inverse relationship was not observed in the current study, since significant down-regulation of *ATF3* only by Unique and H37Rv strains did not result in an increased enrichment of the TLR in both these strains. However, the diversity in the expression of TLRs induced by the different strains may be attributable to the differences observed in this transcriptional factor. Interestingly, the *NFκB* and *NFκB1* transcriptional factors that positively regulate the TLR pathway was induced by only the F15/LAM4/KZN and F11 strains, which maybe suggestive of a protective host immune response as this factor is involved in production of pro-inflammatory cytokines (Giacomini *et al.*, 2010). These results provide further support for the postulation that the F15/LAM4/KZN and F11 strains are associated with lower virulence than the other 3 strains. Whilst the potential of the TLRs for therapeutic purposes is still under investigation (Helbig *et al.*, 2013), TLR antagonists that exploit differential TLR activation may represent an effective strategy to fight Mycobacterial infections.

Strain specific networks induced in host cells may serve as novel targets for anti-tuberculosis treatment by understanding the functional categories involved in these networks. The F15/LAM4/KZN strain showed an increased antimicrobial response network in pulmonary epithelial cells compared to other strains. While epithelial cells may not directly possess a microbicidal effect through the phago-lysosome when compared to macrophages, the ability

to produce cytokines and chemokines through activation of crucial transcriptional factors such as *IRFs* might result in reduced bacterial replication at the infection site. These transcriptional factors (*IRF1*, *IRF8*) were up-regulated within the F15/LAM4/KZN and F11 infected epithelial cells, which may also explain higher immune related pathway enrichment compared to other clinical strains, (Fig. 4, Table 2). They were also shown to be essential in the innate responses to infection (Liu and Ma, 2006; Shin *et al.*, 2011). Gene networks induced by the Beijing, F11, F28 and Unique strains were strain specific, with diverse gene interaction and regulation by different clinical strains of *M. tuberculosis* in pulmonary epithelial cells.

Differentially regulated immune related and lipid metabolism networks were identified in macrophages infected with the Beijing and CDC1551 strains at an earlier time of infection Koo *et al*. (2012). The CDC1551 strain up-regulated the immune related network while the Beijing strain up-regulated the lipid metabolism network at 6 hr post-infection (Koo *et al.*, 2012). Subbian *et al*. (2013) reported higher enrichment of the macrophage activation network in the Beijing (HN878) strain in infected rabbit lungs compared to the CDC1551 strain at 3 hr post-infection. Compared to these studies, we observed differential network regulation by each clinical strain, accompanied by varying levels of activation in pulmonary epithelial cells. Furthermore, the highest scoring network for lipid metabolism was induced by the F28 strain and not the Beijing strain as was reported in macrophages by Koo *et al*. (2012). These differences might be attributed to cell specific response and differences between pulmonary epithelial cells and macrophages when infected by clinical strains as well as differences in sampling time.

In conclusion, this study demonstrated strain specific molecular pathways, and networks in epithelial cells induced by clinical strains representing major strain families in South Africa. Changes in transcriptional factors induced by each strain were able to explain some of the

complex down-stream changes in pathways and networks induced by these strains. This study provides further supporting evidence that hypervirulent strains such as the Beijing strain, invoke lower protective host responses, as displayed by low enrichment of the immune related pathways compared to the F15/LAM4/KZN, F11 and F28 genotypes. An unusual discovery was the similarity in the immune related and cholesterol pathways of the non-clustering Unique genotype to the Beijing strain, which suggests that despite its low transmissibility, this strain may be more virulent than expected, and warrants further study. The strain components that affect changes in these canonical pathways and networks will be explored further in future studies.

CHAPTER 5: GENERAL DISCUSSION

The current study evaluated the *in vitro* response of pulmonary epithelial cells infected by various clinical strains of *M. tuberculosis* by quantifying cytokine production at 24-, 48- and 72- hr post infection, as well as gene expression and pathway regulation at 48 hr. The clinical strains elicited different epithelial cell responses, suggesting the recognition of different virulence factors among these strains. The Beijing and Unique strains induced lower protective responses as evidenced by lower cytokine production, and least enrichment of immune associated pathways compared to other clinical strains. A low gene expression was observed for the Unique strain while the Beijing strain induced higher levels of gene expression (Chapter 3). Therefore, cytokine/chemokine production did not correlate with gene expression by the Beijing strain indicated by lower cytokine production (Chapter 2) and high gene expression (Chapter 3), suggesting the involvement of other mechanisms and interactions that result in lower cytokine production compared to other clinical strains. Furthermore, similarities were also observed between the Beijing and Unique strains to induce increased enrichment of the cholesterol pathways that have been associated with intracellular persistence of *M. tuberculosis* within host cells (Gatfield and Pieters, 2000; Martens *et al.*, 2008) compared to other clinical strains suggesting a preference for lipids as carbon and energy source by both these strains at 48 hr after infection. This hypothesis on differences in lipid preferences by clinical strains remains to be tested in future studies involving other cell types and *in vivo* infection models.

The global prevalence of the Beijing strains (Bifani *et al.*, 2002; Glynn *et al.*, 2002; Nguyen *et al.*, 2012; Streicher *et al.*, 2012) may be due to lower protective immune response induced within the host compared to other clinical strains (Manca *et al.*, 2004). Surprisingly, a similar response was induced by the less prevalent non-clustering Unique strain, which may suggest some virulence attributes in common with the Beijing strain. Further studies need to be

undertaken in order to understand similarities between these strains. Moreover, the *in vitro* (Koo *et al.*, 2012; Manca *et al.*, 2004) and *in vivo* (Manca *et al.*, 1999; Manca *et al.*, 2001; Subbian *et al.*, 2013) studies performed on the Beijing strains revealed induction of delayed TH1 response, low pro-inflammatory cytokines and high anti-inflammatory cytokine production in macrophages (Manca *et al.*, 2004). This behaviour by the Beijing strain may be attributed to the constitutive expression of the DosR regulon responsible for the synthesis of phenolic glycolipid components that have only been identified in the Beijing family (Reed *et al.*, 2004; Sinsimer *et al.*, 2008). These lipid fractions in the Beijing genotype resulted in a lower protective host response and earlier mortality in Beijing infected mice compared to those infected with the CDC1551 and laboratory strains (Manca *et al.*, 1999). This might also explain low cytokine production and enrichment of the immune associated pathways observed in pulmonary epithelial cells in our study. Collectively, these attributes may be responsible for the higher transmissibility of this hypervirulent strain.

The Beijing, F15/LAM4/KZN, F11 and F28 strains were included in this study due to their dominance within the South African population (Chihota *et al.*, 2012; Pillay and Sturm, 2007; Streicher *et al.*, 2012). Compared to the Beijing and Unique strains, the F15/LAM4/KZN, F11 and F28 strains induced higher cytokine/chemokine production, immune associated gene expression and pathway enrichment. These were pro-inflammatory and some of the anti-inflammatory cytokines as well as chemokines, transcriptional factor activation such as *NFκB* and crucial immune associated pathway enrichment that included JAK-STAT, TLR signalling, and cytokine/chemokine signalling pathways required for protective immune response (Desvignes and Ernst, 2009; Khader and Gopal, 2010; Korf *et al.*, 2009). This suggests that the transmission and persistence of F15/LAM4/KZN, F11 and F28 strains within the population may be associated with other factors such as drug resistance, including MDR and XDR, high HIV-TB co-infection rates and failure of the current control measures

and treatment strategies in South Africa, rather than hypervirulence attributes such as those identified in the Beijing strain.

This study has increased our understanding of the role of epithelial cells in the immune response to different clinical strains of *M. tuberculosis*. The abundance of these cells in the lungs ensures first encounter with *M. tuberculosis* and can orchestrate downstream changes within the lungs depending on the infecting strain. Moreover, these findings have the potential to identify useful biomarkers as targets for alternative TB therapeutics including immuno-modulators that take into consideration strain specific pathways, network regulations and strain specific molecular signatures. Several speculations and hypothesis from this study remains to be confirmed in future pathogenesis studies to get a different perspective and better understanding of our findings.

LIMITATIONS, CHALLENGES AND RECOMMENDATIONS

A major limitation in the current study was the lack of knowledge of different virulence factors harboured by the clinical strains that might explain differential cytokine production, gene expression and pathway changes in pulmonary epithelial cells. These virulence factors may range from lipid components, cellular appendages and secretory proteins that can modulate the immune response in a strain specific manner. Another limitation was the lack of literature on previous pathogenesis studies using a wide range of clinical strains that might have enabled understanding the epithelial cells cytokine/gene expression and pathway patterns observed in the current study. This was an *in vitro* study focusing only on one cell type during early infection. Due to financial and time constraints, further experiments to understand the mechanisms underlying the results obtained, could not be performed. Nor could *in vivo* studies be undertaken in order to confirm and support our *in vitro* findings, which cannot be extrapolated to patients.

Another major challenge was the extensive bioinformatics analysis of the vast amount of data generated by the RNA-Seq. The current common transcriptomic microarray technique has an optimized pipeline for analysis, unlike RNA-Seq, for which numerous tools and software packages are required, the application of which poses serious challenges for a laboratory based biologist.

Future recommendations will entail understanding the influence of strain identity in the outcome of infection in mice and human models and individuals infected with clinical *M. tuberculosis* strains of varying pathogenicity that are dominant within the South African population. This will enable a better understanding of tissue pathology, *in vivo* immune cell trafficking and granuloma formation induced in a strain specific manner. Moreover, strain-specific virulence factors that are expressed and required for pathogenesis within different infection models should be identified and characterized. This will provide better understanding of the differential and specific immune responses induced clinical strains of *M. tuberculosis* in pulmonary epithelial cells. Studies to determine differential cytokine/chemokine responses induced by these strains in humans will require well characterized biorepositories. Furthermore, the current RNA-Seq database will be analysed in greater depth to elucidate differential isoform switching and splicing events induced by these clinical strains which might have implications on the functioning of the immune system proteins.

CONCLUSION

This study provides further support that pulmonary epithelial cells are not just a passive protective barrier against *M. tuberculosis* but significantly contribute to the innate immune response through cytokine/chemokine production. These cytokines/chemokines were

differentially induced by different clinical strains of *M. tuberculosis* and can influence downstream changes due to infection by these strains. This study also reveals the in depth transcriptome analysis of pulmonary epithelial cells that had not been previously investigated despite the abundance of these cells in the alveolar space.

Differences in cytokine/chemokine production, global gene expression and pathway specific changes identified in the current study has opened doors for further investigation of associated virulence factors within these clinical strain. Furthermore, identification of strain specific molecular signatures, pathways and transcriptional factors can be explored for potential biomarker applications and host directed therapy in *in vivo* studies.

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LIST OF APPENDICES

APPENDIX A

Supplementary Table 1: The mean ± SEM of the cytokine/chemokine concentrations induced by clinical strains of *M. tuberculosis* at 0, 24, 48 and 72 hr post-infection

Cytokine	Time	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
IL1ra	0 hr	22.24±0.5	23.97±4	19.69±0.75	19.69±0.25	0.31±0.25	31.97±5.75	3.72±0.5
	24 hr	27.48 ± 2	29.26 ± 1.5	10.07±1.25	29.26 ± 2	10.07 ± 0.25	15.56±0.5	14.76 ± 0.25
	48 hr	23.10 ± 2.75	99.94 ± 2	33.80 ± 3.75	53.87 ± 4	15.56 ± 2.5	137.16±4.75	65.96 ± 2
	72 hr	42.22 ± 2	253.58 ± 32.5	117.74±3.5	47.99 ± 3.5	115.49 ± 5.5	91.23 ± 0	116.61 ± 1.25
IL-2	0 hr	3.52±3.5	4.52±3.5	3.52±3.5	3.52±3.5	3.52±3.5	3.52±3.5	3.52±3.5
	24 hr	3.50 ± 3.5	3.50 ± 0.75	3.50 ± 1.25	3.50 ± 0.5	3.50 ± 3.75	3.50 ± 0.5	3.50 ± 2.75
	48 hr	3.50 ± 2.5	15.11 ± 4.75	3.50 ± 0.5	3.52 ± 0.5	3.50 ± 2	23.43 ± 14.75	4.44 ± 4
	72 hr	3.52 ± 3.5	42.48 ± 3.5	8.34 ± 7	3.50 ± 3.25	14.64 ± 7.75	4.73 ± 4.5	9.25 ± 3.25
IL-4	0 hr	1.70±1.25	1.34±0.5	1.49±0.5	1.05±1	0.89±0.5	2.12±2	0.74±1
	24 hr	1.19 ± 0	2.05 ± 0.5	1.41 ± 0.25	1.41 ± 1.75	1.34 ± 0	2.05 ± 0.5	2.25 ± 1.75
	48 hr	1.70 ± 0.25	5.51 ± 2	3.12 ± 1.5	2.19 ± 0	1.63 ± 1	8.64 ± 5.75	6.35 ± 1.5
	72 hr	1.98 ± 0.25	12.24 ± 2.75	5.20 ± 0.25	3.89 ± 0.5	8.12 ± 1	6.95 ± 1	7.83 ± 0.25
IL-6	0 hr	1.15±0.5	1.15±1	1.15±0.5	1.15±0.5	1.15±0.5	4±1.5	1.15±0.5
	24 hr	1.15 ± 0.25	161.4 ± 22.5	627.26 ± 20.5	107.58 ± 21	530.04 ± 77	1531.01 ± 173.25	2292.69 ± 79.75
	48 hr	1.15 ± 1.25	49092.71 ± 525	11172.50 ± 406.75	1376.11 ± 644.5	31.07 ± 9.75	58780.79 ± 676.5	36098.33 ± 436.75

Cytokine	Time	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
IL-7	0 hr	2.77±0	9.93±0.5	2.77±0.25	2.77±1.5	2.77±0.5	2.77±0.75	2.77±0.5
	24 hr	14.45 ± 2.5	12.2 ± 0.5	4.19 ± 1	3.02 ± 2.5	5.35 ± 0.5	12.76 ± 1.25	17.81 ± 3
	48 hr	55.61 ± 2.25	53.99 ± 1.5	25.59 ± 2.5	23.93 ± 3.75	3.02 ± 1.5	132.38 ± 20.75	60.47 ± 1.5
	72 hr	131.33 ± 8.25	113.35 ± 12.75	49.11 ± 14.25	45.31 ± 11	143.45 ± 25.5	66.4 ± 0.25	99.55 ± 5.75
IL-8	0 hr	1.95±0.25	1.95±0.5	1.95±0.5	1.95±0.5	1.95±0.25	1.95±0.5	1.95±1.5
	24 hr	11.30 ± 2.25	97.70 ± 35.75	199.58 ± 30.5	90.06 ± 43.75	282.97 ± 147	990.44 ± 160.25	510.29 ± 66.5
	48 hr	32.63 ± 2	38972.00 ± 182.25	28854.37 ± 110.75	30717.73 ± 569.25	1.95 ± 0.5	24298.97 ± 3083.25	26667.46 ± 2684
	72 hr	77.64 ± 29.25	48804.12 ± 507.5	3100.87 ± 1120.5	29926.60 ± 642.25	32025.49 ± 2983	28469.73 ± 225.5	7500.41 ± 484.5
IL-9	0 hr	5.93±0.25	23.73±2	2.96±2.5	2.70±2	2.96±3.5	8.03±5.75	2.96±1.25
	24 hr	23.13 ± 2	39.78 ± 4	32.26 ± 1	28.58 ± 2	27.35 ± 1	36.00 ± 3	24.93 ± 5
	48 hr	31.64 ± 0.5	79.45 ± 6	44.25 ± 5	59.24 ± 2	9.38 ± 0.5	83.22 ± 20.25	46.18 ± 0
	72 hr	47.47 ± 7	162.49 ± 50	71.29 ± 0	47.79 ± 3.25	76.72 ± 9	48.44 ± 2.25	56.27 ± 5.25
IL-10	0 hr	1.5±0.5	1.5±0.25	1.5±1.25	1.5±0.25	1.5±0.25	1.5±0.25	1.5±0.5
	24 hr	3.79 ± 1.25	1.5 ± 0.5	1.5 ± 0.25	1.5 ± 0.5	1.5 ± 0.5	1.5 ± 1.01	1.5 ± 0.5
	48 hr	45.13 ± 12.25	43.27 ± 13.75	10.44 ± 5	16.63 ± 10	1.5 ± 1.25	79.1 ± 26	48.02 ± 6.5
	72 hr	86.74 ± 21.5	79.23 ± 21.5	34.74 ± 11	30.4 ± 12.25	84.9 ± 24.25	52.85 ± 20	64.81 ± 4.25
IL-12p70	0 hr	9.31±2.5	42.12±2.5	9.56±2.75	9.43±5	9.18±2.5	9.18±0.5	9.18±1.5
	24 hr	80.86 ± 17.5	64.73 ± 12.5	28.73 ± 1.25	29.76 ± 10	39.95 ± 16	72.26 ± 14.5	44.31 ± 8
	48 hr	428.69 ± 39.5	348.43 ± 27.75	116.63 ± 4.5	152.88 ± 42.75	39.95 ± 10	890.63 ± 322.5	431.22 ± 68.5
	72 hr	1090.97 ± 215	779.62 ± 165.75	295.89 ± 23	298.26 ± 107	956.58 ± 229.75	498.38 ± 28	651.17 ± 19
IL-13	0 hr	1.96±0.25	1.96±1	1.96±1	1.96±1	1.96±0.25	1.96±0.25	1.96±0.5

Cytokine	Time	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
	48 hr	39.02 ± 7.42	38.09 ± 18.38	11.71 ± 5.55	11.71 ± 4.4	1.96 ± 1.6	62.83 ± 21.77	37.92 ± 5.66
	72 hr	70.33 ± 28.24	65.09 ± 35.15	26.34 ± 11.42	33.22 ± 13.54	64.22 ± 20.51	43.98 ± 20.15	47.75 ± 19.09
IL-17	0 hr	43.09±1	42.3±1.5	46.25±2	24.81±0.5	23.21±7.5	67.52±28.5	6.25±5
	24 hr	39.52 ± 3.75	48.62 ± 4.5	39.92 ± 0.5	41.11 ± 15.25	6.66 ± 4.25	12.35 ± 3.75	12.76 ± 3
	48 hr	30.39 ± 5.5	61.23 ± 8.5	35.96 ± 3.5	48.23 ± 17.75	31.58 ± 2.75	37.15 ± 0.75	6.25 ± 5
	72 hr	35.16 ± 3	116.34 ± 33.75	109.73 ± 19	56.51 ± 21.5	47.83 ± 4	36.75 ± 1	37.15 ± 5.75
Eotaxin	0 hr	10.74±6.75	9.79±5	10.45±4.75	10±0.5	9.79±1.5	9.88±5.75	9.78±4
	24 hr	8.79 ± 1.5	40.52 ± 2.25	8.79 ± 4	26.68 ± 4.5	8.79 ± 5.75	8.79 ± 2.5	8.79 ± 6
	48 hr	8.79 ± 3.75	162.46 ± 5.25	8.79 ± 4.5	84.07 ± 3.25	8.79 ± 1.25	167.76 ± 13.75	8.79 ± 3.25
	72 hr	68.74 ± 5	256.40 ± 36	160.55 ± 1.25	8.79 ± 1.5	117.2 ± 11.5	8.79 ± 0.5	48.75 ± 5.5
G-CSF	0 hr	8.8±1.5	8.8±0.25	8.8±1.5	8.8±1	8.8±0.75	8.8±2	8.8±0.75
	24 hr	3.54 ± 0.5	221.42 ± 0	421.54 ± 0	121.54 ± 0	289.54 ± 1.5	1107.01 ± 12	2090.42 ± 0.5
	48 hr	7.54 ± 1	4454.84 ± 27	6900.56 ± 0	547.71 ± 11.75	5.54 ± 1.5	32735.56 ± 1660.5	17522.52 ± 651.75
	72 hr	23.54 ± 3	21460 ± 279	250.64 ± 131.5	860.92 ± 17.25	10963.86 ± 460	1527.8 ± 92.5	1309.98 ± 160.75
GM-CSF	0 hr	13.68±3.5	27.40±6	8.31±2.5	14.85±4	7.29±3.5	8.64±4	7.29±5.5
	24 hr	2.45 ± 2.25	4.07 ± 4	5.19 ± 4	2.62 ± 2.30	9.98 ± 7.25	20.99 ± 5.5	36.78 ± 10
	48 hr	3.30 ± 2.00	180.40 ± 30.75	27.90 ± 12	22.22 ± 12.5	134.97 ± 6.5	735.16 ± 10.75	190.98 ± 34.75
	72 hr	42.19 ± 15.25	818.17 ± 230.5	69.70 ± 26.5	7.29 ± 2	219.36 ± 26.5	0.98 ± 1.25	397.07 ± 0
IFN-γ	0 hr	0.03±0	28.08±0.5	30.96±0.75	1.25±0.15	2.5±1.5	3±0.5	8.69±1.5
	24 hr	3.63 ± 0.5	48.35 ± 0.25	62.96 ± 0.5	33.84 ± 0.5	11.00 ± 2	80.60 ± 0.5	54.18 ± 1.75
	48 hr	57.99 ± 1.5	376.48 ± 0.5	140.03 ± 0.5	122.12 ± 0.5	16.65 ± 0.5	747.38 ± 22.5	440.92 ± 5.75

