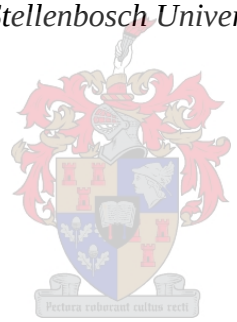
Global transcriptomic investigation of the human macrophage response towards pathogenic/non-pathogenic mycobacteria

Abhilasha Madhvi Mishra

Dissertation presented for the degree of Doctor of Philosophy in Molecular Biology in the Faculty of Medicine and Health Sciences at Stellenbosch University



Supervisor: Dr. Bienyameen Baker
Faculty of Medicine and Health Sciences
Department of Biomedical Sciences

Co-supervisor: Dr. Gina Leisching
Postdoctoral fellow
Faculty of Medicine and Health Sciences
Department of Biomedical Sciences

December 2019

DECLARATION

By submitting this thesis/dissertation, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining it for obtaining any qualification.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Dr. Bienyameen Baker for his patience and continuous support for my Ph.D study. I would like to thank him for his motivation and guidance during this time. I would also like to extend my gratitude to my co-supervisor Dr. Gina Leisching whose encouragement and motivation is much appreciated.

I would like to thank Prof. Ian Wiid for his astute guidance and sagacious scientific inputs, and whose faith and confidence in me and my work helped me to complete my study with an extra polished edge.

My sincere thanks go to Prof. Craig Kinnear, Prof. Novel Chegou and Prof. Colin Kenyon for their scientific inputs, sharing resources and helping with experiments at the right time and right space. I would like to thank them for their precious and valuable time which they gave me and which proved to be very helpful for me.

I would like to thank Prof. Grant Theron for his support and providing help with a trained research nurse since the inception of the study. I would sincerely thank Sister Ruth (Research Nurse) for her professional support during the first phase of my study.

I extend my sincere gratitude to Prof. Gerard Tromp, Prof. David Tabb and Mr. Carel Van Heerden for providing help with statistical analysis throughout my study. Particularly, Prof. Tromp's help was very impactful and critical. I extend my gratitude to Prof. Helena Kuivaniemi and Dr. Cedric Werely for their encouragement and insightful comments and internally reviewing the first draft of my thesis.

I would like to extend my gratitude towards my previous Supervisor Dr. Ranjith Kumar C.T who provided me all the necessary technical knowledge and the basics of a molecular biology laboratory. I would also like to thank Dr. Carine Sao Emani, Carine Kinsolva, Zama Mahlobo, Jeanie Sieberhagen, Haiko Schurz and Dr. Leanie Kleynhans for their help in the laboratory and the support they provided me whenever I needed it.

I thank the three beautiful ladies Dr. Liezel Smith, Dr. Nasiema Allie and Ms. Trudy Snyders who have always stood as a supportive hand to me whenever I turned towards them.

My sincere thanks go to Dr. Jyothi Chabilall who always stood as a supportive guardian and guided me with all the necessary academic support for my doctoral study. Her support and positive guidance is much appreciated.

I would like to thank all the study participants for their voluntary participation, patience and support during the first phase of my study. I would like to thank all the members of TB Drug Discovery Group, my fellow lab mates and the entire Division of Molecular Biology and Human Genetics for the stimulating discussions and helping hands. I am grateful to the funding bodies including South Africa-Medical Research Council (SA-MRC), Centre of Excellence, Stellenbosch University and Harry Crossley Foundation for their significant support in my Research in procuring consumables and equipment.

I thank God for raising me and my family in his hands and blessing my every single step with success and happiness. I extend my thanks to the people next to God, my parents, grandparents and my in-laws. Their best wishes and blessings craft our life with love and success throughout. The time, faith and support provided by them helped me in completing my study with full confidence and enthusiasm.

I would now like to open my heart and let all the appreciation flow to draft beautiful words to thank the two most important persons for filling my life with beautiful colours and everlasting happiness. My all smiles and senses goes to my husband Dr. Hridesh Mishra and our little bundle of joy, the cutest ever baby Master Raghuveer Mishra for making life easy and helping my doctoral study flow smoothly.