Cytokine	Time	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
IP-10	0 hr	10.13±0.5	19.37±0.5	10.13±0.5	10.13±1.5	10.13±2	9.91±2.25	10.13±0.25
	24 hr	4.11 ± 1	95.85 ± 2	62.94 ± 0.75	8.90 ± 0.5	279.08 ± 1.75	795.9 ± 3.25	386.16 ± 0.5
	48 hr	8.90 ± 0.25	9487.01 ± 191.5	2275.50 ± 25	235.62 ± 4	8.90 ± 0.75	9464.02 ± 601.75	4998.51 ± 56.5
	72 hr	28.44 ± 3	29159.77 ± 580.5	2610.58 ± 212	871.72 ± 31.25	24233.67 ± 238.25	3495.49 ± 87.5	10973.59 ± 152.25
MCP-1	0 hr	16.16±2.5	76.77±20.75	1.40±1.25	1.42±0.25	1.42±0.25	1.42±0.5	1.42±0.25
	24 hr	100.48 ± 2.5	661.86 ± 279	472.12 ± 5	327.81 ± 191	1125.35 ± 220.75	3380.68 ± 904	1493.60 ± 167.25
	48 hr	180.77 ± 58	19816.96 ± 209.25	12363.67 ± 477	11294.67 ± 2133.25	14.16 ± 3	8802.30 ± 1593.75	14353.83 ± 903.75
	72 hr	243.12 ± 68	4929.45 ± 131.75	11943.59 ± 382.5	10205.41 ± 239.5	14688.81 ± 166.5	7747.75 ± 186.5	5014.35 ± 379.75
MIP-1α	0 hr	1.34±1	1.34±0.5	1.34±0.25	1.34±0.5	1.34±1	1.34±1.25	1.34±0.5
	24 hr	3.33 ± 1.75	3.33 ± 1	8.44 ± 1.5	3.33 ± 1.5	3.33 ± 2	10.21 ± 0.5	45.88 ± 2.5
	48 hr	7.33 ± 0.5	76.48 ± 6.5	70.22 ± 11.75	12.88 ± 0.75	45.56 ± 0	189.12 ± 21.1	288.75 ± 39
	72 hr	9.33 ± 2.5	511.88 ± 109.5	14.33 ± 2.5	13.33 ± 0.75	141.46 ± 61.5	238.12 ± 64.5	390.56 ± 40.5
PDGF-bb	0 hr	1.15±0.5	6.80±1	1.15±1.25	1.15±0.5	1.15±0.25	1.15±1	1.15±0.25
	24 hr	0.14 ± 1	6.80 ± 0	4.94 ± 0.5	0.14 ± 1	0.14 ± 0.5	7.72 ± 1.25	0.14 ± 0
	48 hr	0.14 ± 1.75	55.15 ± 0.75	19.43 ± 0.5	30.90 ± 0.75	19.43 ± 1.5	106.87 ± 11	45.68 ± 0
	72 hr	31.77 ± 2	101.83 ± 12.5	153.59 ± 16	26.51 ± 4.5	83.27 ± 8	95.10 ± 2.5	81.58 ± 2.5
MIP-1β	0 hr	1.67±0.75	1.67±0.5	1.67±0.75	1.67±0.25	1.67±1	1.67±1.25	1.67±0.5
	24 hr	6.50 ± 0.25	6.50 ± 3.5	10.98 ± 1.5	3.11 ± 3	18.34 ± 2	32.15 ± 3	77.81 ± 2.75
	48 hr	6.44 ± 3	78.02 ± 1.75	64.19 ± 1	10.54 ± 3.25	5.88 ± 2.5	55.11 ± 12.5	96.77 ± 24
	72 hr	6.50 ± 6	125.45 ± 24.5	6.50 ± 6	6.50 ± 2.25	13.96 ± 7.25	6.50 ± 2.5	10.13 ± 1.75
Rantes	0 hr	19.01±2.75	19.18±1	19.88±1	18.30±2.75	19.53±0.75	19.53±1.5	17.41±1.5

Cytokine	Time	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
	48 hr	8.83 ± 0.5	739.56 ± 1.5	425.05 ± 0.25	40.40 ± 0	6.86 ± 0.75	2415.47 ± 557	1236.08 ± 392.5
	72 hr	22.93 ± 2.5	347.27 ± 35.75	29.61 ± 6	23.59 ± 2.25	507.54 ± 92.25	148.19 ± 34	287.24 ± 66.5
TNF-α	0 hr	4.22±0.75	4.60±1.5	10.76±2.5	1.96±0.25	1.59±0.5	1.59±1.25	1.59±0.25
	24 hr	2.71 ± 1.25	4.60 ± 0.75	5.36 ± 3	4.60 ± 4.5	4.60 ± 0.5	19.59 ± 0.5	36.84 ± 2
	48 hr	1.33 ± 1.5	201.11 ± 5.5	30.87 ± 0.5	4.84 ± 2	2.60 ± 1.5	456.28 ± 10.75	98.65 ± 0.5
	72 hr	6.90 ± 1	520.77 ± 31.5	42.45 ± 9.5	13.09 ± 3	258.58 ± 6.5	353.73 ± 0.5	45.67 ± 0.5
VEGF	0 hr	50.75±6.75	252.83±21.5	8.23±5.5	8.23±1.5	8.23±6.5	36.38±5.31	8.23±4
	24 hr	645.08 ± 31.5	494.67 ± 59	256.21 ± 1.25	231.98 ± 77.75	343.74 ± 19.5	447.18 ± 45.75	368.47 ± 26.25
	48 hr	4891.7 ± 85.75	3508.1 ± 22.75	1067.04 ± 59.5	1116.48 ± 286.25	447.48 ± 33.5	10559.76 ± 746	3944.56 ± 2
	72 hr	22110.69 ± 664	12974.23 ± 438.25	3844.29 ± 1303.75	3719.82 ± 917.5	9593.78 ± 156.75	8315.84 ± 140	9294.87 ± 50.5

Supplementary Table 2: Fold changes of 23 analytes detected by Bio-plex system

	M. tuberculosis F15/LAM4/KZN strain			*M. tuberculosis* Beijing strain			*M. tuberculosis* F11 strain			*M. tuberculosis* F28 strain			*M. tuberculosis* Unique strain		
	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
IL1ra	1.06	4.33	3	0.37	1.46	2.79	0.37	0.67	2.74	0.57	5.94	2.16	1.06	2.33	1.14
IL-2	1	4.31	12.07	1	1	2.37	1	1	4.16	1	6.69	0.57	1	1	1
IL-4	1.72	3.24	6.18	1.18	1.84	2.23	1.13	0.96	4.1	1.72	5.08	3.51	1.18	1.29	1.96
IL-6	140.34	42688.69	3038.5	545.44	9715.22	537.56	460.9	27.01	2623.14	1331.31	51113.73	3702.14	93.55	1196.62	215.29
IL-7	0.84	0.97	0.86	0.29	0.46	0.37	0.37	0.05	1.09	0.88	2.38	0.51	0.21	0.43	0.35
IL-8	8.64	1194.36	628.6	17.66	884.29	39.94	25.04	0.06	412.49	87.65	744.68	366.69	7.97	941.4	385.45
IL-9	1.72	2.51	3.42	1.39	1.4	1.5	1.18	0.3	1.62	1.56	2.63	1.02	1.24	1.87	1.01
IL-10	0.4	0.96	0.91	0.4	0.23	0.4	0.4	0.03	0.98	0.4	1.75	0.61	0.4	0.37	0.35
IL-12p70	0.8	0.81	0.71	0.36	0.27	0.27	0.49	0.09	0.88	0.89	2.08	0.46	0.37	0.36	0.27
IL-13	1	0.98	0.93	1	0.3	0.37	1	0.05	0.91	1	1.61	0.63	1	0.3	0.47
IL-17	1.23	2.01	3.31	1.01	1.18	3.12	0.17	1.03	1.36	0.31	1.22	1.04	1.04	1.59	1.61
Eotaxin	4.61	18.48	3.73	1	1	2.33	1	1	1.7	1	1	0.12	3.03	9.56	0.13
G-CSF	62.54	590.83	911.64	119.08	915.19	10.65	81.79	0.73	465.75	312.71	4341.59	64.9	34.33	72.64	36.57
GM-CSF	1.66	54.67	19.39	2.12	8.45	1.65	4.07	40.9	5.2	8.57	222.78	0.02	1.07	6.73	0.17
IFN-γ	13.32	6.49	10.84	17.34	2.41	4.65	3.03	0.29	0.83	22.2	12.89	5.34	9.32	2.11	2.78
IP-10	23.32	1065.96	1025.31	15.31	255.67	91.79	67.9	1	852.1	193.65	1063.37	122.91	2.17	26.47	30.65
MCP-1	6.59	109.63	20.28	4.7	68.39	49.13	11.2	0.08	60.42	33.65	48.69	31.87	3.26	62.48	41.98
MIP-1α	1	10.43	54.86	2.53	9.58	1.54	1	6.22	15.16	3.06	25.8	25.52	1	1.76	1.43
PDGF-bb	48.57	393.93	3.2	35.29	138.79	4.83	1	138.79	2.62	55.14	763.21	2.99	1	220.71	0.83
MIP-1β	1	12.11	19.3	1.7	9.97	1	2.82	0.91	2.15	4.94	8.56	1	0.48	1.64	1
Rantes	3.5	83.76	15.14	6.45	48.14	1.29	3.27	0.78	22.13	7.15	273.55	6.46	3.5	4.58	1.03
TNF-α	1.7	151.21	75.47	1.98	23.21	6.15	1.7	1.95	37.48	7.23	343.07	51.27	1.7	3.64	1.9
VEGF	0.77	0.72	0.59	0.4	0.22	0.17	0.53	0.09	0.43	0.69	2.16	0.38	0.36	0.23	0.17

Supplementary Table 3: Statistical analysis between uninfected and infected epithelial cells and among clinical strains for 23 analytes

IL-1ra

IL-1ra: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected	1	0.5499	0.0179	0.5931	0.0131	0.0286	0.0242
F15/LAM4/KZN		1	0.0102	1.0000	0.0062	0.0131	0.0108
Beijing			1	0.0148	1.0000	0.0552	0.0668
Unique				1	0.0109	0.0219	0.0188
F11					1	0.0102	0.0057
F28						1	0.2844
H37Rv							1

IL-1ra: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0020	0.1481	0.0240	0.1796	0.0023	0.0062
F15/LAM4/KZN			0.0041	0.0093	0.0014	0.0186	0.0069
Beijing				0.0672	0.0560	0.0034	0.0170
Unique					0.0148	0.0055	0.1139
F11						0.0019	0.0040
F28							0.0052
H37Rv							

IL-1ra: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0229	0.0028	0.2886	0.0063	0.0018	0.0010
F15/LAM4/KZN			0.0533	0.0244	0.0525	0.0378	0.0520
Beijing				0.0050	0.7629	0.0173	0.7898
Unique					0.0092	0.0066	0.0029
F11						0.0481	0.8610
F28							0.0028
H37Rv							

IL-2

IL-2: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected	1	1	1	1	1	1	1
F15/LAM4/KZN		1	1	1	1	1	1
Beijing			1	1	1	1	1
Unique				1	1	1	1
F11					1	1	1
F28						1	1
H37Rv							1

IL-2: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.1630	0.7327	0.9945	1.0000	0.3143	0.8605
F15/LAM4/KZN			0.1185	0.1360	0.1531	0.6451	0.2279
Beijing				0.2859	0.6756	0.2919	0.6778
Unique					0.9931	0.3098	0.8407
F11						0.3125	0.8530
F28							0.3400
H37Rv							

IL-2: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0158	0.6007	0.9970	0.3211	0.8516	0.3531
F15/LAM4/KZN			0.0487	0.0147	0.0820	0.0221	0.0200
Beijing				0.5946	0.6076	0.7067	0.9169
Unique					0.3161	0.8452	0.3374
F11						0.3840	0.5870
F28							0.5010
H37Rv							

IL-4

IL-4: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.3480	0.7319	0.9148	0.8517	0.3480	0.6192
F15/LAM4/KZN			0.3708	0.7587	0.4211	1.0000	0.9225
Beijing				1.0000	0.9118	0.3708	0.6815
Unique					0.9728	0.7587	0.7666
F11						0.4211	0.6667
F28							0.9225
H37Rv							

IL-4: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.1993	0.4490	0.4732	0.9520	0.3512	0.0924
F15/LAM4/KZN			0.4400	0.2486	0.2248	0.6583	0.7688
Beijing				0.6160	0.4954	0.4510	0.2673
Unique					0.6661	0.3800	0.1192
F11						0.3527	0.1201
F28							0.7371
H37Rv							

IL-4: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0654	0.0118	0.0760	0.0270	0.0404	0.0036
F15/LAM4/KZN			0.1255	0.0962	0.2945	0.2124	0.2513
Beijing				0.1438	0.1053	0.2317	0.0176
Unique					0.0633	0.1116	0.0195
F11						0.4951	0.8049
F28							0.4832
H37Rv							

IL-6

IL-6: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0192	0.0011	0.0368	0.0205	0.0126	0.0012
F15/LAM4/KZN			0.0042	0.2225	0.0442	0.0159	0.0015
Beijing				0.0032	0.3468	0.0353	0.0024
Unique					0.0339	0.0147	0.0014
F11						0.0341	0.0039
F28							0.0574
H37Rv							

IL-6: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0001	0.0013	0.1665	0.0931	0.0001	0.0001
F15/LAM4/KZN			0.0003	0.0003	0.0001	0.0077	0.0028
Beijing				0.0060	0.0013	0.0003	0.0006
Unique					0.1722	0.0003	0.0005
F11						0.0001	0.0001
F28							0.0013
H37Rv							

IL-6: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0042	0.0006	0.0220	0.0001	P<0.0001	P<0.0001
F15/LAM4/KZN			0.0062	0.0050	0.1737	0.0803	0.1654
Beijing				0.0116	0.0002	0.0001	P<0.0001
Unique					0.0003	0.0002	P<0.0001
F11						0.0015	0.0012
F28							0.0155
H37Rv							

IL-7

IL-7: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.4706	0.0625	0.0838	0.0703	0.6069	0.4802
F15/LAM4/KZN			0.0189	0.0692	0.0105	0.7178	0.2064
Beijing				0.7063	0.4085	0.0332	0.0499
Unique					0.4572	0.0734	0.0632
F11						0.0315	0.0547
F28							0.2604
H37Rv							

IL-7: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.6099	0.0123	0.0185	0.0026	0.0666	0.2141
F15/LAM4/KZN			0.0104	0.0176	0.0017	0.0638	0.0925
Beijing				0.7480	0.0163	0.0362	0.0069
Unique					0.0353	0.0358	0.0120
F11						0.0249	0.0014
F28							0.0745
H37Rv							

IL-7: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.3581	0.0378	0.0246	0.6954	0.0158	0.0872
F15/LAM4/KZN			0.0783	0.0561	0.4018	0.0665	0.4278
Beijing				0.8524	0.0840	0.3489	0.0816
Unique					0.0716	0.1953	0.0486
F11						0.0943	0.2351
F28							0.0288
H37Rv							

IL-8

IL-8: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0323	0.0254	0.2140	0.2059	0.0258	0.0173
F15/LAM4/KZN			0.0972	0.8846	0.3368	0.0310	0.0264
Beijing				0.1764	0.6344	0.0400	0.0512
Unique					0.3354	0.0324	0.0341
F11						0.0829	0.2942
F28							0.1095
H37Rv							

IL-8: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		P<0.0001	P<0.0001	0.0003	0.0045	0.0158	0.0100
F15/LAM4/KZN			0.0004	0.0052	P<0.0001	0.0416	0.0446
Beijing				0.0847	P<0.0001	0.2778	0.5011
Unique					0.0003	0.1772	0.2779
F11						0.0157	0.0100
F28							0.6209
H37Rv							

IL-8: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0001	0.1144	0.0005	0.0086	P<0.0001	0.0042
F15/LAM4/KZN			0.0007	0.0019	0.0310	0.0007	0.0003
Beijing				0.0023	0.0119	0.0020	0.0691
Unique					0.5626	0.1657	0.0013
F11						0.3566	0.0148
F28							0.0006
H37Rv							

IL-9

IL-9: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0652	0.0551	0.1938	0.1998	0.0703	0.7700
F15/LAM4/KZN			0.2097	0.1292	0.0947	0.5286	0.1462
Beijing				0.2416	0.0739	0.3585	0.2871
Unique					0.6375	0.1758	0.5678
F11						0.1117	0.6818
F28							0.1980
H37Rv							

IL-9: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0155	0.1288	0.0055	0.0010	0.1258	0.0024
F15/LAM4/KZN			0.0459	0.0856	0.0073	0.8748	0.0312
Beijing				0.1085	0.0201	0.2027	0.7379
Unique					0.0017	0.3598	0.0240
F11						0.0677	0.0004
F28							0.2090
H37Rv							

IL-9: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.1504	0.0769	0.9707	0.1243	0.9071	0.4205
F15/LAM4/KZN			0.2097	0.1493	0.2334	0.1503	0.1690
Beijing				0.0190	0.6081	0.0100	0.1043
Unique					0.0942	0.8845	0.3033
F11						0.0929	0.1887
F28							0.3040
H37Rv							

IL-10

IL-10: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.2311	0.2143	0.2311	0.2311	0.2902	0.2311
F15/LAM4/KZN			1	1	1	1	1
Beijing				1	1	1	1
Unique					1	1	1
F11						1	1
F28							1
H37Rv							

IL-10: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.9288	0.1199	0.2133	0.0712	0.3587	0.8542
F15/LAM4/KZN			0.1540	0.2577	0.0941	0.3473	0.7844
Beijing				0.6355	0.2249	0.1221	0.0445
Unique					0.2721	0.1541	0.1191
F11						0.0965	0.0197
F28							0.3659
H37Rv							

IL-10: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.8280	0.1642	0.1505	0.9599	0.3677	0.4224
F15/LAM4/KZN			0.2068	0.1872	0.8772	0.4638	0.5782
Beijing				0.8168	0.2003	0.5107	0.1255
Unique					0.1827	0.4395	0.1175
F11						0.4152	0.5002
F28							0.6178
H37Rv							

IL-12p70

IL-12p70-24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.5315	0.0971	0.1267	0.2266	0.7415	0.1979
F15/LAM4/KZN			0.1033	0.1605	0.3467	0.7320	0.3027
Beijing				0.9279	0.5568	0.0960	0.1942
Unique					0.6432	0.1373	0.3737
F11						0.2732	0.8302
F28							0.2335
H37Rv							

IL-12p70: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.2383	0.0158	0.0418	0.0108	0.2910	0.9774
F15/LAM4/KZN			0.0144	0.0617	0.0090	0.2359	0.3791
Beijing				0.4878	0.0198	0.1385	0.0445
Unique					0.1237	0.1515	0.0748
F11						0.1188	0.0299
F28							0.2981
H37Rv							

IL-12p70: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.3701	0.0667	0.0808	0.7109	0.1119	0.1785
F15/LAM4/KZN			0.1017	0.1348	0.5960	0.2363	0.5219
Beijing				0.9847	0.1035	0.0306	0.0070
Unique					0.1217	0.2121	0.0832
F11						0.1863	0.3163
F28							0.0457
H37Rv							