ABSTRACT

Background: Tuberculosis (TB) is a major cause of infection-related mortality worldwide. In 2017 an estimated 1.3 million people who were HIV-negative died of TB. An estimated 5-10% of infected individual develop active TB during their lifetime, while the remaining 90% (of infected population) successfully control the bacteria. Also, some of the close household contacts of TB patients remain uninfected and healthy. Studying host immune response towards *Mycobacterium tuberculosis* (*M. tb*) can unfold the reason behind this enigma.

Methods: We conducted a detailed investigation of *in vitro* host response from human monocyte derived macrophages (hMDMs) towards different strains of mycobacteria (grown in detergent-free media), i.e. pathogenic (*M. tb* R179) and non-pathogenic (*M. smegmatis* and *M. bovis* BCG). The host response was measured post-infection (at mRNA and protein levels) using AmpliSeq, quantitative real time polymerase chain reaction (qRT-PCR), multiplex ELISA (Luminex), intracellular mycobacterial survival and cytotoxicity assay. Biological network analysis (ingenuity pathway analysis IPA) was performed to understand the gene regulatory network involved in the pathophysiology associated with the host-immune system.

Based on false discovery rate (FDR) and biological functions, we selected an inter-related gene family of interferon induced protein with tetratricopeptides (*IFIT1, IFIT2* and *IFIT3*) from the list of 19 potential differentially expressed genes (DEGs) for knock-up (vector-based over-expression)/down experiments. This gene family is known to form a protein complex during viral infection to act against the antigen. Study encompassing their role against bacteria is not well established. Therefore, we performed knocking-up of *IFITs* via vector-based transfection and knocking-down via small interfering RNA (siRNA) approach to investigate their effect upon mycobacteria inside the host macrophages.

Results: AmpliSeq analysis found 19 DEGs at 12 hours post-infection across all three strains. We observed lower number of mycobacterial CFUs and higher host response (at both RNA and protein level) in hMDMs infected with *M. smegmatis* as compared to other two strains. Biological network analysis revealed interferon-interleukin associated signalling pathways as most prominent among the 19 differentially expressed genes. We found a differed host response towards all three strains, which may attribute to their pathogenicity. Messenger RNA and protein level comparisons at different time points, depicted strong role of interferon and interleukin associated gene network. This network was able to successfully counter *M. smegmatis* but succumb to *M. bovis* BCG and *M. tb* R179.

Most importantly, across all three strains, intra-cellular bacterial growth and survival measured through colony forming units (CFUs) decreased significantly upon knocking up of *IFITs* (*IFIT1*, *IFIT2* and *IFIT3*), while we recorded an increase in CFUs upon knocking down of *IFITs* in the host macrophages. Using multiplex ELISA, we found higher expression of key pro-inflammatory cytokines (i.e. IDO1, IFN-γ, IL-6, and IL-23) during knock-up (vector-based over-expression) of *IFITs* resulting in reduction of mycobacteria.

Conclusion: Differentially expressed *IFITs* showed a strong effect against mycobacteria, which can be used as a promising therapeutic target adjunct to anti-TB therapy. This knowledge will broaden the scope of host drug targets for resistance free bacteriostatic immuno-therapy.

OPSOMMING (ABSTRAK)

Agtergrond: Tuberkulose (TB) is ‘n hoofoorsaak van infeksieverwante sterftes wêreldwyd. ‘n Benaderde 1.3 miljoen MIV-negatiewe mense is in 2017 dood aan TB. ‘n Benaderde 5-10% van geïnfekteerde individue ontwikkel aktiewe TB in hul leeftyd, terwyl die oorblywende 90% (van die geïnfekteerde bevolking) die bakterie suksesvol beheer. Sommige huisgenote van TB pasiënte bly ook ongeïnfekteerd en gesond. Die rede vir hierdie enigma kan ontbloot word deur die gasheer immuunreaksie te bestudeer.

Metodes: Ons het deeglik ondersoek ingestel na die *in vitro* gasheerreaksie (van menslike monosiet-afgeleide makrofae) op verskillende stamme van mikobakterieë (opgegroei in ontsmettingsmiddelvrye media), d.i. patogenies (R179) en nie-patogenies (*M..smegmatis* en *M. bovis* BCG). Die gasheer reaksie is na infeksie gemeet (boodskapper RNS en proteïenvlakke) met AmpliSeq, reële tyd PKR, veelvuldige baan ELISA (Luminex), biologiese netwerk analise (Ingenuity Pathway Analysis), intrasellulêre mikobakteriële oorlewing en sitotoksisiteit eksperimente.