IL-13

IL-13: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		1	1	1	1	1	1
F15/LAM4/KZN			1	1	1	1	1
Beijing				1	1	1	1
Unique					1	1	1
F11						1	1
F28							1
H37Rv							

IL-13: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.9413	0.0800	0.0870	0.0395	0.4093	0.9169
F15/LAM4/KZN			0.1013	0.1082	0.0515	0.4000	0.9881
Beijing				1.0000	0.1293	0.1464	0.0593
Unique					0.1727	0.1480	0.0673
F11						0.1081	0.0257
F28							0.3835
H37Rv							

IL-13: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.9181	0.2855	0.3578	0.8772	0.5268	0.5758
F15/LAM4/KZN			0.4044	0.4866	0.9849	0.6543	0.7069
Beijing				0.7352	0.2480	0.5258	0.4374
Unique					0.3344	0.7009	0.5980
F11						0.5544	0.6162
F28							0.9044
H37Rv							

IL-17

IL-17: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.2605	0.9254	0.9286	0.0285	0.0361	0.0307
F15/LAM4/KZN			0.1946	0.6832	0.0211	0.0251	0.0220
Beijing				0.9449	0.0162	0.0183	0.0123
Unique					0.1615	0.2085	0.2097
F11						0.4212	0.3617
F28							0.9397
H37Rv							

IL-17: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0930	0.4829	0.4383	0.8644	0.3475	0.0832
F15/LAM4/KZN			0.1108	0.5768	0.0800	0.1060	0.0307
Beijing				0.5676	0.4288	0.7712	0.0397
Unique					0.4518	0.5965	0.1506
F11						0.1899	0.0472
F28							0.0257
H37Rv							

IL-17: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.1388	0.0606	0.4291	0.1268	0.6650	0.7880
F15/LAM4/KZN			0.8802	0.2735	0.1814	0.1425	0.1468
Beijing				0.2048	0.0859	0.0617	0.0673
Unique					0.7298	0.4555	0.4761
F11						0.1151	0.2668
F28							0.9516
H37Rv							

EOTAXIN

Eotaxin-24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0072	1.0000	0.0637	1.0000	1.0000	1.0000
F15/LAM4/KZN			0.0203	0.1106	0.0358	0.0111	0.0384
Beijing				0.0971	1.0000	1.0000	1.0000
Unique					0.1339	0.0738	0.1398
F11						1.0000	1.0000
F28							1.0000
H37Rv							

Eotaxin: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0018	1.0000	0.0043	1.0000	0.0079	1.0000
F15/LAM4/KZN			0.0020	0.0061	0.0012	0.7532	0.0016
Beijing				0.0054	1.0000	0.0082	1.0000
Unique					0.0021	0.0273	0.0037
F11						0.0075	1.0000
F28							0.0078
H37Rv							

Eotaxin: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0355	0.0031	0.0075	0.0070	0.0609	0.1149
F15/LAM4/KZN			0.1170	0.0205	0.0664	0.0205	0.0294
Beijing				0.0002	0.0644	P<0.0001	0.0025
Unique					0.0113	1.0000	0.0198
F11						0.0111	0.0330
F28							0.0186
H37Rv							

G-CSF

G-CSF: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		P<0.0001	P<0.0001	P<0.0001	P<0.0001	0.0001	P<0.0001
F15/LAM4/KZN			P<0.0001	P<0.0001	0.0005	0.0002	P<0.0001
Beijing				P<0.0001	0.0001	0.0003	P<0.0001
Unique					P<0.0001	0.0001	P<0.0001
F11						0.0002	P<0.0001
F28							0.0001
H37Rv							

G-CSF: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		P<0.0001	P<0.0001	0.0005	0.3828	0.0026	0.0014
F15/LAM4/KZN			0.0001	P<0.0001	P<0.0001	0.0034	0.0025
Beijing				P<0.0001	P<0.0001	0.0041	0.0037
Unique					0.0005	0.0027	0.0015
F11						0.0026	0.0014
F28							0.0135
H37Rv							

G-CSF: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0002	0.2264	0.0004	0.0018	0.0038	0.0153
F15/LAM4/KZN			0.0002	0.0002	0.0026	0.0002	0.0003
Beijing				0.0441	0.0020	0.0155	0.0364
Unique					0.0021	0.0193	0.1089
F11						0.0025	0.0025
F28							0.3611
H37Rv							

GM-CSF

GM-CSF: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.7578	0.6111	0.9627	0.4258	0.0892	0.0788
F15/LAM4/KZN			0.8614	0.7831	0.5494	0.1306	0.0935
Beijing				0.6335	0.6214	0.1458	0.0992
Unique					0.4353	0.0911	0.0796
F11						0.3499	0.1622
F28							0.3007
H37Rv							

GM-CSF: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0290	0.1805	0.2736	0.0027	0.0002	0.0327
F15/LAM4/KZN			0.0438	0.0413	0.2852	0.0034	0.8408
Beijing				0.7742	0.0159	0.0005	0.0472
Unique					0.0153	0.0005	0.0447
F11						0.0004	0.2540
F28							0.0044
H37Rv							

GM-CSF: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0275	0.4632	0.1513	0.0285	0.1146	0.0018
F15/LAM4/KZN			0.0302	0.0249	0.0461	0.0246	0.0841
Beijing				0.1433	0.0574	0.1223	0.0065
Unique					0.0153	0.1159	P<0.0001
F11						0.0144	0.0215
F28							P<0.0001
H37Rv							

IFN-γ

IFN-γ: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0002	0.0001	0.0005	0.0701	P<0.0001	0.0013
F15/LAM4/KZN			0.0015	0.0015	0.0029	0.0003	0.0809
Beijing				0.0006	0.0016	0.0016	0.0404
Unique					0.0080	0.0002	0.0079
F11						0.0009	0.0038
F28							0.0047
H37Rv							

IFN-γ: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		P<0.0001	0.0004	0.0006	0.0015	0.0011	0.0002
F15/LAM4/KZN			P<0.0001	P<0.0001	P<0.0001	0.0037	0.0079
Beijing				0.0016	P<0.0001	0.0014	0.0004
Unique					P<0.0001	0.0013	0.0003
F11						0.0009	0.0002
F28							0.0057
H37Rv							

IFN-γ: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0008	0.0012	0.0063	0.4852	0.0005	0.0005
F15/LAM4/KZN			0.0020	0.0012	0.0010	0.0023	0.0047
Beijing				0.0044	0.0025	0.0107	0.0026
Unique					0.0109	0.0015	0.0010
F11						0.0016	0.0011
F28							0.0031
H37Rv							

IP-10

Uninfected	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
F15/LAM4/KZN		0.0006	0.0005	0.0504	P<0.0001	P<0.0001	P<0.0001
Beijing			0.0042	0.0006	0.0002	P<0.0001	P<0.0001
Unique				0.0003	P<0.0001	P<0.0001	P<0.0001
F11					P<0.0001	P<0.0001	P<0.0001
F28						P<0.0001	0.0003
H37Rv							P<0.0001
Uninfected							

IP-10: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0004	0.0001	0.0003	1.0000	0.0040	0.0001
F15/LAM4/KZN			0.0007	0.0004	0.0004	0.9743	0.0020
Beijing				0.0002	0.0001	0.0069	0.0005
Unique					0.0003	0.0042	0.0001
F11						0.0040	0.0001
F28							0.0178
H37Rv							

IP-10: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0004	0.0067	0.0014	P<0.0001	0.0006	0.0002
F15/LAM4/KZN			0.0005	0.0004	0.0158	0.0005	0.0011
Beijing				0.0148	0.0002	0.0611	0.0010
Unique					0.0001	0.0013	0.0002
F11						0.0001	0.0005
F28							
H37Rv							

MCP-1

MCP-1: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.1819	0.0002	0.3561	0.0434	0.0683	0.0141
F15/LAM4/KZN			0.5667	0.4273	0.3225	0.1028	0.1249
Beijing				0.5289	0.0978	0.0845	0.0258
Unique					0.1119	0.0807	0.0443
F11						0.1363	0.3150
F28							0.1765
H37Rv							

MCP-1: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0001	0.0016	0.0349	0.1031	0.0326	0.0041
F15/LAM4/KZN			0.0048	0.0578	0.0001	0.0206	0.0276
Beijing				0.6732	0.0015	0.1656	0.1908
Unique					0.0340	0.4481	0.3175
F11						0.0314	0.0039
F28							0.0938
H37Rv							

MCP-1: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0010	0.0011	0.0006	0.0002	0.0007	0.0065
F15/LAM4/KZN			0.0033	0.0027	0.0005	0.0065	0.8523
Beijing				0.0613	0.0223	0.0101	0.0060
Unique					0.0042	0.0149	0.0074
F11						0.0013	0.0018
F28							0.0231
H37Rv							

MIP-1α

MIP-1α: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		1.0000	0.1569	1.0000	1.0000	0.0634	0.0051
F15/LAM4/KZN			0.1052	1.0000	1.0000	0.0254	0.0040
Beijing				0.1376	0.1776	0.3793	0.0060
Unique					1.0000	0.0490	0.0047
F11						0.0793	0.0056
F28							0.0051
H37Rv							

MIP-1α: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0088	0.0332	0.0254	0.0003	0.0132	0.0187
F15/LAM4/KZN			0.6869	0.0104	0.0417	0.0363	0.0330
Beijing				0.0397	0.1709	0.0389	0.0330
Unique					0.0008	0.0140	0.0194
F11						0.0209	0.0248
F28							0.1537
H37Rv							

MIP-1α: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0444	0.2929	0.2651	0.1649	0.0712	0.0111
F15/LAM4/KZN			0.0452	0.0450	0.0983	0.1640	0.4079
Beijing				0.7385	0.1749	0.0741	0.0114
Unique					0.1726	0.0734	0.0113
F11						0.3914	0.0774
F28							0.1833
H37Rv							

PDGF-bb

PDGF-bb: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0270	0.0502	1.0000	1.0000	0.0418	1.0000
F15/LAM4/KZN			0.1192	0.0270	0.0111	0.5649	0.0111
Beijing				0.0502	0.0210	0.1749	0.0210
Unique					1.0000	0.0418	1.0000
F11						0.0301	1.0000
F28							0.0301
H37Rv							

PDGF-bb: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0012	0.0088	0.0038	0.0140	0.0107	0.0016
F15/LAM4/KZN			0.0006	0.0019	0.0022	0.0426	0.0089
Beijing				0.0061	1.0000	0.0155	0.0007
Unique					0.0207	0.0204	0.0037
F11						0.0157	0.0036
F28							0.0309
H37Rv							

PDGF-bb: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0311	0.0171	0.3973	0.0247	0.0025	0.0041
F15/LAM4/KZN			0.1255	0.0297	0.3376	0.6503	0.2531
Beijing				0.0167	0.0590	0.0688	0.0470
Unique					0.0252	0.0056	0.0086
F11						0.2936	0.8589
F28							0.0621
H37Rv							

MIP-1β

MIP-1β: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		1.0000	0.0985	0.3771	0.0278	0.0135	0.0015
F15/LAM4/KZN			0.3605	0.5386	0.0990	0.0308	0.0039
Beijing				0.1435	0.0986	0.0242	0.0022
Unique					0.0517	0.0207	0.0030
F11						0.0619	0.0033
F28							0.0079
H37Rv							

MIP-1β: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0023	0.0030	0.4518	0.8991	0.0632	0.0648
F15/LAM4/KZN			0.0206	0.0030	0.0018	0.2112	0.5174
Beijing				0.0040	0.0021	0.5443	0.3078
Unique					0.3736	0.0747	0.0706
F11						0.0610	0.0638
F28							0.2636
H37Rv							

MIP-1β: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0421	1.0000	1.0000	0.5110	1.0000	0.6201
F15/LAM4/KZN			0.0421	0.0402	0.0487	0.0403	0.0425
Beijing				1.0000	0.5110	1.0000	0.6201
Unique					0.4294	1.0000	0.3308
F11						0.4333	0.6587
F28							0.3563
H37Rv							

Rantes

Rantes: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0256	0.0062	0.0564	0.0263	0.0070	0.0005
F15/LAM4/KZN			0.0096	1.0000	0.3829	0.0123	0.0003
Beijing				0.0331	0.0059	0.2467	0.0005
Unique					0.7007	0.0277	0.0010
F11						0.0093	0.0002
F28							0.0010
H37Rv							

Rantes: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		P<0.0001	P<0.0001	0.0005	0.1604	0.0496	0.0237
F15/LAM4/KZN			P<0.0001	P<0.0001	P<0.0001	0.0950	0.1232
Beijing				P<0.0001	P<0.0001	0.0702	0.0520
Unique					0.0007	0.0508	0.0250
F11						0.0495	0.0237
F28							0.1833
H37Rv							

Rantes: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0033	0.4121	0.8626	0.0215	0.0352	0.0297
F15/LAM4/KZN			0.0034	0.0033	0.2252	0.0074	0.0255
Beijing				0.4467	0.0222	0.0409	0.0316
Unique					0.0216	0.0354	0.0298
F11						0.0421	0.1244
F28							0.1172
H37Rv							

TNF-α

TNF-α: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.3242	0.5005	0.7249	0.2955	0.0063	0.0047
F15/LAM4/KZN			0.8288	1.0000	1.0000	0.0036	0.0044
Beijing				0.9011	0.8260	0.0428	0.0129
Unique					1.0000	0.0804	0.0225
F11						0.0022	0.0041
F28							0.0140
H37Rv							

TNF-α: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0008	0.0029	0.2955	0.6102	0.0006	0.0003
F15/LAM4/KZN			0.0011	0.0009	0.0008	0.0022	0.0029
Beijing				0.0062	0.0031	0.0006	0.0001
Unique					0.4648	0.0006	0.0005
F11						0.0006	0.0003
F28							0.0009
H37Rv							

TNF-α: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0037	0.0652	0.1894	0.0007	P<0.0001	0.0008
F15/LAM4/KZN			0.0047	0.0039	0.0147	0.0338	0.0044
Beijing				0.0984	0.0028	0.0009	0.7672
Unique					0.0008	P<0.0001	0.0086
F11						0.0047	0.0009
F28							P<0.0001
H37Rv							

VEGF

VEGF: 24 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.1535	0.0065	0.0389	0.0148	0.0705	0.0213
F15/LAM4/KZN			0.0561	0.1148	0.1358	0.5898	0.1899
Beijing				0.7848	0.0465	0.0529	0.0507
Unique					0.2979	0.1398	0.2382
F11						0.1730	0.5284
F28							0.2742
H37Rv							

VEGF: 48 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0041	0.0007	0.0062	0.0004	0.0171	0.0081
F15/LAM4/KZN			0.0007	0.0141	0.0002	0.0110	0.0027
Beijing				0.8813	0.0119	0.0062	0.0004
Unique					0.1460	0.0071	0.0101
F11						0.0054	P<0.0001
F28							0.0125
H37Rv							

VEGF: 72 hr	Uninfected	F15/LAM4/KZN	Beijing	Unique	F11	F28	H37Rv
Uninfected		0.0075	0.0064	0.0038	0.0030	0.0024	0.0027
F15/LAM4/KZN			0.0220	0.0119	0.0184	0.0096	0.0141
Beijing				0.9449	0.0484	0.0763	0.0528
Unique					0.0242	0.0384	0.0261
F11						0.0260	0.2112
F28							0.0223
H37Rv							

Supplementary Table 4: Colony forming units obtained from infected pulmonary epithelial cells by clinical strains of *M. tuberculosis*.

	24 hr ($x10^6$)			48 hr ($x10^6$)			72 hr ($x10^6$)		
F15/LAM4/KZN	1.56	1.66	1.58	2.64	2.77	2.65	2.44	2.37	2.55
F11	0.3	0.3	0.3	16	13	14	0.24	0.27	0.23
F28	0.7	0.4	0.2	13	10	9.4	10	13	9.9
Beijing	3.7	3.7	4.6	2.7	2.6	3.6	0.03	0.026	0.042
Unique	0.98	0.8	1.1	5.6	5.3	4.8	2.2	2	1.9
H37Rv	0.2	0.2	0.4	1.7	1.6	1.9	0.2	0.1	0.3

Supplementary table 5: Statistical analysis of the cfu/ml for clinical strains of *M. tuberculosis* at 24, 48 and 72 hr post-infection

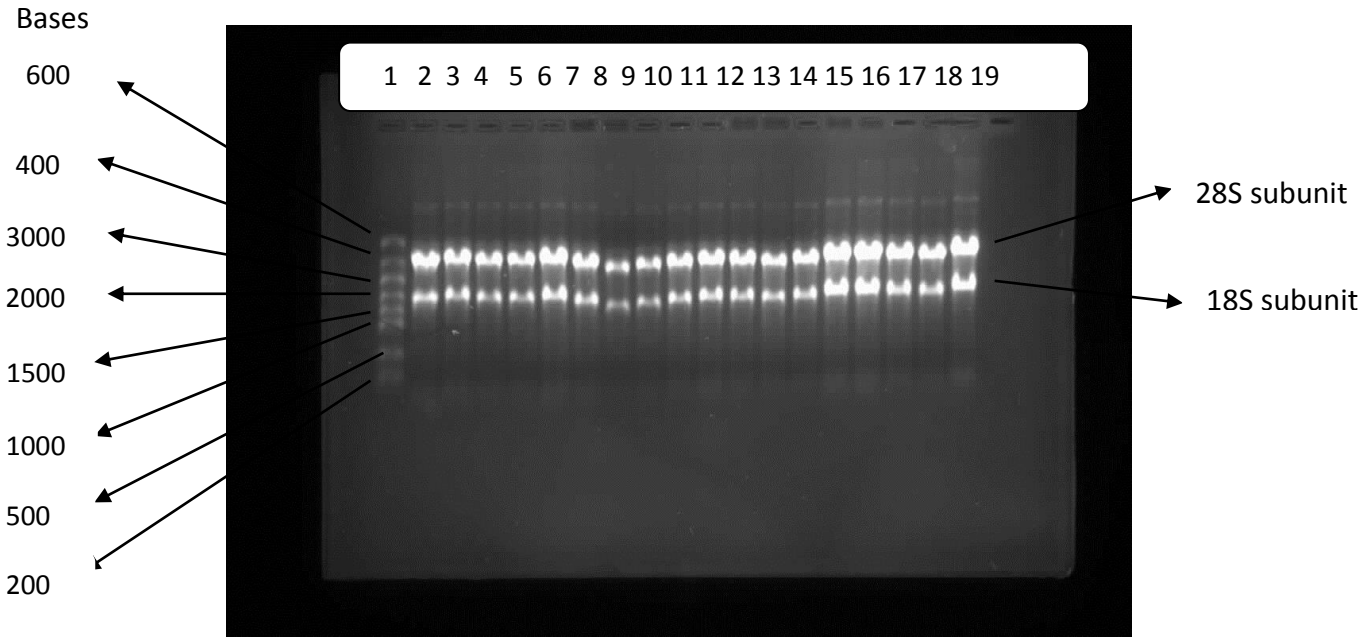
24 hr	F15/LAM4/KZN	Beijing	F11	F28	Unique	H37Rv
F15/LAM4/KZN	1	0.0164	0.0017	0.0177	0.0302	0.0038
Beijing		1	0.0052	0.0139	0.0060	0.0039
F11			1	0.6220	0.0101	0.1835
F28				1	0.1091	0.4975
Unique					1	0.0056
H37Rv						1

48 hr	F15/LAM4/KZN	Beijing	F11	F28	Unique	H37Rv
F15/LAM4/KZN	1	0.4985	0.0061	0.0189	0.0083	0.0158
Beijing		1	0.0072	0.0271	0.0517	0.0340
F11			1	0.0220	0.0073	0.0048
F28				1	0.0261	0.0156
Unique					1	0.0075
H37Rv						1

72 hr	F15/LAM4/KZN	Beijing	F11	F28	Unique	H37Rv
F15/LAM4/KZN	1	0.0004	0.0007	0.0152	0.0739	P<0.0001
Beijing		1	0.0045	0.0086	0.0020	0.0888
F11			1	0.0089	0.0018	0.5286
F28				1	0.0132	0.0097
Unique					1	0.0043
H37Rv						1