Ons het ‘n onderling-verwante geenfamilie van interferon geïnduseerde proteïen met tetratrigopeptiede *(IFIT1*, *IFIT2* en *IFIT3)* gekies uit die lys van 19 moontlike verskillend-uitgedrukte gene vir ons oor-uitdrukking, en onderdrukking eksperimente. Hierdie geenfamilie is bekend daarvoor om ‘n proteïenkompleks te vorm om gedurende virale infeksie teen die antigeen op te tree. Hul rol in bakteriële infeksie is nie goed bevestig nie. Daarom het ons oor-uitdrukking van *IFITs* deur vektor-gebaseerde transfeksie en onderdrukking deur klein onderdrukkende RNS (siRNA) uitgevoer om hul effek op mikobakterieë in gasheer makrofae te ondersoek.

Resultate: AmpliSeq analise het 19 verskillend-uitgedrukte gene teen 12 ure na infeksie oor al drie stamme gevind. Ons het ‘n laer getal kolonie-vormende eenhede en ‘n hoër gasheerreaksie (in beide RNS- en proteïenvlak) in menslike monosiet-afgeleide makrofae

geïnfekteer met *M. smegmatis* opgemerk, as in die ander twee stamme. Biologiese netwerk analise het gewys dat interferon-interleuken verwante seinweë ‘n belangrike rol speel tussen die 19 verskillend-uitgedrukte gene.

Ons het verskillende gasheerreaksies teenoor die 3 stamme gevind, wat moontlik aan elk se patogeniese aard verwant is. Boodskapper RNS en proteïenvlak vergelykings by verskillende tydpunte, het die belangrike rol van ‘n interferon en interleuken verwante geennetwerk gewys. Hierdie netwerk kon *M. smegmatis* suksesvol beheer, maar nie *M. bovis* BCG en R179 nie.

Interessant genoeg, het intra-sellulêre bakteriële groei en oorlewing, soos gemeet in kolonie-vormende eenhede, beduidend verminder wanneer *IFITs* oor-uitgedruk is, terwyl dit vermeerder het wanneer *IFIT1*, *IFIT2* en *IFIT3* onderdruk is in gasheer makrofae. Intrasellulêre groei en oorlewing van mikobakterieë is bevestig deur boodskapper RNS uitdrukking met kwantifiserende PKR en proteïen uitdrukking met “Western blot”, te ondersoek. ‘n Paar sleutel-molekules is opgemerk in die sel sitokien uitdrukking wat ons met veelvuldige-baan ELISA ondersoek het. Dit het IDO1, IFN-γ, IL-6, en IL-23 ingesluit, wat bekend is as pro-inflammatoriese sitiokiene.

Afsluiting: Hierdie uitslae sal ons kennis verbreed oor gasheer middel-teikens vir weerstandvrye bakteriostatiese immuunterapie aanvullend tot die huidige chemoterapie.

RESEARCH OUTPUTS

Patent (*Provisional patent filed*)

Abhilasha Madhvi, Bienyameen Baker-Employing molecules to induce expression of *IFIT1*, *IFIT2* and *IFIT3* for the treatment of tuberculosis and other bacterial infections in humans and animals. [SU-DISC-2019-0021-South Africa-provisional patent PA172249/P; SPOORSA_ sa _cases. 0153678.PA172249/P]

Research Articles

1. Comparison of human monocyte derived macrophages and THP1-like macrophages as *in vitro* models for *M. Tuberculosis* infection, Comparative Immunology, Microbiology and Infectious Diseases(2019), doi: https://doi.org/10.1016/j.cimid.2019.101355
   Madhvi A, Mishra H, Leisching G, Mahlobo P, Baker B,

2. **The Gene Expression Omnibus Database – NCBI – NIH**
   AmpliSeq data have been deposited in the NCBI Gene Expression Omnibus (GEO) database with experiment series accession number [GSE122619].
   Abhilasha Madhvi, Bienyameen Baker ***(Appendix X)***

3. Activation of Interferon-Interleukin Associated Gene Network during *in vitro* Host Response to Different Mycobacterial Strains Grown in Detergent-Free Media
   Abhilasha Madhvi, Hridesh Mishra, Novel N. Chegou, Gerard Tromp, Carel J. Van Heerden, R.D Pietersen, Gina Leisching, Bienyameen Baker
   (*Under peer review in Virulence*)

4. Interferon-induced Tetratricopeptide (*IFITs*) Proteins: Their role in Mycobacterial infection and survival
   Abhilasha Madhvi, Hridesh Mishra, Novel N. Chegou, Carel J. Van Heerden, Gerard Tromp, Bienyameen Baker
   (Manuscript under preparation)

Funding Obtained

1. Postgraduate Research Funding 2018 from Harry Crossley Foundation (HCF)
2. Postgraduate Research Funding 2019 from Harry Crossley Foundation (HCF)
3. KIC Travel Grant 2018 from National Research Foundation (NRF), South Africa
4. Travel Grant 2018 from Faculty of Medicine and Health Sciences (FMHS), Stellenbosch University, South Africa.
5. Travel Grant 2019 from Faculty of Medicine and Health Sciences (FMHS), Stellenbosch University, South Africa.
6. Travel Grant 2017 from Overseas Conference Grant (OCG), Stellenbosch University, South Africa.

Presentations at national and international conferences

a) **Selected for Oral presentation:** Global transcriptomic investigation of the human macrophage response to infection with pathogenic/non-pathogenic mycobacteria
Abhilasha Madhvi, Hridesh Mishra, Novel Chegou, Gerard Tromp, Carel Van Heerden, Gina Leisching, Bienyameen Baker; the 50th union world conference on lung health, Hyderabad, India (scheduled to be held in November 2019)

b) **Oral presentation:** Activation of interferon-interleukin gene network during *in vitro* host response to different mycobacterial strains.
Abhilasha Madhvi, Novel Chegou, R.D Pietersen, Carel Van Heerden, Gina Leisching, Bienyameen Baker; Pittcon, Orlando, Florida, United States of America (USA, February 2018)

c) **Poster presentation:** Differentially expressed genes in host response from the human macrophage response to infection with mycobacteria.
Abhilasha Madhvi, R.D Pietersen, Carel Van Heerden, Gina Leisching, Bienyameen Baker; 5th SA TB Conference, Durban, South Africa (June 2018)

d) **Poster presentation:** Global transcriptomic investigation of the human macrophage response to infection with pathogenic/non-pathogenic mycobacteria
Abhilasha Madhvi, Novel Chegou, Gerard Tromp, Carel Van Heerden, Gina Leisching, Bienyameen Baker; 62nd Annual Academic Day at Stellenbosch University, Cape Town, South Africa (August 2018)

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

º	Degree
%	Percentage
µg	Micro gram
µL	Microliter
µl	micro liter
µm	Micrometer
16s	16 Svedberg Unit
18s	18 Svedberg Unit
AIDS	Acquired Immuno Deficiency Syndrome
ANOVA	Analysis of variance
AP1	Activator protein 1
BC	Before Christ
BCG	Bacillus
BGH	Bovine growth hormone
BMDMs	Bone Marrow-derived Macrophages
BMI	Body Mass Index
bp	Base Pair
BSA	Bovine serum albumin
BSL3	Bio Safety Level 3
CAF	Central analytical facility, Stellenbosch University
CaMKII	Calcium/Calmodulin-dependent Protein Kinase II
cDNA	Complementary De-oxy Ribo Nucleic Acid
CFU	Colony Forming Unit
CHUK	Conserved helix-loop-helix ubiquitous kinase
CMV	Cytomegalovirus
CO2	Carbon Deoxide
CPM	Count per million
CRs	Cell-surface receptors
CSF	Cerebrospinal fluid
DEGs	Differentially expressed genes
DH5α	E.coli cells