APPENDIX B

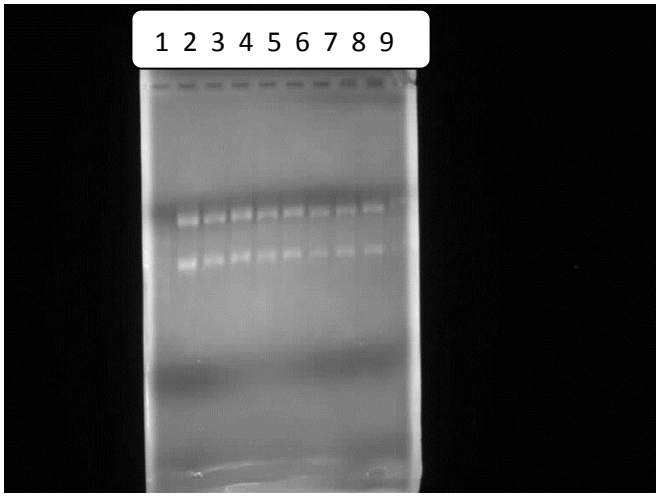
MOP gels and nanodrop readings of the RNA samples



Integrity of the A549 uninfected controls (t=0-12 h)

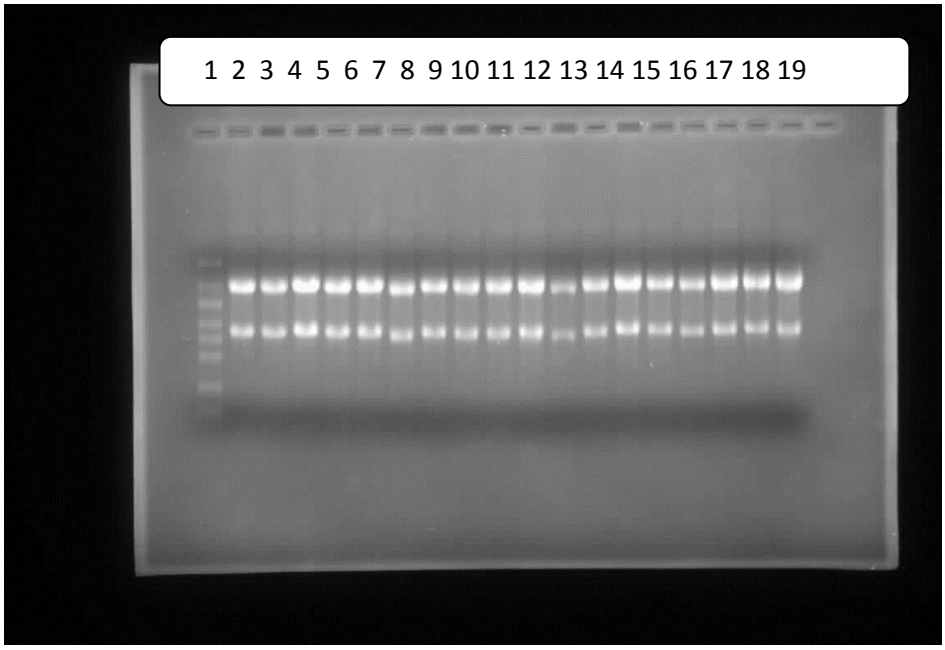
Well	Concentration (ng/µl)	Purity (260/280)
1-Marker		
2-T=0 (a)	1041.2	2.07
3-T=0 (b)	1040.2	2.06
4-T=0 (c)	807.1	2.05
5-T=1 (a)	865.4	2.08
6-T=1 (b)	1216.4	2.07
7-T=1 (c)	841.8	2.05
8-T=2 (a)	439.6	2.05
9-T=2 (b)	639.5	2.02
10-T=2 (c)	849.6	2.06
11-T=4 (a)	1040.7	2.07
12-T=4 (b)	963.5	2.05

13-T=4 (c)	779.3	2.05
14-T=8 (a)	936	2.06
15-T= 8 (b)	1491.5	2.07
16-T=8 (c)	1688	2.08
17-T=12 (a)	1167.3	2.07
18-T=12 (b)	1040.3	2.07
19-T=12 (c)	1532.3	2.07



RNA integrity of the A549 uninfected controls (t=24-72 h)

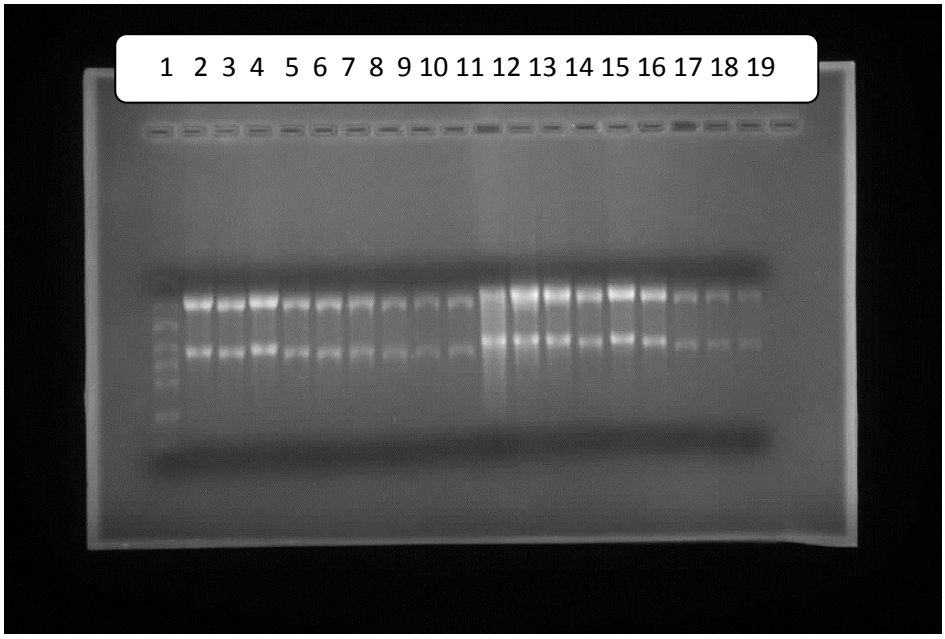
Well	Concentration (ng/µl)	Purity (260/280)
1-Marker		
2-T=24 (a)	1033.9	2.08
3-T=24 (b)	1109.2	2.07
4-T=24 (c)	947.8	2.07
5-T=48 (a)	626.6	2.08
6-T=48 (b)	594.3	2.06
7-T=48 (c)	403.6	2.07
8-T=72 (a)	525.4	2.07
9-T=72 (b)	544.9	2.07
10-T=72 (c)	578.8	2.06



RNA integrity of the A549 cells infected with the F15/LAM4/KZN (t=0-12 h) strain

Well	Concentration (ng/µl)	Purity (260/280)
1-Marker		
2-T=0 (a)	752.7	2.05
3-T=0 (b)	881.8	2.06
4-T=0 (c)	1065.3	2.08
5-T=1 (a)	970.2	2.07
6-T=1 (b)	993.3	2.06
7-T=1 (c)	684.3	2.07
8-T=2 (a)	826.1	2.08
9-T=2 (b)	965.1	2.06
10-T=2 (c)	930.1	2.06
11-T=4 (a)	957.1	2.08
12-T=4 (b)	889.2	2.07
13-T=4 (c)	899.4	2.06
14-T=8 (a)	1091.7	2.07

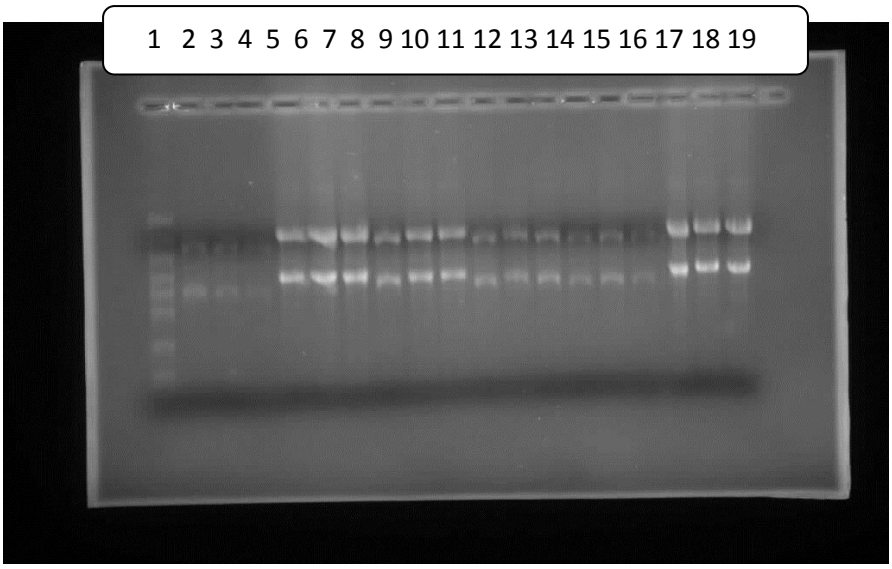
15-T= 8 (b)	850	1.99
16-T=8 (c)	658.1	2.06
17-T=12 (a)	1011.9	2.08
18-T=12 (b)	1067.7	2.08
19-T=12 (c)	1028.6	2.08



RNA integrity of the A549 cells infected with the F15/LAM4/KZN (t=24-72 h) and F11 (t=0-48 h) strains

Well	Concentration (ng/µl)	Purity (260/280)
1-Marker		
2-T=24 (a)	868	2.08
3-T=24 (b)	664.7	2.06
4-T=24 (c)	972.4	2.06
5-T=48 (a)	573.6	2.07
6-T=48 (b)	528.2	2.07
7-T=48 (c)	454.8	2.05
8-T=72 (a)	315.2	2.08
9-T=72 (b)	257.2	2.09
10-T=72 (c)	307.3	2.1
11-T=0 (a)	1159.6	2.07
12-T=0 (b)	1374.0	2.07
13-T=0 (c)	1087	2.05

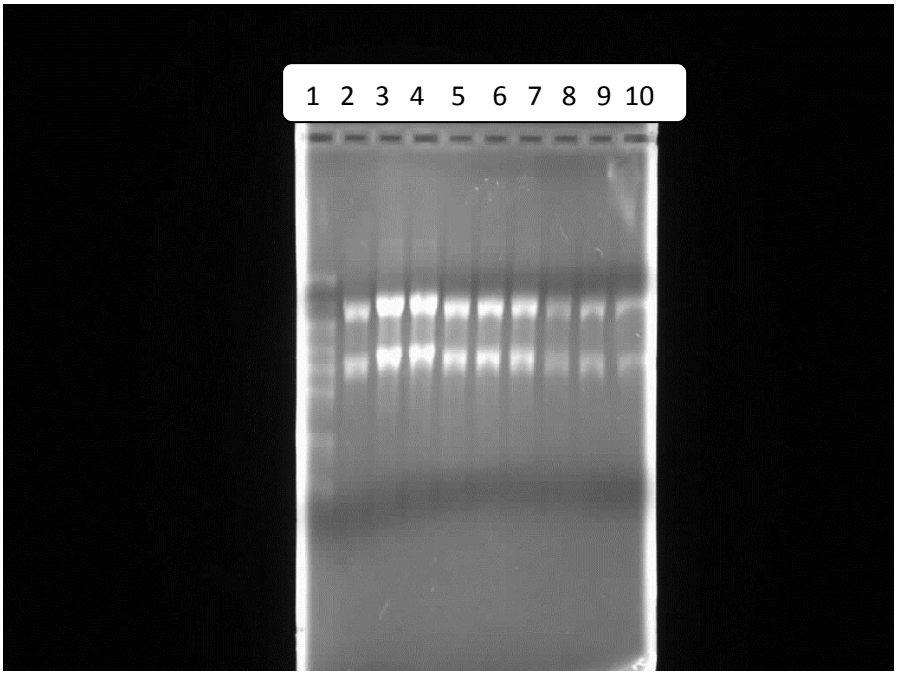
14-T=24 (a)	653	2.02
15-T=24 (b)	927	2.06
16-T=24 (c)	597.8	2.04
17-T=48 (a)	311.8	2.12
18-T=48 (b)	268.3	2.12
19-T=48 (c)	172.8	2.11



RNA integrity of the A549 cells infected with the F11 (t=72 h), F28 (t=0-72 h) and H37Rv (t=0) strains

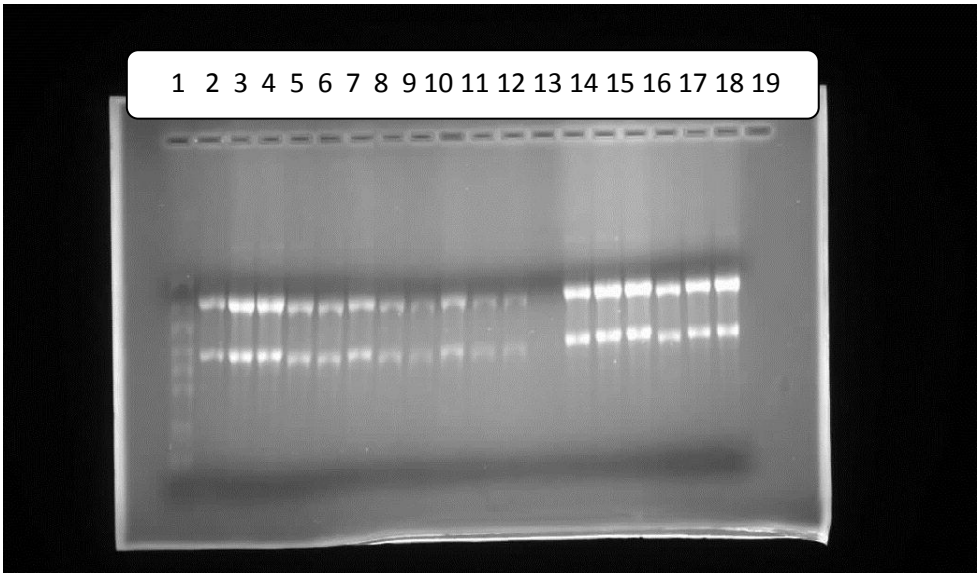
Well	Concentration (ng/µl)	Purity (260/280)
1-Marker		
2-T=72 (a)	169.8	2.05
3-T=72 (b)	152.3	2.06
4-T=72 (c)	144.0	2.13
5-T=0 (a)	1191.9	2.05
6-T=0 (b)	1026.9	2.06
7-T=0 (c)	940.1	2.05
8-T=24 (a)	478.8	2.07
9-T=24 (b)	626.7	2.05
10-T=24 (c)	630.4	2.04
11-T=48 (a)	310.8	2.11
12-T=48 (b)	341.6	2.11
13-T=48 (c)	337.7	2.13
14-T=72 (a)	173.4	2.06

15-T=72 (b)	231.7	2.06
16-T=72 (c)	154.6	2.04
17-T=0 (a)	872.4	2.05
18-T=0 (b)	951.5	2.06
19-T=0 (c)	933.5	2.04



RNA integrity of the A549 cells infected with the H37Rv (t=24-72) strain

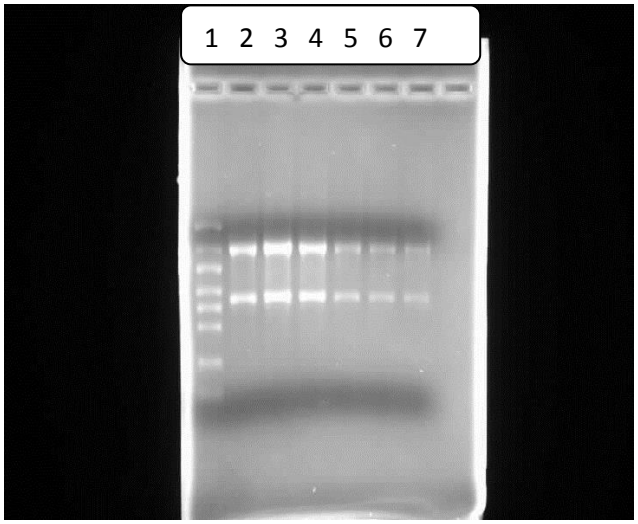
Well	Concentration (ng/µl)	Purity (260/280)
1-Marker		
2-T=24 (a)	461.6	2.07
3-T=24 (b)	797.5	2.06
4-T=24 (c)	764.9	2.07
5-T=48 (a)	484.5	2.06
6-T=48 (b)	472.2	2.06
7-T=48 (c)	536.3	2.06
8-T=72 (a)	237.0	2.35
9-T=72 (b)	190.2	2.44
10-T=72 (c)	174.6	2.49



RNA integrity of the A549 cells infected with the Beijing (t=0-72 h) and Unique (t=0-24 h) strains

Well	Concentration (ng/µl)	Purity (260/280)
1-Marker		
2-T=0 (a)	872.6	2.05
3-T=0 (b)	1032.3	2.05
4-T=0 (c)	1053.2	2.05
5-T=24 (a)	683.5	2.08
6-T=24 (b)	592.9	2.07
7-T=24 (c)	658.5	2.07
8-T=48 (a)	349.3	2.1
9-T=48 (b)	250.5	2.13
10-T=48 (c)	516	2.07
11-T=72 (a)	239.2	2.09
12-T=72 (b)	250	2.11
13-T=72 (c)	101	2.09
14-T=0 (a)	952.1	2.05

15-T=0 (b)	1191.1	2.06
16-T=0 (c)	1222.6	2.08
17-T=24 (a)	614.2	2.06
18-T=24 (b)	754.6	2.08
19-T=24 (c)	899.9	2.08



RNA integrity of the A549 cells infected with the Unique (t=48-72) strain

Well	Concentration (ng/µl)	Purity (260/280)
1-Marker		
2-T=48 (a)	418.8	2.07
3-T=48 (b)	621.9	2.06
4-T=48 (c)	445.1	2.06
5-T=72 (a)	225.8	2.09
6-T=72 (b)	195.1	2.1
7-T=72 (c)	192.7	2.1

Supplementary Fig. 1. RNA integrity of uninfected and infected A549 pulmonary epithelial cells at 48 hr.