DM2	Diabetes mellitus 2
dsRNA	Double Stranded Ribo Nucleic Acid
EC_{50}	Half maximal effective concentration
ECL	Enterochromaffin-like
EEA1	Early Endosome Antigen 1
EPTB	Extrapulmonary tuberculosis
ERK1/2	Extracellular signal–regulated kinases 1/2
FBS	Foetal Bovine Serum
FCS	Foetal calf Serum
FDR	False discovery rate
FMHS	Faculty of Medicine and Health Sciences
g	gram
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
gDNA	Genomic De-oxy Ribose Nucleic Acid
gDNA	Genomic DNA
GTPase	Guanosine triphosphatase
GWAS	Genome Wide Association Study
HAM	Human alveolar macrophages
HBCs	High burden countries
HbhA	heparin binding hemagglutinin
HEPES	4-(2-Hydroxy Ethyl)-1-Piperazineethanesulfonic acid
HIV	Human Immuno deficiency Virus
HMDMs	Human Monocyte-derived Macrophages
HREC	Health Research Ethics Committee
Hs EIF2AK2	Eukaryotic translation initiation factor 2 alpha kinase 2
Hs GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Hs IDO1	Indoleamine 2,3-dioxygenase
Hs IFI44	Interferon induce protein
Hs IFI44L	Interferon induce protein
Hs IFIT1	Interferon induced protein with tetratricopeptide
Hs IFIT2	Interferon induced protein with tetratricopeptide
Hs IFIT3	Interferon induced protein with tetratricopeptide

Hs IFNγ	Interferon gamma
Hs IL12B	Interleukin -12-beta
Hs IL1β	Interleukin-1-beta
Hs IL23A	Interleukin-23-alpha
Hs IL6	Interleukin-6
Hs IL8	Interleukin-8
Hs ISG15	Interferon stimulated gene-15
Hs MT1A	Metastasis-associated protein-1-alpha
Hs MX1	Interferon induced GTP binding protein-1
Hs MX2	Interferon induced GTP binding protein-2
Hs RSAD2	Radical S-Adenosyl Methionine Domain-Containing protein 2
Hs TRIB3	Trible homolog 3
Hs UBC	Polyubiquitin-C
HSCs	Hematopoietic Stem Cells
IFNAR	The interferon-α/β receptor
IFN$\boldsymbol{\alpha}$	Interferon α
IFN$\boldsymbol{\beta}$	Interferon β
IPA	Ingenuity pathway analysis
IQR	Inter quartile range
JAK/STAT	Janus Kinase/Signal Transducer and Activator of Transcription
JNK	c-Jun N-terminal kinases
Kg	Kilogram
LAMP	Loop-mediated isothermal amplification
M. Smegmatis	*Mycobacterium smegmatis*
M1	Inflammatory macrophages M1
M2	Anti inflammatory macrohages M2
MAPK	Mitogen-activated protein kinase
MDA-5	Melanoma Differentiation-Associated protein 5
MDMs	Monocyte-derived Macrophages
MDR	Multidrug resistance
MIQUE	Minimum information for publication of quantitative real-time PCR expreriment

ml	millilitre
mM	Millimolar
MOI	Multiplicity of Infection
MOPS	3-(N-morpholino) propane sulfonic acid
mRNA	Messenger Ribo Nucleic Acid
MTB	*Mycobacterium tuberculosis*
MyD88	Myeloid differentiation primary response 88
NaCl	Sodium Chloride
NK	Natural killer cells
OADC	Oleate-Albumin-Dextrose-Catalase
OD	Optical density
P13K	Phosphoinositide 3-kinases
Partek E/M	Partek expectation-maximization
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Saline
pcDNA	Mammalian expression vector
PCR	Polymerase Chain Reaction
PE-PPGRS	Pro-Glu-polymorphic GC-rich sequences
pg	pico gram
pH	Pouvoir hydrogène
pM	Pico Molar
PRR	Pattern recognition receptor
PVDF	Polyvinylidene fluoride
QC	Quality control
qRT-PCR	quantitative Polymerase Chain Reaction
Rab5	Ras-related-protein 5
RBCs	Red Blood Cells
REL	proto-oncogene c-Rel
RIG-I	Retinoic acid-inducible gene-I
RIN	Ribo Nucleic Acid Integrity Number
RLT Buffer	Lysis buffer for cells and tissues (RNAeasy)™
rmp	round per minute