Supplementary Table 1: Fold changes of 292 genes that are shared by all clinical and laboratory strains of *M. tuberculosis*

Gene	**F15/LAM4/KZN**	**F11**	**F28**	**Beijing**	**Unique**	**H37Rv**
ACSL4	3.95838	4.6745	3.64264	4.38683	2.69558	4.10799
ACSL5	4.58701	5.21155	4.30048	4.20811	3.12627	4.19116
ADAM12	2.42838	4.13885	3.04329	4.04269	3.11493	4.66463
ADAMTS9	6.22784	7.02038	5.32867	6.83322	4.97676	6.79308
ADRB1	-2.2721	-2.29451	-2.24894	-2.56921	-2.28405	-2.89337
AHSG	4.02042	6.53805	2.69623	5.56956	8.53399	4.0639
ANGPTL6	3.14373	3.3847	2.09323	3.11962	2.03544	2.69788
ANPEP	3.73722	3.17803	2.90657	4.09735	2.94155	5.11184
ANXA8L2	2.25175	2.45336	2.42793	2.55801	2.04274	2.69691
AOC3	-2.94626	-3.24766	-2.12678	-2.8296	-3.4655	-3.00166
APOE	-3.19	-4.07381	-4.19631	-4.38964	-3.32049	-3.35683
APOL1	5.45298	6.42466	4.56305	5.86027	3.80488	5.57883
APOL2	4.07432	4.96193	2.93634	4.60532	3.1803	4.23785
ASS1	4.27331	4.34895	3.93794	4.10729	3.04284	3.70079
ATP8B5P	3.9557	4.74668	3.00928	4.47799	3.62639	3.65453
BATF2	6.11848	6.78011	4.74164	6.26566	4.47917	5.39188
BCL11B	2.51099	4.31509	2.94439	4.55339	3.5058	4.65524
BCL2	-2.83312	-3.91041	-3.41378	-3.80404	-3.60599	-3.93982
BCL2A1	8.06049	8.22911	6.55853	6.49439	3.13755	6.63116
BCL2L10	-3.55142	-3.28198	-2.14562	-4.22732	-2.51236	-2.44587
BMPER	4.03973	4.66484	3.92502	4.85557	3.57934	5.34057

Gene	F15/LAM4/KZN	F11	F28	Beijing	Unique	H37Rv
BST2	9.7693	9.43184	8.23892	8.88134	6.25715	7.55367
C11orf86	4.29992	5.6979	4.0394	4.59557	3.72714	4.55155
C11orf96	5.66344	7.04047	4.82876	6.44274	3.70353	5.27796
C15orf48	8.08707	8.48302	7.94532	7.37778	4.01893	7.25585
C15orf59	-2.55366	-3.05516	-2.18932	-2.87253	-2.80165	-2.69079
C19orf66	3.28763	3.59127	2.33067	3.2656	2.21606	2.97096
C1orf116	3.03719	3.57772	3.01244	3.55947	3.59936	2.8864
C1R	6.48452	6.78924	5.84026	6.59977	4.76978	6.03363
C1RL	3.23041	3.45233	2.96796	3.3705	2.85479	2.79296
C1S	5.12931	5.47894	4.41702	5.50867	4.02233	4.71449
C3	4.74323	5.07983	4.34649	5.41176	4.21993	4.6084
C4orf33	3.29961	3.37358	2.01048	3.15943	2.5044	2.81636
C6orf223	2.4958	3.05224	2.29333	3.25658	2.27782	2.98117
CA9	5.39769	8.07752	5.4973	8.34871	9.41324	7.54376
CABLES1	-3.80536	-3.5787	-2.12735	-3.16049	-2.67859	-2.38645
CAPN5	-3.65628	-4.49155	-2.55737	-3.70291	-2.50324	-3.73097
CARD9	-2.4034	-3.69586	-2.27297	-3.82755	-4.01661	-2.76954
CATSPERB	4.45359	3.84058	3.88304	4.281	2.13068	2.87125
CCL2	5.62672	5.40018	3.4407	3.78896	2.73086	2.2771
CCL20	6.57196	6.98019	5.33902	6.16308	3.3272	5.60776
CCL5	7.39263	9.01358	7.74906	6.05803	2.61885	7.06112
CD274	6.50107	6.80733	3.77581	5.41016	2.45992	3.99845
CD38	7.27713	7.55589	5.0335	6.73637	4.69999	5.65978

Gene	F15/LAM4/KZN	F11	F28	Beijing	Unique	H37Rv
CD68	4.13386	4.72129	3.01219	4.24285	2.03879	3.79736
CDA	2.84561	4.0074	3.86327	3.99375	2.92631	4.39304
CDCP1	3.44433	4.28639	4.15668	4.13	2.56783	4.96767
CDH24	-2.51608	-3.62037	-2.82694	-2.92197	-2.14061	-3.48186
CDKL2	-2.47484	-4.22421	-2.33364	-4.83609	-2.2792	-2.75572
CEACAM5	3.29609	4.72502	4.6511	4.7551	2.31839	2.66775
CEACAM7	4.09309	4.13843	4.49313	5.60659	3.35412	3.80277
CFB	8.94072	9.51435	8.17166	8.94312	6.70465	7.80353
CHI3L2	3.97803	5.55162	3.09243	4.70855	2.85864	3.0718
CLEC4E	10.0401	10.866	10.4537	9.69265	6.62845	9.63749
CLMP	4.54489	5.3177	5.39004	4.67276	2.08349	6.51259
CMPK2	9.82841	9.57136	8.73052	9.07116	7.84263	8.74077
CNTD2	-3.58449	-3.61801	-3.31387	-4.81994	-4.39259	-2.99902
COL11A2	-4.02084	-3.53354	-3.19964	-3.69393	-4.10295	-2.17089
COL22A1	5.8534	6.80898	7.39715	6.52053	3.77623	7.53007
CP	3.87704	5.27025	3.18158	4.82398	5.03523	3.86226
CPNE7	-2.3358	-3.41343	-2.84403	-3.09551	-3.07814	-2.48979
CREB3L1	-3.62588	-4.46191	-3.91431	-3.89378	-2.84576	-3.50759
CSF3	11.6252	11.5005	10.7491	10.7267	6.223	10.3787
CST1	2.36079	4.69368	2.64546	6.3706	5.35331	3.43419
CTGF	-3.29598	-3.91388	-2.72646	-3.63402	-2.63318	-3.04932
CTSS	4.94386	5.29067	4.17324	4.82297	2.17639	4.51767
CXCL1	5.18477	5.18501	3.70049	4.83121	2.52621	3.73081

Gene	F15/LAM4/KZN	F11	F28	Beijing	Unique	H37Rv
CXCL5	5.4222	5.74236	4.4797	5.2362	3.29586	4.56433
CXCR4	3.29595	4.45584	2.52922	4.18055	2.26864	4.24144
CYBA	2.61896	2.99482	2.55259	2.86666	2.05341	2.08381
CYP1B1	2.85337	3.49937	4.64684	2.4258	2.69928	2.34892
DBP	-4.25491	-3.64113	-2.6304	-4.42328	-2.3311	-3.36198
DDX58	6.1742	6.39318	4.4398	5.92034	4.49532	5.34728
DDX60	4.62992	4.46514	3.92795	4.27028	3.80854	4.0533
DDX60L	4.17384	4.1082	3.00028	3.70346	2.4309	3.57489
DNAJC15	2.84201	4.80114	2.51798	4.36118	3.55543	2.67835
DNER	4.0336	5.10917	4.6895	4.24017	2.34449	4.91705
DPP4	2.60868	3.87621	2.20501	3.09557	2.04599	3.93235
DTX3L	3.27252	3.34869	2.60117	2.8231	2.17672	2.69634
DYSF	-3.41077	-4.71238	-3.41209	-5.35588	-4.98365	-3.2417
EBI3	7.2643	8.20801	7.42441	7.78099	4.89773	7.09824
EHF	3.47197	3.10815	3.44949	4.45457	3.69705	2.99713
ELOVL3	5.2203	6.10314	3.51523	5.86063	4.12786	4.77186
EMR1	4.23579	4.45266	3.41657	4.45263	2.51453	3.71563
F3	4.65366	5.82124	2.54297	5.28425	2.52108	5.26718
FAM101B	-3.91432	-4.42917	-2.42466	-4.54447	-2.83428	-3.18192
FAM160A1	4.87014	5.83471	3.79878	3.98482	3.03275	4.03869
FAM83A	4.69329	5.7548	2.62541	4.13835	2.95639	3.55805
FBLN5	3.96781	4.26232	3.40902	3.4345	3.21781	2.10597
FBXO2	3.44392	5.40935	4.01022	5.10254	3.66195	5.119

Gene	F15/LAM4/KZN	F11	F28	Beijing	Unique	H37Rv
FGFBP1	2.98948	5.2104	3.65701	5.90129	5.53592	3.80621
FOXL1	3.36561	3.89136	3.17679	3.3449	2.93956	3.46313
FST	4.00848	5.0379	3.12299	4.04571	2.76038	3.97895
G0S2	5.4731	5.16002	3.64206	3.56304	2.00487	4.22193
GALNT4	3.06363	4.17459	2.08393	3.46603	2.85739	2.71115
GALNT9	2.33978	2.24432	2.13532	3.47305	2.20072	2.44807
GBP1	7.79806	7.99964	5.7317	6.58797	3.81045	5.90878
GBP2	5.37777	5.82931	4.07202	4.44165	2.9621	3.65578
GBP3	6.02686	6.33353	4.34802	4.88946	2.54602	3.89551
GBP4	8.48438	9.1585	5.53233	6.44988	3.63379	5.59099
GDA	5.20339	5.03012	4.03444	4.9995	4.41428	3.0221
GFRA2	2.97677	3.924	3.71281	3.51862	2.64529	4.21138
GJB2	4.71124	4.47995	3.46376	2.43904	2.37774	2.31164
GPNMB	-3.23619	-4.90539	-3.63404	-4.33854	-3.58118	-2.94994
GPR1	-2.74016	-3.28379	-3.30808	-2.87969	-4.03549	-3.1584
GPX3	2.66362	3.29866	3.13502	3.05631	2.18772	2.74042
GREB1	-2.71908	-3.37242	-2.56534	-3.17313	-2.42593	-2.98821
GRHL1	-2.67305	-2.66404	-2.33282	-2.15781	-2.25819	-3.00657
GZMM	-4.12809	-2.99939	-3.01936	-3.29688	-3.41642	-2.30903
HAS3	4.46629	5.41776	3.68094	3.98851	2.73254	3.81616
HCAR2	6.01071	5.73337	4.14437	4.74861	3.20962	5.80178
HCP5	6.57748	7.06735	6.1255	6.7487	3.1811	5.72478
HDAC10	-2.77263	-3.14877	-2.31946	-2.6833	-2.44031	-2.37654

Gene	F15/LAM4/KZN	F11	F28	Beijing	Unique	H37Rv
HELZ2	4.84988	5.2415	3.73662	4.88688	3.62689	4.37329
HERC6	4.90654	4.96638	4.33971	4.83021	3.92532	4.55885
HGFAC	-4.23882	-3.68373	-2.75778	-3.51273	-3.17106	-2.98804
HIST2H2BE	3.9838	4.90726	3.66976	4.69264	2.46978	4.99846
HLA-B	5.69857	6.36506	5.32149	5.91625	2.80449	5.19389
HLA-C	4.35218	4.90315	3.83143	4.54592	2.21421	3.73218
HLA-F	7.39195	8.12363	6.96022	7.59064	4.68399	6.89822
HLA-H	4.01722	4.89657	3.61834	4.25391	2.17088	3.49238
HLF	-2.62511	-4.82672	-2.45427	-4.22021	-2.23561	-5.40838
HS3ST1	2.84156	2.96885	2.45339	3.23348	2.19021	2.13925
HSH2D	5.89837	6.00122	3.93275	5.61431	3.6541	4.82221
IFI27	10.3865	10.788	9.54881	10.252	6.52275	9.22465
IFI35	5.20574	5.10196	4.09265	4.77546	3.42489	4.16494
IFI44	7.81452	7.86926	6.9094	7.49244	6.42383	7.21887
IFI44L	9.02034	8.3579	8.71222	7.71998	6.22354	8.10954
IFI6	8.55478	9.21346	7.65467	9.08478	7.30238	8.372
IFIH1	6.195	6.40279	4.68331	5.56606	3.91455	4.97993
IFIT1	8.85533	9.42574	7.37901	8.80019	7.43871	8.04116
IFIT2	9.01452	10.0375	6.42742	8.54799	6.27028	7.65086
IFIT3	7.42127	7.82087	5.82482	7.05397	5.21392	6.28102
IFITM1	9.37177	9.40937	8.0916	8.66969	7.52286	8.16817
IFITM3	5.47643	5.21115	3.78776	4.32528	3.52157	3.60785
IGF2	2.52797	3.61982	3.17794	2.20357	3.55836	4.06361

Gene	F15/LAM4/KZN	F11	F28	Beijing	Unique	H37Rv
IGFBPL1	-3.09333	-4.43148	-3.64504	-2.99524	-2.04306	-3.96548
IKBKE	4.86609	4.56715	3.45225	3.9339	2.16923	2.7732
IL1A	9.23967	8.63389	6.82572	5.36213	2.23801	5.29544
IL1B	9.2956	9.59165	7.36795	7.1204	3.19683	6.80114
IL22RA1	5.4566	6.60646	4.0923	6.01573	3.94251	5.62374
IL23A	9.95342	9.56809	8.25134	7.35562	3.95109	6.62714
IL8	5.13022	5.25733	3.87253	4.56741	2.55849	3.7053
INHBA	6.35451	7.30686	5.3559	5.74011	3.20622	6.63873
IRF7	4.87605	5.18348	4.39423	4.97046	3.78262	4.32491
ISG15	7.90817	8.15541	6.97261	7.49978	6.09678	7.01933
ISG20	4.67573	4.75929	3.1819	4.12862	2.05592	3.17168
ITGA5	2.90524	5.07577	3.74963	4.78114	3.13682	5.28682
ITGB3	3.56899	5.26593	3.44729	4.00458	2.2963	5.39828
ITGB8	5.79489	6.76607	5.74623	5.40711	3.58073	5.23746
ITIH5	2.72264	3.88437	2.59621	3.70665	3.22892	2.55354
KCND1	-2.90584	-3.50748	-2.93052	-4.23238	-4.12142	-3.12429
KCNIP3	-4.40677	-3.09004	-2.09744	-3.2623	-2.36122	-2.69373
KCNJ16	3.93341	4.01218	3.07509	5.15361	4.28359	2.88018
KCNJ6	3.24962	4.9136	3.05164	4.80389	4.86519	5.50016
KCP	-2.10554	-4.48721	-2.74145	-3.81267	-4.14168	-3.36816
KIAA1199	4.75096	7.71954	5.67979	7.86358	6.08633	7.37219
KIF17	-4.89504	-4.38012	-3.72602	-4.79125	-3.73343	-3.19792
KRT15	2.19432	4.17472	3.18819	4.62781	4.31742	4.41618

Gene	F15/LAM4/KZN	F11	F28	Beijing	Unique	H37Rv
KRT20	-3.65138	-3.22803	-3.55445	-2.64182	-2.03963	-4.35341
LAD1	5.17918	5.39188	4.75306	4.55621	3.41456	4.31402
LAMB3	5.94448	5.7955	6.20149	4.99123	2.34816	4.97517
LAP3	4.60272	4.73553	2.55847	4.03179	2.5731	3.27638
LAPTM5	3.46269	4.67746	3.76505	5.79801	2.93662	5.81553
LBP	4.95256	4.88496	3.12187	6.8939	5.81088	4.93017
LGALS3BP	3.3034	3.57698	2.41664	3.51965	2.05865	2.76856
LGALS9	6.33747	6.54596	4.04213	5.76936	2.11638	3.27212
LMO2	5.02772	5.69118	2.44776	3.95305	2.20771	2.99131
LOC100127888	2.69196	4.74959	3.24361	4.86277	2.91223	4.47163
LOC100130705	-2.28312	-4.00003	-2.66338	-3.13196	-2.98372	-2.55071
LOC100134229	2.77111	4.70208	2.49144	3.92562	2.32665	4.29658
LOC100505817	2.12855	2.99098	2.15203	3.13219	2.38807	2.72792
LOXL2	2.87602	4.40313	3.44112	4.68655	3.68669	4.95803
LTB	9.08195	9.46265	8.10109	8.40743	5.96664	7.72957
LYPD3	5.37693	6.44069	4.51979	5.18442	2.15538	5.20106
MAN1A1	3.26566	4.38479	3.11572	3.83792	3.23198	3.56518
MASP2	-2.52544	-3.82112	-3.24622	-3.1606	-2.94336	-2.61716
MAST4	2.7503	3.2324	2.2235	2.95793	2.39689	2.16773
MCIN	-2.73153	-2.53638	-2.10915	-3.20787	-2.0693	-2.4163
MED12L	-2.08553	-2.85095	-2.87357	-3.68062	-2.7363	-2.8543
MEGF6	2.45881	3.99371	2.56233	4.07927	4.03509	3.75223
MMP1	4.77265	6.72387	6.67857	6.75329	6.33112	9.42979

Gene	F15/LAM4/KZN	F11	F28	Beijing	Unique	H37Rv
MMP10	5.32139	7.36226	7.83428	7.03721	5.66926	9.46858
MSX2	3.64794	4.36058	3.45555	4.0038	2.28927	4.01506
MTMR11	2.71358	3.82276	2.4903	3.11409	2.36356	3.11075
MUC1	4.45334	5.6208	2.83129	5.15506	4.67253	3.57716
MVD	2.75434	3.43576	2.10178	3.68272	2.59111	2.49792
MX1	7.68353	7.08719	6.45521	6.65092	5.68558	6.43515
MX2	9.34028	8.51022	7.11293	7.83319	5.71701	7.19506
NDNF	4.4388	6.13985	4.14258	6.3387	5.49958	6.097
NOD2	9.58624	9.80697	8.63226	8.15133	5.4466	8.4917
NOS1	4.78184	5.92403	4.66469	5.40357	4.23546	5.30479
NPC1L1	-3.48476	-3.80918	-4.19626	-3.35785	-3.56751	-3.85185
NR0B1	-2.79938	-4.1633	-2.80838	-4.23988	-3.15353	-4.64247
NR4A3	-2.91418	-2.20468	-4.13023	-2.07687	-3.5548	-2.26189
OAS1	6.12865	6.92308	5.27503	6.19615	4.69339	5.52235
OAS2	10.9123	10.2406	8.93686	9.4609	6.94032	8.75582
OAS3	6.33779	6.15345	5.07524	5.86842	4.66839	5.35202
OASL	10.7421	11.1366	8.88389	10.2211	7.82173	9.58162
ODF3B	4.00589	3.75389	3.10406	3.40191	2.01543	3.17271
OLR1	8.97527	8.75587	7.3439	5.88324	2.87619	6.67438
PABPC1L	-2.49703	-4.99357	-2.78382	-4.2822	-3.56145	-4.00903
PARP10	5.90146	6.20511	4.84748	5.81507	4.07613	5.18009
PARP9	4.62089	4.6175	3.7039	4.33843	3.53262	4.11935
PDE6G	-2.23568	-3.06576	-2.1747	-2.51301	-2.03476	-2.72345

Gene	F15/LAM4/KZN	F11	F28	Beijing	Unique	H37Rv
PDGFRL	3.61365	3.55376	2.10143	3.0103	2.71981	2.19449
PDZK1IP1	5.20316	5.37374	5.53676	5.9479	2.72668	5.09096
PF4	2.21911	3.19934	2.09094	3.12071	2.46706	2.52283
PI3	9.92374	10.4957	9.447	10.4082	7.01702	9.95645
PID1	4.84739	6.00216	5.40363	5.09751	3.9071	6.16937
PLAUR	3.69387	4.35606	2.6762	3.79767	2.29634	3.37603
PLEKHA4	8.47011	9.1429	6.17247	7.86261	4.82129	6.93525
PLSCR1	3.41937	3.48019	2.76303	3.10407	2.29793	2.77892
PML	4.0045	4.1959	3.05432	3.73515	2.00893	3.1177
PNLIPRP3	3.64847	3.02385	4.33425	3.52344	2.56971	4.21838
PPM1K	4.01785	4.35561	2.73201	3.4401	2.31575	3.11198
PROZ	-2.2045	-2.88009	-2.05253	-3.40155	-2.07588	-2.00306
PRSS3	2.58847	4.75953	3.34354	4.52275	4.35995	4.37955
PSD4	3.44412	4.32119	3.00318	4.18979	2.95147	3.66443
PSMB8	3.40469	3.67144	2.72607	3.37408	2.15453	2.73788
PSMB9	5.65754	6.01987	4.39809	5.50179	3.44069	4.55202
PTPRN2	2.04938	3.65125	3.38862	3.36962	2.44837	3.16936
PYGM	-3.48238	-2.93891	-2.15924	-4.26245	-3.04283	-2.43552
QRICH2	-2.6286	-3.36814	-2.30108	-3.24858	-3.45188	-2.61254
RAB39B	-2.22736	-4.74665	-2.35238	-4.85479	-3.92696	-4.18238
RARRES3	4.24749	3.46259	2.62548	3.93967	2.26687	2.11832
RHBDL1	-4.6976	-4.76263	-3.25809	-3.22808	-2.99206	-3.49055
ROBO1	3.35987	3.86387	2.6439	3.15492	2.80996	2.98906

Gene	F15/LAM4/KZN	F11	F28	Beijing	Unique	H37Rv
RSAD2	11.1901	11.6607	9.8543	10.1216	7.10421	9.73009
SAA2	8.55086	8.87495	7.89733	8.6781	4.49714	8.04398
SAMD9	5.91556	6.45489	4.63149	5.75945	3.54918	5.03136
SAMD9L	7.05924	7.02816	5.11301	6.16611	4.07701	5.43095
SAMHD1	5.32759	5.11483	3.4612	4.56117	3.30225	4.26762
SECTM1	4.81208	5.06717	2.20077	4.78264	3.12673	3.65927
SERINC2	2.27512	3.67037	2.88228	3.35095	2.50355	3.54926
SERPINA1	3.22736	3.90795	2.39457	3.54201	4.44399	2.89534
SGPP2	4.86111	5.64548	4.533	3.94557	2.24975	4.14024
SLC16A3	3.05087	4.08247	2.71857	4.12848	3.49948	3.7335
SLC1A2	5.25174	6.70661	5.32342	6.47058	5.56499	6.60215
SLC22A11	4.00707	5.10318	3.20308	5.74807	3.28977	2.88308
SLC26A9	4.70519	5.29209	3.6237	5.46949	4.33358	3.27505
SLC2A6	5.34331	5.01116	4.38117	4.4887	2.83803	3.56571
SLC39A8	4.41603	5.15255	4.11708	4.68785	2.27823	3.99856
SLC4A11	-2.77382	-3.21029	-2.87533	-3.00901	-2.92562	-3.227
SLCO4A1	2.47349	4.73001	3.2094	4.94501	2.87813	4.32945
SLCO5A1	3.44765	5.09583	2.88891	3.42782	2.95191	2.76025
SLPI	5.30593	6.44015	5.1329	6.17823	4.3658	5.13369
SOD2	3.86058	4.04596	3.69789	3.71891	2.2209	3.64458
SORCS2	2.79196	3.50012	2.86832	4.30532	4.00705	4.44218
SP110	4.13537	4.27048	3.02163	3.87085	2.51426	3.56094
SPINT1	4.7597	6.05232	5.30972	5.96211	3.80053	5.92033

Gene	F15/LAM4/KZN	F11	F28	Beijing	Unique	H37Rv
SQRDL	3.10575	3.359	2.78348	3.095	2.49467	2.91096
SRD5A3	2.18603	3.3449	2.38658	3.43629	2.44136	3.40295
ST6GALNAC3	-3.75664	-5.45571	-2.71177	-4.56078	-3.22363	-3.43285
STARD4	3.2311	3.48251	2.04145	3.10602	2.0322	2.40559
STAT1	3.94012	3.91864	3.2584	3.85935	3.22591	3.59764
STAT2	3.46345	3.59953	2.02872	3.25538	2.15336	2.95356
STC1	3.88451	5.94009	4.02361	5.65436	4.94857	6.34535
TAP1	4.0578	4.83575	3.15572	4.2529	2.35929	3.42186
TAP2	4.34592	4.58011	3.2974	4.10521	2.19446	3.37296
TESC	2.19799	3.57709	2.01924	3.68038	3.63826	3.27149
TFF1	3.60969	5.49969	3.36021	5.24948	3.76317	3.39083
TGM2	4.22632	5.15263	3.16398	4.90842	3.94929	4.48574
THBS1	-3.05487	-4.16676	-2.82488	-3.90581	-2.72181	-2.92188
THEMIS2	5.31021	5.86529	3.69702	5.33357	3.87973	5.47124
TLR5	2.51243	3.89953	2.35796	3.99378	3.02013	4.71785
TMEM132A	4.4022	4.88249	4.27709	5.0482	2.71305	4.5048
TMEM154	3.06033	3.49541	4.89545	4.14589	3.00949	4.95251
TMEM163	3.33567	3.80447	3.10925	3.50336	2.8534	2.32414
TMEM37	2.73084	4.80121	2.35987	5.66043	6.44741	4.90926
TMPRSS3	6.08766	7.25866	6.2309	6.90952	4.37527	6.11699
TNFAIP6	10.3053	11.3011	8.28298	8.52132	6.04027	7.726
TNFAIP8L3	2.81186	3.19418	2.23478	2.93351	2.4988	3.49422
TNFRSF1B	7.05931	7.28341	6.32511	5.86302	2.14918	5.20692

Gene	F15/LAM4/KZN	F11	F28	Beijing	Unique	H37Rv
TNFSF10	8.07049	9.10647	4.46502	7.74642	5.54602	5.70674
TNS1	3.55131	5.75804	3.76863	5.63436	5.30416	6.55993
TNS4	2.55351	3.8976	2.08444	4.46035	3.69519	4.11298
TRANK1	6.08728	6.40029	4.63777	5.64694	3.61973	4.96546
TREX1	3.84333	4.20731	2.96098	3.65333	2.2723	3.1416
TRIB2	5.46671	7.96902	5.16906	6.82896	5.0678	5.34207
TRIM14	4.02516	3.87513	2.77427	3.70408	3.0826	3.60997
TRIM22	8.45266	8.41371	6.43282	7.76301	4.94956	6.97281
TRIM34	2.60088	3.28127	2.43023	3.60657	2.9045	3.29488
TRIM67	-2.54104	-4.86817	-2.74583	-3.67823	-3.5388	-3.48696
UBA7	4.84925	5.03407	3.15953	5.14431	3.13935	4.0566
UBE2L6	4.25121	4.08085	2.92867	3.65172	2.1498	3.08569
USP18	6.35555	6.15658	4.59405	5.72925	4.3954	5.20125
VDR	3.26064	3.81633	2.97066	3.67467	2.44194	3.39074
WIPF3	-2.20796	-3.39175	-2.0669	-3.77636	-2.23013	-3.79753
XAF1	9.0612	8.48864	7.45917	7.92858	4.46922	7.12313
XDH	6.27968	5.4469	5.57217	5.04422	2.46414	4.72157
XKRX	3.63513	4.73891	4.15878	5.16407	3.61984	4.98235

Supplementary Table 2: Gene combination for different *M. tuberculosis* strain families and strain specific genes

Names	total	elements
Beijing F11 F28 H37Rv F15/LAM4/KZN Unique	292	CLMP CREB3L1 ITGA5 XDH APOL2 CXCR4 ADAM12 C1RL GZMM C15orf48 SOD2 GDA KIAA1199 CMPK2 DYSF DNAJC15 CLEC4E CD38 HS3ST1 APOE PF4 BCL2L10 C3 RARRES3 PPM1K BCL2 ASS1 ANXA8L2 UBE2L6 HLF C15orf59 NDNF KRT15 CDH24 EMR1 SORCS2 IFI27 LAMB3 KCNJ6 TNFAIP6 ADRB1 CARD9 FOXL1 OAS1 IL23A GRHL1 IL1A FST TNFAIP8L3 IL8 IRF7 HERC6 XAF1 CA9 HCAR2 SECTM1 XKRX OLR1 LYPD3 TLR5 GALNT9 LOC100505817 USP18 MVD GPR1 ITGB8 TRANK1 PSMB8 TRIM14 APOL1 MAST4 NR4A3 BCL11B TMEM132A PI3 THEMIS2 GBP4 GFRA2 RHBDL1 MMP1 ST6GALNAC3 STAT1 PDE6G MEGF6 CD68 BCL2A1 FGFBP1 FAM83A PDZK1IP1 DBP HAS3 C1S MAN1A1 EBI3 BATF2 COL11A2 SP110 TRIM34 SAMHD1 TAP2 KIF17 CHI3L2 CEACAM5 TMPRSS3 SLC2A6 MASP2 GBP1 QRICH2 PSD4 KRT20 OAS2 PLSCR1 LAD1 STARD4 THBS1 PID1 SRD5A3 MCIN CATSPERB MED12L SLC39A8 CCL5 MX1 PNLIPRP3 LOC100130705 LAP3 IFIH1 AHSG HDAC10 IL1B SLCO5A1 C11orf96 NOD2 ATP8B5P HLA-C ODF3B IFITM1 KCNJ16 HGFAC PARP9 MMP10 CDA TFF1 PLAUR FAM101B FBXO2 CPNE7 TMEM37 ACSL4 HIST2H2BE CYP1B1 KCP IFIT3 CNTD2 CYBA EHF C1R COL22A1 ITIH5 PROZ HCP5 SQRDL C6orf223 LOC100127888 TRIM22 GPNMB KCNIP3 TGM2 NOS1 HLA-H KCND1 ELOVL3 DPP4 GREB1 UBA7 TNFSF10 DTX3L MUC1 LBP ADAMTS9 NR0B1 TAP1 CST1 SERPINA1 WIPF3 MSX2 IFIT2 C11orf86 SERINC2 AOC3 OAS3 SLC22A11 CTSS STC1 01-Mar SLC1A2 LTB LGALS3BP SAMD9L CSF3 LMO2 DDX60 HELZ2 CCL20 ROBO1 SLPI GBP2 TNS1 ACSL5 C4orf33 PRSS3 G0S2 CDKL2 OASL BMPER CCL2 GALNT4 CTGF TMEM154 ISG15 MX2 RAB39B VDR SLCO4A1 HLA-F SGPP2 PDGFRL C19orf66 PML IFITM3 TRIM67 PTPRN2 BST2 INHBA IFI6 LOXL2 IGF2 NPC1L1 PABPC1L IFI44L IFIT1 GJB2 PARP10 TNFRSF1B LGALS9 TNS4 SPINT1 SLC16A3 ISG20 IL22RA1 CXCL1 IKBKE HSH2D SLC26A9 ANPEP ANGPTL6 CEACAM7 CAPN5 IFI35 LOC100134229 TMEM163 GBP3 STAT2 RSAD2 DDX60L HLA-B CXCL5 LAPTM5 CFB DDX58 PLEKHA4 CDCP1 GPX3 ITGB3 CABLES1 SAMD9 SAA2 F3 PYGM DNER TESC IGFBPL1 C1orf116 TREX1 SLC4A11 CD274 CP FAM160A1 MTMR11 TRIB2 IFI44 PSMB9 FBLN5
Beijing F11 F28 H37Rv F15/LAM4/KZN	217	FSTL1 PLCE1 TNIP3 MEFV STOX1 GGT3P CXCL3 HOXA13 CCL22 FAM129C EFNB1 03-Sep COL1A1 GPR37L1 NEIL1 GBP7 GMPR BTN3A1 CD82 STARD13 IL24 NPR1 ADRA2A HLA-J HHLA2 RASSF5 PTGES MFAP5 HIST1H2BJ NPHS1 STAT5A MTSS1L IFI16 FHDC1 MUC2 NGFR CACNA1H EPS8L3 DSE LRRC16B CASP1 WNT5A PTGS2 B2M HAS2 SLC16A2 CILP2 PLCXD3 CHST2 HIST1H2AG AMPD3 LOC100288432 CARD16 RPH3A FHOD3 COL5A3 IDO1 ERAP2 LOC100287314 BRCA2 PARP12 CYP4F22 HS3ST3B1 IRAK2 FLJ16779 DHRS12 KIRREL2 LIPG FEZ1 SCARA3 AKR1B1 HLA-G HRH1 TNFRSF14 TNIP1 SMTN NT5C3 CA8 HERC5 IL32 LAMC2 KCNJ2-AS1 MLKL SSTR5-AS1 C12orf70 KIAA1462 GBP5 SEMA6B CACNG6 IRF9 MT2A CYFIP2 PASK TYMP INSIG1 KCNE4 TRIM5 SAT1 ROS1 C1QTNF1 SPINK1 ZSCAN18 KLK14 GK BIK RGS16 ACVRL1 PDLIM7 HRK MOB3C MAML2 CREB3L3 CYP2U1 CD55 LOC339666 RERG GGT8P LOC728392 HLA-E FLT3LG BTN3A2 TRIM31 OSMR SEMA3E RAP1GAP ADORA2A KCNH2 ADCY5 HIST1H2BC OVGP1 AIM1L PRRG4 RAB26 HCAR3 SNX10 PARP14 MSI1 PIK3R3 CDCA7L RCOR2 SLC43A3 CARD6 KIAA1217 FA2H NCCRP1 NALCN CERS1 HLA-A MPP1 STX11 DPYSL5 IRAK3 GARNL3 TBX1 TRIM69 DPY19L2 KCNE1L TMEM106A RHCG SLC6A14 FAM92A1 DFNA5 IL6 TLR3 ZMAT3 RNF213 L1CAM GIMAP2 HIST1H3H 03-Mar SEPP1 RASGRP1 SLC16A6 NLRC5 VNN1 SLC16A13 IFIT5 OSR1 RALA RNF144B AMH NTN1 MDK TNFAIP8 ICAM1 FLJ35282 CA2 TTYH2 BCAS1 PAPL CNTNAP2 PAX8 BTN3A3 SEMA3D ZEB2 SERPINE1 HLA-L PTAFR SMTNL1 DNAJB5 MATN2 GLIPR1 KLK10 P2RX4 LRP4 GPR156 PTPLAD2 IL11 LCN2 ADAM22 DLX1 PSMB10 APOL3 SSTR5 ARHGAP22 IL7 BIRC3
Beijing F11 F28 H37Rv Unique	21	HSD17B2 MIR17HG WNK4 RHOV KCNMB3 ITGA2 LRP1 SLAMF7 DOC2B IGFBP3 GJA1 DSCAML1 TMEM45A CREG2 NUPR1 HSPA6 TMEM158 EGLN3 DOK7 PPP1R3G KCNN4
Beijing F11 F28 F15/LAM4/KZN Unique	9	RFPL3S SPATA18 RARRES1 VNN2 AQPEP SPEF1 SFMBT2 CCDC154 TLR9

Beijing F11 H37Rv F15/LAM4/KZN Unique	52	ARRB2 KCNG1 SIDT1 MREG SUSD2 CPNE4 NKD1 NAT8L ISM1 DPYD SH3PXD2A BCL2L15 FXYD2 PHF21B EEPD1 PCSK9 FAM83B HOXA2 PI4K2B TOX2 MSMO1 P4HA1 ALDH1A3 NEURL AIFM3 DDO HMGCS1 CEACAM1 GGT5 AOC2 SEC16B SYPL2 NTNG2 RET IFITM2 WDR17 C2CD4B NXPH4 C17orf104 HK2 SNAI3-AS1 IL33 TLL1 EXOC3L1 C10orf54 RBM43 USP44 FAM155B FDPS WDR66 HLX C2CD4A
Beijing F28 H37Rv F15/LAM4/KZN Unique	3	RGL1 RGS7 STK33
F11 F28 H37Rv F15/LAM4/KZN Unique	5	SH2D7 ADAM11 VPREB3 IL16 WNT5B
Beijing F11 F28 H37Rv	34	C11orf93 F7 04-Mar AGTRAP KLRC1 SH3BGRL2 RRAS GPR160 LSAMP EFEMP1 STARD8 NID2 MAF MROH6 EPHB2 PDE3B C14orf182 ST3GAL1 SLC47A1 GJB3 OXCT1 SLC16A9 HS6ST2 CENPA PRUNE2 PLEKHH2 CCDC40 TUBA4A PLEK2 DDC RHOBTB3 ZNF385A ATP8B1 ADD2
Beijing F11 F28 F15/LAM4/KZN	34	STYK1 OTUD3 ECE1 CEBPD IL15RA PARP8 LOC728431 EXOC3L4 TTC39B UXS1 CMTM4 SLC43A2 ST3GAL4 IL10RA FFAR2 ATRNL1 STOML1 STAT4 GRIK4 OLFM1 LINC00525 KCNK1 KLHL4 SCEL SERPINF2 SLITRK6 LSS PDIA5 LOC645638 SHROOM3 FLJ22184 ATP6V1B1 TUBB1 CTSC
Beijing F11 F28 Unique	2	CEACAM6 AQP3
Beijing F11 H37Rv F15/LAM4/KZN	50	ANKRD13B PNPT1 GFPT2 A4GALT TMEM117 NEBL RTN4RL1 IL17D SGK1 PRDM13 PIFO CDK18 FAM171B GNAZ NKX3-1 RASGRP3 PDZD2 TMEM229B GATA2 HAPLN3 CD47 PLEKHF1 BLZF1 TDRD7 SYNM PRTG CARD11 TAPBPL STRIP2 MASTL MYPN GSTO2 EFNA1 LCA5 DENND5A MYD88 HDX SERPINE2 TCP10L BTC LGMN TRIM21 TNFSF13B SH2B3 WTAP BEND6 KCNB1 ZNFX1 CACNA1I GATA4
Beijing F11 H37Rv Unique	91	WISP2 GYS1 C4orf3 ALDOC P4HA3 ADAMTS16 PLD6 BACE2 AKNA GALNT18 PSORS1C3 CSTA S100A3 BEX2 PHGDH CBS TNFRSF19 ITGB2 ULBP1 PLOD2 FIGN LOC728228 SLC11A1 MAGI2-AS3 SLC2A1 UBE2QL1 EBP FAM13A NDRG1 DCBLD1 MCOLN3 PLAC1 CYTIP SUSD4 MAFB LOX SLC39A10 BNIP3L HILPDA CD22 TMIE INHA PFKFB4 KCNMB4 ESR2 MIR210HG LOC154761 PGK1 IQGAP2 LGR6 FAM115C SYNE2 PTPDC1 FAM129A ITGB4 LGALS1 ATP1B1 MBOAT1 SLC2A3 RASGRP2 PDE9A TMEM47 LDHA PLD5 ACVR1C MT1X SEMA4B SPAG4 EFCAB3 C16orf74 ABCG2 LOC100422737 CDH16 AK4 ARRDC3 PSAT1 PRODH SLC43A1 ASNS WWC2-AS2 FAM189A2 GLT25D2 PDK1 ZMAT4 PPFIA4 ANKRD55 NPL CRISPLD2 AR FJX1 SCN5A
Beijing F11 F15/LAM4/KZN Unique	30	CECR2 TMEM92 FCGBP KCNQ5 GC MUC5B WNT6 NNMT VASH2 KRT80 C19orf26 PREX1 TCN2 CYR61 DTNA CFH PKD1L2 AIM1 PLA2G4A RIMKLB SLC7A9 ACAT2 ITIH2 FLRT2 MORC4 LY6E CITED4 CFHR3 ANKRD1 TP63
Beijing F28 H37Rv F15/LAM4/KZN	3	MIOX C6orf58 SESN3
Beijing F28 H37Rv Unique	2	LTBP2 MCAM
Beijing F28 F15/LAM4/KZN Unique	2	RBM44 SLC23A3
Beijing H37Rv F15/LAM4/KZN Unique	2	REM2 PPYR1
F11 F28 H37Rv F15/LAM4/KZN	21	ACSM3 AGPHD1 ACADL PHACTR1 CDSN AKAP2 GYG2 CNTN1 HIST1H2BK KIF1A NEURL1B CCDC78 SLC22A4 AICDA EPHA5 ASIC1 MAP7D2 LOC654433 LOC440896 PPP4R4 PPIL6

F11 F28 H37Rv Unique	2	CALB2 LY96
F11 H37Rv F15/LAM4/KZN Unique	8	EPSTI1 GNG2 VNN3 RIMS4 TMEM171 UBD ITGB2-AS1 CSF2
F28 H37Rv F15/LAM4/KZN Unique	2	BACH2 GNB3
Beijing F11 F28	6	YJEFN3 KIF5C AK5 C12orf36 ZNF883 TRERF1
Beijing F11 H37Rv	63	FIBIN XK CDK20 TLR8-AS1 SYT12 SLC10A4 PGM2L1 CCDC113 PECR EMR2 BMP2 SLCO1B1 GPD1L IRAK1BP1 MDGA1 TMEM178B HOXD8 FOXP2 NKAIN4 OSGIN1 SLC25A27 SLC6A8 IFNLR1 IL4R KLHL23,PHOSPHO2-KLHL23 CYB5D1 SP8 NSUN7 SOBP CPT1A ROR1 XYLT1 BEAN1 SLC5A11 KIF7 ZBED3 JAM3 MUC16 CLEC2B ZMYND8 NOTUM SLCO1B7 GATSL3 CNRIP1 ZGLP1 SGTB TMEM169 SEMA7A RBPMS2 DYNC2H1 ARRDC4 HOXA7 AMIGO1 SEMA6A CAMKMT P4HA2 PLK2 TBC1D30 KCNAB2 TNFRSF11A MAP9 MGC32805 HOXB2
Beijing F11 F15/LAM4/KZN	78	TMEM139 LAMP3 FNDC8 PIK3AP1 DAPP1 HABP4 APOBEC3B TJP3 TMEM140 CYP3A5 MEIS1 LDLR SYDE2 AQP11 C5orf56 EDA2R ABHD16B SLC37A1 APOBEC3F TMEM62 NRTN NAMPT CXCL2 DANCR PRKD2 NFE2L3 KLF5 LOC728743 BCAT2 UNC13A PDE4B ASIC4 KIAA1211 CFLAR FTSJD2 CTSO TRADD BID TGFB2 ALOX5 ZNF704 APOL6 CYP21A1P CASP10 CSF1 CKB TAPBP ZC3H12A RIMS3 SDC4 CYB561D2 LIPA CDC42EP2 SLC52A3 RRBP1 SRGN CASP7 PELI1 ZBP1 ISYNA1 MST1R MOV10 KIF26A DSC3 SOCS7 TMEM173 NR5A2 SNX30 NMNAT3 NMI NOX1 TNFAIP2 ARHGEF17 TCTN2 PARP3 UNC93B1 GSDMD FAM81A
Beijing F11 Unique	55	C9orf152 PON3 CCDC64 SPTSSA ID1 CST3 SLC9A3 PFKL CDHR1 IGFLR1 FUOM SQLE ENTPD2 TPPP3 GPX2 LFNG IGFBP5 MMP14 ADAM28 LINC00304 LINC00087 LOXL1-AS1 HCN4 BCRP3 ZNF503-AS2 RGL4 CFD NRXN2 FRMD3 CLGN VEPH1 GRB7 TNFSF15 FAM215A FSTL4 EDN3 VTN IL15 BEX4 KCNJ14 ACSS2 B3GNT3 TCN1 ID4 FOS IDI1 PRR15 SALL4 NBR2 DOCK3 BCAT1 ANXA4 LOC100268168 RNF128 TINCR
Beijing F28 H37Rv	5	SAMSN1 GREB1L AOX1 MDH1B SPOCK1
Beijing F28 F15/LAM4/KZN	4	PROC GGT1 TFAP2E CDHR3
Beijing F28 Unique	4	CRABP1 SNORD32A SPTSSB KCNQ2
Beijing H37Rv F15/LAM4/KZN	2	SAMD13 TNFSF14
Beijing H37Rv Unique	15	ANLN RIPK4 FGF17 CCL26 ADAMTSL4 CDCA2 GPR63 CRYAB ITPR1 ABLIM2 COL13A1 LOC100505695 LOC100131551 JMY MYOM3
Beijing F15/LAM4/KZN Unique	5	SEMA4A HAVCR1 RBP4 ABAT DACT1
F11 F28 H37Rv	15	LINC00520 CCDC34 DSCC1 QPRT SAPCD2 ARHGEF26 CD302 RUNX2 FOSB HIST1H2BD EFNB3 ARNTL2 ABI3BP PRR11 HIST2H2BF
F11 F28 F15/LAM4/KZN	35	KLRG2 ZBTB20 TREML3P L3MBTL4 DOCK4 KCNH3 RASGEF1B CD74 CCDC136 NAV3 TLR2 GAS6 ETV1 ADAMTS7 CERS4 NBN TNFRSF11B EFHD1 ACP2 RIPK2 MAP2K2 RGL3 PFKM ADSSL1 CCRL2 ITGAM SLC11A2 TNC CX3CL1 PRSS23 MRGPRX3 CMBL VCAM1 FANK1 TUBB4A
F11 F28 Unique	1	METTL7B
F11	17	FXYD6 DLX2 NRARP GYLTL1B LUM TFAP4 BOC GDF1 IL2RG ZNF774 CCDC147 C7orf10

H37Rv F15/LAM4/KZN		LRRC55 SP100 PHYHIPL TRAF1 CTSH
F11 H37Rv Unique	7	TRIM29 GREM2 CALCR TRPC6 HIST1H4H PITPNC1 PTCH2
F11 F15/LAM4/KZN Unique	5	GPR124 ZNF469 MAMSTR LAT2 TMEM51-AS1
F28 H37Rv F15/LAM4/KZN	5	PATL2 B4GALNT2 SPOCK3 ANK1 TMEM145
F28 H37Rv Unique	2	HABP2 EGR4
F28 F15/LAM4/KZN Unique	4	KCNH1 ESRP1 LAG3 ZNF300P1
Beijing F11	53	ZNF799 EPB41L4B MMP7 ITM2B LINC00261 ARHGAP39 ROR2 LRRC48 LONRF2 SNHG9 IER3 C1GALT1C1 FOXN2 HSD17B7 TBC1D10A HSD17B7P2 SLC25A15 HMGCR LMO7 RCOR1 PGF RGS11 ZNF853 SPTBN4 SULT2B1 HSD17B14 TTC26 LOC151009 RNF122 TC2N ZYG11A INTU RNF180 CELF6 TMEM165 FAM169A ABTB2 NAT6 SPATA6 MATN3 CASC2 CHKA KIAA0040 FRMD8 SLC2A12 IGFBP1 WDR35 CYP51A1 HJURP COL17A1 EFNA3 DUSP16 CYP24A1
Beijing F28	1	GOLGA8A
Beijing H37Rv	26	COL7A1 MYCBPAP UCN2 DMGDH DDIT4L PDGFA KCTD11 CDR1 ARSG SHB GLI2 LINC-ROR CMIP TBX2 ECM1 KLRC2 ANK2 RORA SORBS1 GUCY1A2 KLRC3 PORCN MYO1F FSTL3 NPTX1 LOC100289137
Beijing F15/LAM4/KZN	3	ACP5 ABCG1 ZNF488
Beijing Unique	34	KCNK3 ELANE TM4SF4 KIAA1161 LINC00239 LINC00324 GAL3ST1 C22orf23 BPIFB1 SLC29A4 SEC14L2 ALB NGF CLDN2 NUDT18 FADS2 EMID1 P2RY1 CIDEC ANGPT1 ALDH3A1 SLC1A1 C14orf1 RAB3IL1 TMCC1 NHLRC4 SEMA4G NPR2 HHIP ATP6V0D2 KLF15 SEC14L4 ADH6 TMEM141
F11 F28	21	MIR4461 LRRC6 FRAS1 SYTL1 KIF4A GAL DACT2 LOC643401 BAAT NEK2 PLK1 SEC31B CDC25C ANKRD20A12P PBK ASPM S1PR1 C6orf165 CENPF KIAA0825 IL11RA
F11 H37Rv	40	NFIA PCDHAC1 SMO LOC645249 GSTM4 TSPAN7 CXCR7 ADCY3 NECAB2 TPD52L1 ALDH5A1 G6PD PLEKHG5 SPTBN2 CYP27C1 PCYOX1L DAGLA LINC00312 ZNF697 ADAT2 PAX9 PTPRB MERTK SLC1A5 MPC1 FGFR2 RTN4RL2 FAM212B ALDH1B1 RNFT2 DUSP6 PSORS1C1 PLEKHN1 CCDC88C CCDC171 CRTAC1 MLXIPL WDR86 GAD1 ARL13A
F11 F15/LAM4/KZN	107	SH3BP4 SPECC1L KNDC1 GTPBP1 SERPINB9 TNFRSF9 INPP5J RGS14 IL18BP SPDYE5 MIA2 CLDN9 ZFPM2 DIXDC1 TRIM45 SNCAIP NFKBIZ JAK2 USP43 GCH1 PTP4A3 NYAP1 TNFAIP3 CSAG1 PTGER4 CGNL1 FOXN3 TMEM38A ADORA2A-AS1 HOXB4 C1RL-AS1 EHD4 RNF24 CCDC85C STK32B LRRC20 NCK1 DLC1 COL26A1 FNDC3B NFKB1 HLA-DOB CDRT15P1 PSMA6 IFNAR2 FBXO6 NCOA7 SLC30A3 PSME2 IL18R1 TRAFD1 FAM222A PHF11 FAM171A2 DCHS2 IRF1 TTLL6 HAGHL B4GALT1 CD40 MPV17L KHK ELOVL7 KLF6 MAP3K8 SH3KBP1 RNF112 NPM2 PDK2 EBF1 EREG ADRB2 SLC17A7 IL12A ACTN2 PBX3 PSTPIP2 OPTN LYN BRIP1 PION ADORA1 GDPD3 HADH ZNF367 TRIM38 CASZ1 PPAP2B LOC100506714 VSX1 CYLD PPAPDC2 CBLN3 IFNGR1 FAM122C PLEKHS1 SLC25A28 PDZK1 FDXR IRF8 RNF157 HES4 SYTL3 DENND2D VAV3 LINC00410 BTG3
F11 Unique	11	NOXO1 ZNF467 XPOT KIAA1045 LOC100129726 LOC100289019 AGTR1 ZDHHC11 GRAPL CCDC85A RUSC1-AS1
F28 H37Rv	20	DNAH17 VWA5A PSG4 FAIM3 C5 ESPNL THSD7A RAPGEF5 DEPDC7 BTNL9 AKR1B10 GLB1L2 GALM EGR3 LHX1 FGA IGDCC4 PKDCC CHRNA9 KLF10
F28 F15/LAM4/KZN	31	SORL1 PCOLCE HSD11B2 SLCO2B1 LINC00605 BCAM ATG7 NEFL FGF12 SULF2 C2orf72 HTRA1 INHBE CYB5R2 RFTN1 MRAP2 CACNG4 HDAC9 STK32A AVPR2 BHLHA15 PTPRD PRTN3 SOX8 SPTB NFATC2 CTDSPL LPPR3 PDE8B MFNG LOC100505865
F28 Unique	11	FGFR3 AGT MGC16121 APOH TMEM88 ANXA13 NCF2 FGB ANKRD30BL OLAH SELENBP1
H37Rv F15/LAM4/KZN	15	TMEM52 EN1 TNF PPP1R14D MGC39372 DHX58 LINC00173 SIX3 PLA1A PTK7 GREM1 ATP8A1 HOXA4 CXCL6 ZFP57
H37Rv Unique	16	UGT2B7 SYT13 RCAN1 PRIMA1 UGT1A7 SH3D21 SLCO1B3 ATF3 CACNA1B TUBB3 NAGS AZU1 UPK1A-AS1 LOC100507387 SBK2 SERTAD4-AS1

F15/LAM4/KZN Unique	7	LOC653712 CYP2F1 C7orf61 TRIML2 ST6GALNAC1 KLHDC7B PDE2A
Beijing	52	SERPINF1 PAQR8 CPEB1 TM6SF1 IGSF8 CATSPER1 SLC51B PAWR UPK3B SPDL1 MCM10 IL21R FAM129B DHCR7 CA12 CITED2 ZNF114 TNPO1 CDC45 COL16A1 ESCO2 BREA2 FAM111B GLP2R ZC3H12B C3orf72 RCBTB1 TEX19 WTIP MAPT MGAT5B MFSD2A TTLL7 SEMA3B INO80C TBL1X CLDN15 BCMO1 MEIG1 STIL DMBT1 C2CD2 TSPAN1 FLOT1 EDN2 GKAP1 IGFBP4 TTC9 SYT9 KIF3C NUSAP1 DHRS2
F11	255	KCNJ2 GCSH TMSB10 NKX2-5 FAM91A2 CCNB1 CYTH1 CCDC28B SLC12A8 MNS1 C20orf27 DHRS11 BRCA1 CDC25B IPW BIRC5 STK17A B3GNT7 NPW FAXC CLCN4 EFHC1 GLIS2 BBS1 VASH1 KIF14 ATP10A PPM1H ARFRP1 TTC13 SYTL4 EDAR KCND3 POLR3G WFIKKN1 TEX9 GPATCH11 APOBEC3A HOXA5 CLIP2 CHTF18 RABAC1 PCDHA12 TNFRSF10C FLYWCH2 GTSE1 NUF2 RAB4B FAM228B USP51 FKTN FBXO15 FN3K DEPDC1 ANKRD65 CAPN8 MYBL1 IQGAP3 DIAPH3 EIF2AK2 GPC4 PKIG MRAS FAM8A1 IFT172 STK36 CES3 CALCB ADHFE1 RPGRIP1L ARL4D SIRPA CEP55 ITPKB QTRT1 UBXN11 SCLT1 SYTL2 SYNE1 C2 SOCS1 KGFLP1 FAM66C MESP1 XRN1 IRF2 C7orf63 SAMD15 BFSP1 MB21D2 COL4A1 CLIP3 C2orf15 DYNC2LI1 CLEC2D N4BP1 CCDC71L AGPAT5 PREPL HSBP1L1 MAPK8IP3 KATNAL2 WDR90 SH3BGR CENPK ZNF404 PMS1 TLE4 NINJ1 TIMP4 ZNF804A RABL5 PSCA DTX3 C3orf55 TMEM56 C20orf96 TRIM36 C11orf70 RCN2 SAV1 MAST3 LOC100288181 HYAL1 L3MBTL1 SLC44A4 C17orf97 LRP3 SUSD5 LOC100129534 NID1 RFX5 KCNAB3 EML1 THAP10 NPHP1 FBN2 SLC16A7 SPATA17 SFR1 LMLN GOLGA2P5 TFCP2L1 FZD2 C12orf61 ZFP69B TNNI3 FAM216A DUOX2 ERMP1 DMPK DLL1 ZNF771 C2orf81 CROT HOXA3 SCNN1D ZC3HAV1 ANKRD42 STEAP4 C4A,C4B_2 DDX26B RNF19B LPCAT2 DUOXA1 FAM83E ZNF792 PARPBP IFNGR2 KBTBD11 SIPA1L1 PYCR1 SPTBN5 TUB PNRC1 TOM1 CDC20 NMT2 GLI3 BCL2L13 WASF3 SEPT5-GP1BB ACHE SLC9B2 CXorf38 PVRL2 ENKD1 EPHX2 ELOVL4 PSME1 MTX3 C1QL4 SCLY TMEM67 WDR31 ZBTB42 SNHG3 ST3GAL2 FAM108C1 SHCBP1 LOC643669 CC2D2A METAP1D HIVEP2 NUDT11 SYT6 NUBPL PCGF5 FOXRED2 LIFR STXBP6 SERPINB1 CCDC80 MMAA ETHE1 AUNIP STRBP RAPGEF3 PNMAL1 CCL28 FAM188B PC RELA TMEM231 NIPAL3 OGFR TMEM121 MFSD12 LOC339535 CYB5RL FAM83D OSCP1 GLRB C6orf132 MORN4 CNP SSX2IP PIP5KL1 TIMM21 MORN2 XYLB C15orf38-AP3S2 DDR1 ETV6 RECQL4 MLPH CDKN3 FGD6 PTPN13 SLC18B1 FMN2 FAM64A MAMLD1 PMS2 GPLD1
F28	55	MMP2 NR4A2 CA11 MTRNR2L4 CDK1 CDS1 CYP1A1 SRD5A2 C10orf107 MTRNR2L10 CPVL MTRNR2L8 CHDH TYMS PROS1 KRT34 FRMD4A PDK4 MIRLET7DHG KIF12 NR4A1 APOD HIST1H2AC SRPX DLGAP5 PALD1 TMEM59L GFRA1 ABCC8 GSTM2 SLC4A4 REEP2 ATP1A3 PRKCDBP LOC284454 SYNGR3 MTRNR2L1 CADM2 ANXA9 MAP4K1 LIN7A EDIL3 MTRNR2L2 NCKAP5 VWDE C10orf91 MTRNR2L3 LINC00707 NR1H4 SMPDL3B CCDC150 HPX MALAT1 ACTA2 PEG10
H37Rv	125	CMTM8 CSPG4 NAP1L2 GPR143 PCSK1 SNX33 FRY TLR1 HTR7 CABP1 PLIN2 FAM167B ZNF239 C1QL1 BIRC7 FAM110C ADAM19 DNAH11 MCOLN2 TPBG SLAIN1 SOWAHB GALR2 JPH2 TUBA1A ABCC2 COL5A2 LGALS4 UPK2 OLIG1 PODXL HCG27 DMBX1 SLC5A3 THEG MYC BEST3 TMEM255B KLHDC7A MPZ FAM153B LOC100507156 C1QL2 KCNG3 S100A2 ST5 LOC729683 EN2 MAPRE2 ODC1 MYZAP KIRREL3 SH3BP1 SNHG12 ACACB DOK5 RSPO3 COL11A1 HIST1H1C ADM5 PPARGC1B SOX18 AGR2 EYA2 MIR137HG HMGB3 DOCK2 FSCN2 LRRC8C LDLRAD4 HIST1H3B HAS2-AS1 CPLX1 PAG1 STAC2 LOC388942 EGR2 FRMD6 SIRPB1 PDGFB HLA-DMB S1PR4 GPR176 TSPAN33 TGFBR1 WAS PCDHAC2 MIR3648 SHC3 CUX2 RGS9BP SLC7A8 SNAI3 HMGA2 LYPD6 GRB10 MOXD1 TGFB3 SRXN1 PYGL MTMR9LP ZBTB1 RAB3B MEG9 FNDC5 EPHX1 ZDHHC23 KIF15 RGS9 PSORS1C2 MEG3 SYT14 MICALL2 STC2 PPP1R14C ELL2 ZNF30 DAW1 COL5A1 PTGS1 PMEPA1 PAQR9 SCG5 SFRP1 KRT23
F15/LAM4/KZN	138	LOC285758 NAALAD2 ATP9B SP140L CERK BBC3 RAB40B FIBCD1 ZMYND15 GHR ING5 ADAM23 LIF LOC100132707 CPAMD8 OLFML2A PPL RASSF4 CADPS2 XKR9 BAI1 KANK3 PRRT1 PLA2G4C GGTLC2 CD200 C12orf39 SLC9A2 DNAJC1 IGFN1 BAIAP3 GRTP1 SOCS3 C10orf114 GPM6A ZNF165 SPEG LOC731424 NXN LRRC32 IL20 PALM ZSCAN12P1 BTN2A2 ADAMTS15 FSCN1 UPB1 LINC00273 HRASLS2 ARMCX1 TCAP HYAL4 GCKR BARX1 HEY1 ADTRP THRA GJC2 SLFN11 FBXL16 MAP4K2 GGTLC1 CELSR2 AGMO PGM5 MYCL1 RIMKLA FLJ42875 PMP22 FLT4 KAZN YPEL3 SOX2 SMAD6 TMEM81 BCL2L14 CBFA2T3 SEC24D PLAU KCNF1 B4GALNT4 NAPSB PLCG1 DNAAF3 GDF6 C5orf38 DDAH1 RASD2 DUSP2 BDKRB2 SLC4A3 PIGR NRP2 ERAP1 TMPRSS6 CTXN1 CCL17 CNR1 FAR2 RSPH1 WARS SAMD1 LOC113230 DRAM1 ARRB1 FOXN4 PTX3 MAST1 TPRA1 JAG2 CLUHP3 PHF17 AQP7P1 LOC100506178 B3GNT4 GAL3ST2 LRCH2 HAND1 KIFC2 LOC100134368 FGF18 CDK5R1 DKK1 CDKN1C RHBDL2 LYNX1 PITPNM3 STARD9 SAP25 PPP1R3C KCTD12 FBXL18 CNIH3 MGEA5 JPH3 TNFRSF10D PRKCA AQP7

Unique	185	AEN KIAA0895L PADI1 UPK1A NOSTRIN AK7 TRIQK TENM4 TSPAN13 PGBD5 PRDM16 ATOH8 NKX6-1 RAB3A WT1-AS SDC2 ALDH1L2 PCSK6 FN1 MLIP UNC5CL CHRM4 RRAD TM4SF20 ATP8B4 FYN LXN KLF2 UPK1B NTN4 FLJ35390 CD8A SFTPD F2 CFHR1 LOC100130357 REPS2 CD83 IGFBP2 UGT2A3 SCNN1B NPNT FGFR4 NTS TMEFF2 MALL LOC283710 ZDHHC8P1 SMIM1 FILIP1 RPS6KA2 ITGA10 LGALS2 CHGB VIL1 ASB4 SLC23A1 ASL ZNF365 ANGPTL4 GDNF FAAH2 CYP27B1 KRT4 GYS2 SERPINI1 NUDT8 DBI F13B MOCOS SLC3A2 SNCG TMEM97 TMEM200C PRPH SGCD LPPR1 CCDC69 SLC6A12 F5 OLFML3 NREP GABRA5 PPP1R1A ANKRD33B RORC CPE VGF ST3GAL5 TPST1 BHLHE41 KCNJ8 ZNF503 CACNA1G PROSAPIP1 SCN3B SLC22A18AS RENBP LDHD SERPINA6 VSTM2L SOX21 FAM46B ZNF274 WDR54 GJB6 CHST1 FGG ABCA1 TSPAN8 IDH2 GPI SCML2 DEGS2 HAL MMAB NPSR1-AS1 EGR1 THSD1 CPB2 TM7SF2 CERKL ACRBP SCGN MRC2 PNPLA1 CDH6 USP2 ASRGL1 HPGD GCHFR LHPP GLYCTK LOC730102 HEG1 CCND3 NEB ASGR1 UGT2B15,UGT2B17 PLIN1 SLC7A2 IRF4 MYLIP TMEM45B FUT1 PCDH1 ARHGAP18 TTR C8orf44-SGK3,SGK3 S100A4 MYO1A ADM2 IL1R2 ERBB3 INSL4 FMO5 C4orf47 ST6GAL2 FAM43A PADI2 PRDX2 CLYBL PCDH9 TMEM191A CYP2C8 SLC29A2 DHDH FAM162A CHRD TLE6 SCUBE2 4SEP NIM1 ZNF462 MIF GPRC5B VIPR1 HKDC1 SNED1 ACOX2 RBP1 TM4SF5 FOSL1 USH1C SPOCD1

Supplementary Table 3: Pathways (Kegg and Reactome) associated with each clinical and laboratory *M. tuberculosis* strain combinations in pulmonary epithelial cells at 48 hr post-infection

Strain combinations	**Number of genes**	**Activated pathway**
Beijing F11 F28 H37Rv F15/LAM4/KZN	217	Genes involved in Cytokine Signaling in Immune system Genes involved in Immune System Genes involved in Interferon gamma signaling Cytokine-cytokine receptor interaction Pathways in cancer Genes involved in Interferon Signaling Genes involved in Signaling by Interleukins Axon guidance Jak-STAT signaling pathway Genes involved in Endosomal/Vacuolar pathway
Beijing F11 F28 H37Rv Unique	21	No overlaps
Beijing F11 F28 F15/LAM4/KZN Unique	9	No overlaps
Beijing F11 H37Rv F15/LAM4/KZN Unique	52	Terpenoid backbone biosynthesis Phenylalanine metabolism beta-Alanine metabolism Genes involved in Cholesterol biosynthesis
Beijing F28 H37Rv F15/LAM4/KZN Unique	3	No overlaps
F11 F28	5	No overlaps

H37Rv F15/LAM4/KZN Unique		
Beijing F11 F28 H37Rv	34	No overlaps
Beijing F11 F28 F15/LAM4/KZN	34	No activated pathways
Beijing F11 F28 Unique	2	NO OVERLAPS
Beijing F11 H37Rv F15/LAM4/KZN	50	NO OVERLAPS
Beijing F11 H37Rv Unique	91	Genes involved in Metabolism of amino acids and derivatives Genes involved in Amino acid synthesis and interconversion (transamination) Genes involved in Metabolism of carbohydrates Genes involved in Glucose metabolism Genes involved in Transmembrane transport of small molecules Genes involved in Transport of glucose and other sugars, bile salts and organic acids, metal ions and amine compounds Genes involved in Glycolysis Glycine, serine and threonine metabolism Genes involved in SLC-mediated transmembrane transport Glycolysis / Gluconeogenesis
Beijing F11 F15/LAM4/KZN Unique	30	Genes involved in Regulation of Complement cascade
Beijing F28 H37Rv F15/LAM4/KZN	3	No overlaps
Beijing F28	2	NO OVERLAPPING PATHWAYS

H37Rv Unique		
Beijing F28 F15/LAM4/KZN Unique	2	No overlaps
Beijing H37Rv F15/LAM4/KZN Unique	2	No overlaps
F11 F28 H37Rv F15/LAM4/KZN	21	No overlaps
F11 F28 H37Rv Unique	2	No overlaps
F11 H37Rv F15/LAM4/KZN Unique	8	No overlaps
F28 H37Rv F15/LAM4/KZN Unique	2	No overlaps
Beijing F11 F28	6	No overlaps
Beijing F11 H37Rv	63	No overlaps
Beijing F11 F15/LAM4/KZN	78	Apoptosis Genes involved in Extrinsic Pathway for Apoptosis Genes involved in Apoptosis RIG-I-like receptor signaling pathway
Beijing F11 Unique	55	No overlaps
Beijing	5	No overlaps

F28 H37Rv		
Beijing F28 F15/LAM4/KZN	4	No overlaps
Beijing F28 Unique	4	No overlaps
Beijing H37Rv F15/LAM4/KZN	2	No overlaps
Beijing H37Rv Unique	15	No overlaps
Beijing F15/LAM4/KZN Unique	5	No overlaps
F11 F28 H37Rv	15	No overlaps
F11 F28 F15/LAM4/KZN	35	Genes involved in Integrin cell surface interactions Genes involved in MyD88:Mal cascade initiated on plasma membrane Genes involved in Activated TLR4 signalling Genes involved in Toll Receptor Cascades
F11 F28 Unique	1	No overlaps
F11 H37Rv F15/LAM4/KZN	17	No overlaps
F11 H37Rv Unique	7	No overlaps
F11 F15/LAM4/KZN Unique	5	No overlaps
F28 H37Rv F15/LAM4/KZN	5	No overlaps
F28	2	No overlaps

H37Rv Unique		
F28 F15/LAM4/KZN Unique	4	No overlaps
Beijing F11	53	Genes involved in Cholesterol biosynthesis
Beijing F28	1	No overlaps
Beijing H37Rv	26	No overlaps
Beijing F15/LAM4/KZN	3	No overlaps
Beijing Unique	34	No overlaps
F11 F28	21	Genes involved in Mitotic G2-G2/M phases
F11 H37Rv	40	Butanoate metabolism
F11 F15/LAM4/KZN	107	Genes involved in Immune System Leishmania infection Genes involved in Cytokine Signaling in Immune system Genes involved in Adaptive Immune System Toll-like receptor signaling pathway Genes involved in Signaling by the B Cell Receptor (BCR) Genes involved in Interferon gamma signaling Cytokine-cytokine receptor interaction Genes involved in Interferon Signaling Genes involved in Antigen Activates B Cell Receptor Leading to Generation of Second Messengers
F11 Unique	11	No overlaps
F28 H37Rv	20	No overlaps
F28 F15/LAM4/KZN	31	No overlaps
F28 Unique	11	No overlaps
H37Rv	15	No overlaps

F15/LAM4/KZN		
H37Rv Unique	16	Genes involved in Glucuronidation Ascorbate and aldarate metabolism Pentose and glucuronate interconversions Porphyrin and chlorophyll metabolism Drug metabolism - other enzymes Starch and sucrose metabolism Steroid hormone biosynthesis Retinol metabolism Metabolism of xenobiotics by cytochrome P450 Genes involved in Phase II conjugation
F15/LAM4/KZN Unique	7	No overlaps
Beijing	52	No overlaps
F11	255	No overlaps
F28	55	No overlaps
H37Rv	125	Chronic myeloid leukemia
F15/LAM4/KZN	138	Cytokine-cytokine receptor interaction
Unique	185	Genes involved in Common Pathway Starch and sucrose metabolism Genes involved in Developmental Biology Pentose and glucuronate interconversions Complement and coagulation cascades Genes involved in Formation of Fibrin Clot (Clotting Cascade) Metabolism of xenobiotics by cytochrome P450 Drug metabolism - cytochrome P450 Porphyrin and chlorophyll metabolism Retinol metabolism

APPENDIX C

Supplementary Table 1: Top Bio-function induced by clinical strains of *M. tuberculosis* in pulmonary epithelial cells at 48 hr post-infection

	F15/LAM4/KZN	Beijing	F11	F28	Unique	H37Rv
Diseases and Disorders	Dermatological Diseases and Conditions Inflammatory Response Immunological Disease Cancer Infectious Disease	Cancer Dermatological Diseases and Conditions Inflammatory Response Immunological Disease Connective Tissue Disorders	Cancer Dermatological Diseases and Conditions Inflammatory Response Immunological Disease Inflammatory Disease	Dermatological Diseases and Conditions Immunological Disease Cancer Inflammatory Response Connective Tissue Disorders	Dermatological Diseases and Conditions Cancer Connective Tissue Disorders Inflammatory Disease Skeletal and Muscular Disorders	Cancer Dermatological Diseases and Conditions Immunological Disease Metabolic Disease Endocrine System Disorders
Molecular and Cellular Functions	Cellular Movement Cellular Function and Maintenance Cell-To-Cell Signaling and Interaction Cell Death and Survival Cellular Growth and	Cellular Movement Cell Death and Survival Cell-To-Cell Signaling and Interaction	Cellular Movement Cell Death and Survival Cellular Function and Maintenance Cell-To-Cell	Cellular Movement Cell-To-Cell Signaling and Interaction Cellular Function and Maintenance	Cellular Movement Cell-To-Cell Signaling and Interaction Cell Death and Survival	Cellular Movement Cell Death and Survival Cellular Growth and Proliferation Cellular Function

	Proliferation	Cellular Function and Maintenance Cellular Growth and Proliferation	Signaling and Interaction Cellular Growth and Proliferation	Cell Death and Survival Cellular Growth and Proliferation	Cellular Growth and Proliferation Cellular Function and Maintenance	and Maintenance Cellular Development
Physiological System Development and Function	Immune Cell Trafficking Organismal Survival Hematological System Development and Function Tissue Morphology Cardiovascular System Development and Function	Immune Cell Trafficking Hematological System Development and Function Organismal Survival Cardiovascular System Development and Function Organismal Development	Immune Cell Trafficking Hematological System Development and Function Tissue Morphology Organismal Survival Organismal Development	Immune Cell Trafficking Hematological System Development and Function Organismal Survival Tissue Morphology Tissue Development	Immune Cell Trafficking Hematological System Development and Function Tissue Development Cardiovascular System Development and Function Tissue Morphology	Immune Cell Trafficking Tissue Morphology Organismal Survival Cardiovascular System Development and Function Hematological System Development and Function

Supplementary Table 2: Immune related canonical pathway enrichment by clinical strains of *M. tuberculosis* in pulmonary epithelial cells at 48 hr after infection

Pathway	F15/LAM4/KZN	Beijing	F11	F28	Unique	H37Rv
Superpathway of Cholesterol Biosynthesis	2.31	8.58	2.20	0.473	5.77	0.56
Interferon Signaling	13.0	8.21	14.90	9.07	9.00	8.87
Antigen Presentation Pathway	9.31	7.05	7.92	8.90	3.13	7.65
Cholesterol Biosynthesis I Cells	0.784	5.52	1.25	0.376	2.80	0.476
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	0.784	5.52	1.25	0.376	2.80	0.476
Cholesterol Biosynthesis III (via Desmosterol)	0.784	5.52	1.25	0.376	2.80	0.476
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	11.60	5.34	7.20	7.99	3.58	6.79
Communication between Innate and Adaptive Immune	8.34	5.31	6.45	6.07	4.64	6.64

cells						
Complement System	2.68	3.28	2.05	3.49	3.45	1.52
Activation of IRF by Cytosolic Pattern Recognition Receptors	5.70	2.93	3.88	3.57	2.31	3.99
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	2.76	2.66	2.20	2.03	0.832	1.62
Coagulation System	3.22	2.42	1.45	6.02	4.12	2.05
Toll-like Receptor Signaling	6.25	2.41	6.85	3.06	2.45	4.60
IL-6 Signaling	4.07	2.09	4.19	3.53	1.64	2.53
Role of JAK1 and JAK3 in Î³c Cytokine Signaling	1.61	1.96	2.84	1.35	0	1.34
IL-8 Signaling	2.45	1.93	3.10	3.24	2.37	2.03
IL-12 Signaling and Production in Macrophages	5.73	1.88	3.90	2.00	0.953	0.951
Role of IL-17F in Allergic Inflammatory Airway Diseases	4.62	1.87	3.70	3.42	2.67	3.42
iNOS Signaling	3.87	1.87	5.53	2.05	1.45	1.98
Apoptosis Signaling	3.90	1.82	3.30	2.45	0.778	1.37

Pathway	F15/LAM4/KZN	Beijing	F11	F28	Unique	H37Rv
IL-10 Signaling	2.34	1.75	1.90	1.22	1.64	1.47
IL-17 Signaling	2.65	1.60	3.31	2.57	0.742	1.47
JAK/Stat Signaling	2.65	1.60	3.86	2.03	0	0.760
Inhibition of Matrix Metalloproteases	0.422	1.60	1.67	1.68	2.29	0.81
IL-17A Signaling in Airway Cells	3.68	1.48	2.66	2.34	0.893	1.67
Role of Cytokines in Mediating Communication between Immune Cells	4.97E+00	2.84	3.32	2.71	2.67	3.89
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	3.68	0.581	1.88	1.77	1.13	0.753
Chemokine Signaling	0.980	0.622	0.786	0.775	0.464	0.255
Role of JAK1, JAK2 and TYK2 in Interferon Signaling	2.75	0.380	4.65	0.586	0.578	0.444
STAT3 Pathway	1.28	0.366	1.82	0.741	0	1.04
TGF-β Signaling	1.25	2.31	3.41	2.52	1.15	3.80
IL-22 Signaling	1.36	0.783	0.616	1.13	0.578	0.893
IL-1 Signaling	1.49	0.778	2.18	0.764	0.482	1.60

CD40 Signaling	1.98	0.474	2.23	0.889	0	0.909
IL-9 Signaling	2.60	0.490	1.51	0.787	0	1.52
IL-15 Production	3.23	1.15	3.37	0.514	0.507	0.782
IL-15 Signaling	4.83	1.41	4.85	2.83	0.529	2.10
Macropinocytosis Signaling	1.06	2.21	1.29	0.830	1.20	2.06
Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F	6.77	3.38	4.83	4.24	4.21	5.83
CCR5 Signaling in Macrophages	0.457	0	0	0	0.489	0
Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F	6.74	3.59	4.82	4.57	4.54	5.93

Supplementary Table 3: Fold changes of transcriptional factors induced by clinical and laboratory strain of *M. tuberculosis* in pulmonary epithelial cells

Transcriptional factor	**Beijing**	**F11**	**F28**	**H37Rv**	**F15/LAM4/KZN**	**Unique**
ANKRD42	-1.31624	**-2.224**	-0.70367	-1.33518	-0.95399	-0.57043
ATF3	-1.46846	-1.27407	-1.77016	**-2.374**	-0.29603	**-2.081**
BATF2	**6.26566**	**6.78011**	**4.74164**	**5.39188**	**6.11848**	**4.47917**
BHLHE41	-1.18292	-1.17118	-0.20386	-1.22901	0.552223	**-3.232**
BRCA1	-0.81331	**-2.671**	-1.27526	-1.57859	-0.79514	-1.44868
BRCA2	**2.50623**	**3.14293**	**2.02802**	**2.15945**	**2.644**	0.719255
CEBPD	**2.113**	**2.626**	**2.002**	1.06204	**2.4**	1.73186
CYLD	1.8499	**2.94458**	1.87692	1.76919	**2.732**	0.714753
DLX2	-1.9363	**-2.332**	-1.22938	**-3.411**	**-2.0553**	-1.50343
EGR1	1.64209	1.04452	0.462746	-0.14957	0.83448	**2.12**
EGR2	0.253242	-0.29562	-1.28717	**-2.545**	-0.42441	1.25639
EGR3	-0.76433	-1.81442	**-4.6268**	**-3.762**	-1.19186	-1.77984
EGR4	-1.23919	-1.78827	**-3.0126**	**-2.322**	-0.53603	**-2.547**
EHF	**4.455**	**3.108**	**3.449**	**2.997**	**3.472**	**3.697**
EN2	-1.35832	-1.42227	-1.32118	**-2.172**	-1.20161	-0.84752
ETV1	-1.3231	**-2.738**	**-2.2817**	-1.98439	**-2.4835**	0.873586
FOS	**2.769**	**2.055**	0.964956	1.33353	1.27653	**3.437**
FOSB	-0.89998	**-2.122**	**-3.165**	**-2.508**	0.099227	-1.33528
FOSL1	-1.25557	-1.57015	-1.09346	-1.78749	0.286369	**-2.809**
GATA4	**-2.628**	**-3.5262**	-1.7942	**-3.2236**	**-2.4977**	-1.38942
GLI2	**2.742**	1.66535	1.17454	**2.582**	0.626844	1.77806
GLI3	1.55962	**3.031**	0.989144	1.00101	1.98886	0.389004

	Beijing	F11	F28	H37Rv	F15/LAM4/KZN	Unique
GLIS2	-1.50506	**-2.099**	-1.33223	-1.77808	-1.93927	-1.48243
HLF	**-4.2202**	**-4.8267**	**-2.454**	**-5.4084**	**-2.625**	**-2.2356**
HLX	**-3.089**	**-2.7053**	-0.94901	**-2.2064**	**-2.382**	**-2.242**
HOXA13	**-2.687**	**-3.1311**	**-2.778**	-3.3167	**-4.636**	-0.96046
HOXB4	1.25523	**2.29782**	0.400646	1.44178	**2.822**	0.111904
ID1	**2.38786**	**2.38983**	0.201078	1.04026	0.222385	**4.144**
IFI16	**3.383**	**3.735**	**2.664**	**3.018**	**3.741**	1.67682
IRF1	1.37584	**2.612**	0.676299	0.617528	**2.389**	-0.0758
IRF2	1.53882	**2.06**	0.971891	1.30886	1.7327	1.02094
IRF4	-1.40574	-0.16653	-1.88312	-0.35561	-0.96875	**-2.766**
IRF7	**4.97**	**5.183**	**4.394**	**4.325**	**4.876**	**3.783**
IRF8	0.912598	**2.433**	0.292412	0.224041	**4.039**	0.836009
IRF9	**2.211**	**2.475**	**2.001**	**2.026**	**2.523**	1.76763
KLF2	1.64544	1.09368	0.782139	0.689906	1.55768	**2.109**
KLF5	**2.13**	**2.65688**	1.23915	1.59668	**2.255**	1.62259
MEIS1	**2.103**	**2.46205**	1.92614	1.87632	**2.114**	0.127751
MLXIPL	-0.92051	-2.00202	-1.90801	**-2.405**	-1.15241	-0.86513
MSX2	**4.004**	**4.36058**	**3.45555**	**4.015**	**3.648**	**2.289**
MYC	-1.61707	-1.81933	-1.36362	**-2.098**	-0.78508	-1.54251
NFATC2	-0.04327	-1.54053	**-2.293**	-1.40233	**-2.599**	0.116637
NFIA	-1.84496	**-2.342**	-1.36849	**-2.206**	-1.07003	-0.55598
NFκB1	1.8614	**2.445**	1.31193	1.41108	**2.437**	0.966108
NFκBIZ	1.86866	**2.451**	1.11767	1.44594	**2.981**	0.655676
NKX2-5	-1.48733	**-2.016**	-1.0206	-1.66453	-1.32669	-0.92762

	Beijing	F11	F28	H37Rv	F15/LAM4/KZN	Unique
NKX3-1	**2.146**	**2.943**	1.7856	**2.381**	**2.29**	0.322095
NLRC5	**3.483**	**4.019**	**2.313**	**3.026**	**3.891**	1.72056
NUPR1	**-4.086**	**-3.358**	**-2.422**	**-3.716**	-1.05337	**-3.885**
OLIG1	0.385812	0.458463	0.650469	**2.249**	-2.71173	-1.04232
PML	**3.735**	**4.196**	**3.054**	**3.118**	**4.004**	**2.009**
PPARGC1B	-1.67048	-1.78019	-1.35593	**-2.823**	-1.80411	-1.4299
RCAN1	-1.99902	-1.25413	-0.03823	**-2.361**	0.552412	**-2.308**
RELA	1.5834	**2.452**	1.05041	1.20769	1.68582	-0.10485
RFX5	1.65724	**2.195**	0.955985	1.20887	1.8343	0.720721
RUNX2	1.52381	**2.352**	**2.568**	**2.962**	1.56374	0.852909
SOX2	0.549389	0.871384	0.421959	-0.97818	**2.029**	1.39203
SOX8	1.93979	1.25864	**-2.072**	-0.05618	**-2.02072**	-0.0038
SP100	1.9346	**2.337**	1.31503	**2.03**	**2.194**	1.16224
STAT1	**3.859**	**3.919**	**3.258**	**3.598**	**3.94**	**3.226**
STAT2	**3.255**	**3.6**	**2.029**	**2.954**	**3.463**	**2.153**
STAT4	**2.245**	**2.436**	**3.51**	1.78371	**3.016**	0.506687
STAT5A	**2.822**	**3.603**	**3.051**	**2.55**	**3.247**	0.748591
TBL1X	**-2.301**	-1.97643	-1.46303	-1.90291	-1.76855	-1.01849
TFCP2L1	-1.09935	**-2.044**	-1.76717	-1.75329	-1.90992	-0.91843
TP63	**3.411**	**3.985**	1.3385	1.6377	**4.1**	**3.642**
VDR	**3.675**	**3.816**	**2.971**	**3.391**	**3.261**	**2.442**

Bold numbers-significant transcriptional factors

Shaded numbers-Strain specific transcriptional factors