RNAi	Ribo Nucleic Acid Interference
RNAseq	Ribo Nucleic Acid Sequencing
RPE Buffer	RNA Precipitating Elution Buffer
RPMI	Roswell Park Memorial Institute
RPMI-1640	Roswell Park Memorial Institute Medium-1640
rRNA	Ribosomal ribonucleic acid
s	Seconds
SAPE	Streptavidin-R-Phycoerythrin
saRNA	Small Activating Ribo Nucleic Acid
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
siRNA	Small Inhibiting Ribo Nucleic Acid
SNPs	Single Nucleotide Polymorphism
SSF	Syringe Settle Filtrate
TACO	tryptophan aspartate-containing coat protein
TB	Tuberculosis
TBST	Tris-buffered saline tween
TCR	T cell receptor
TDM	Trehalose dimycolate
TE Buffer	Tris EDTA Buffer
TLR	Tall like receptor
TMAP	Texas Medication Algorithm Project
UBC	Polyubiquitin-C
UI	Un-Infected
V-ATPase	Vacuolar-ATPase
WHO	World Health Organization
WST	Water Soluble Tetrazolium
XDR	Xtreme Drug Resistance
Z Score	Standard score

List of figures

List of tables

CHAPTER 1

CHAPTER 1: INTRODUCTION

1.1 Tuberculosis

> *If the importance of a disease for mankind is measured from the number of fatalities which are due to it, then tuberculosis must be considered much more important than those most feared infectious diseases, plague, cholera, and the like. Statistics have shown that 1/7 of all humans die of tuberculosis.*
>
> *—Robert Koch (1882),* (*Die Ätiologie der Tuberculose*)

The above stated quote by Dr. Koch is more than a century old, but it remains true to this date as tuberculosis (TB) continues to be the major cause of high morbidity and mortality worldwide. This global public health menace is caused by a group of closely related intracellular gram-positive bacilli known as *Mycobacterium tuberculosis* (*M. tuberculosis*) (Dye, 2006). Mycobacteria are non-motile, non-sporulating, weakly gram-positive, acid-fast bacilli that appear as straight or slightly curved rods under a microscope. Dimensionally it is 1 to 4 μm long and 0.3 to 0.6 μm wide.

Mycobacteria are within the order Actinomycetales, which it shares with bacteria such as *Corynebacterium*, *Nocardia*, and *Rhodococcus*. It belongs to genus Mycobacterium, family Mycobacteriacea and phylum Actinobacteria. These bacteria also express unique mycolic acids in the cell envelope that play a critical role in the structure and function of the cell wall (Barry et al., 1998). The waxy cell wall confers many of the unique characteristics of this genus: acid-fastness, extreme hydrophobicity, resistance to drying, acidity/alkalinity as well as distinctive immune-stimulatory properties (Sakamoto et al., 2012).

M. tuberculosis is a member of the slow-growing pathogenic mycobacterial species, characterized by a 12 to 24-hour division rate and prolonged culture period on agar of up to 21 days. Why *M. tuberculosis* divides so slowly is not well understood. Proposed

mechanisms include restriction of nutrient uptake through the highly impermeable cell wall and slow rates of RNA synthesis (Harshey et al., 1977).

1.2 Epidemiology of TB

Globally, TB is one of the top ten causes of mortality and a leading cause of infection related death from a single agent. According to the World Health Organization (WHO), in 2017, there were 10 million incident cases of TB. TB resulted in 1.3 million deaths among human immunodeficiency virus HIV negative and 300 000 deaths among HIV positive individuals globally. Of 10 million people infected with TB, 5.8 million were men and 3.2 million were women, about 1 million children developed TB. **Figure 1.1** describes the global incidence of TB in 2017 and shows that South Africa is among the high burden countries (in dark-green).

World-wide majority (90%) of TB patients were adult (≥15 years) and 9% were people with HIV (72% of these in Africa alone). Of all TB cases world-wide, about two-third (66%) were present in eight countries namely; India (27%), China (9%), Indonesia (8%), Philippines (6%), Pakistan (5%), Nigeria (4%), Bangladesh (4%) and South Africa (3%). These eight countries together with 22 others make the 30 high burden countries (HBC) in WHO list (WHO | Global tuberculosis report 2018).

The emergence of HIV and the resulting acquired immune deficiency syndrome (AIDS) has aggravated the TB epidemic (Barnes et al., 1991). TB is an opportunistic infection, which can affect anyone but those with weakened immune systems are at greater risk. HIV infection results in depletion of the CD4 helper cells, which are one of the main defensive force against foreign antigens (pathogen microbes) (Vijayan et al., 2017). Infection with both HIV and TB is known as HIV/TB coinfection, and this coinfection is established as the main burden of infectious disease in resource-limited countries.

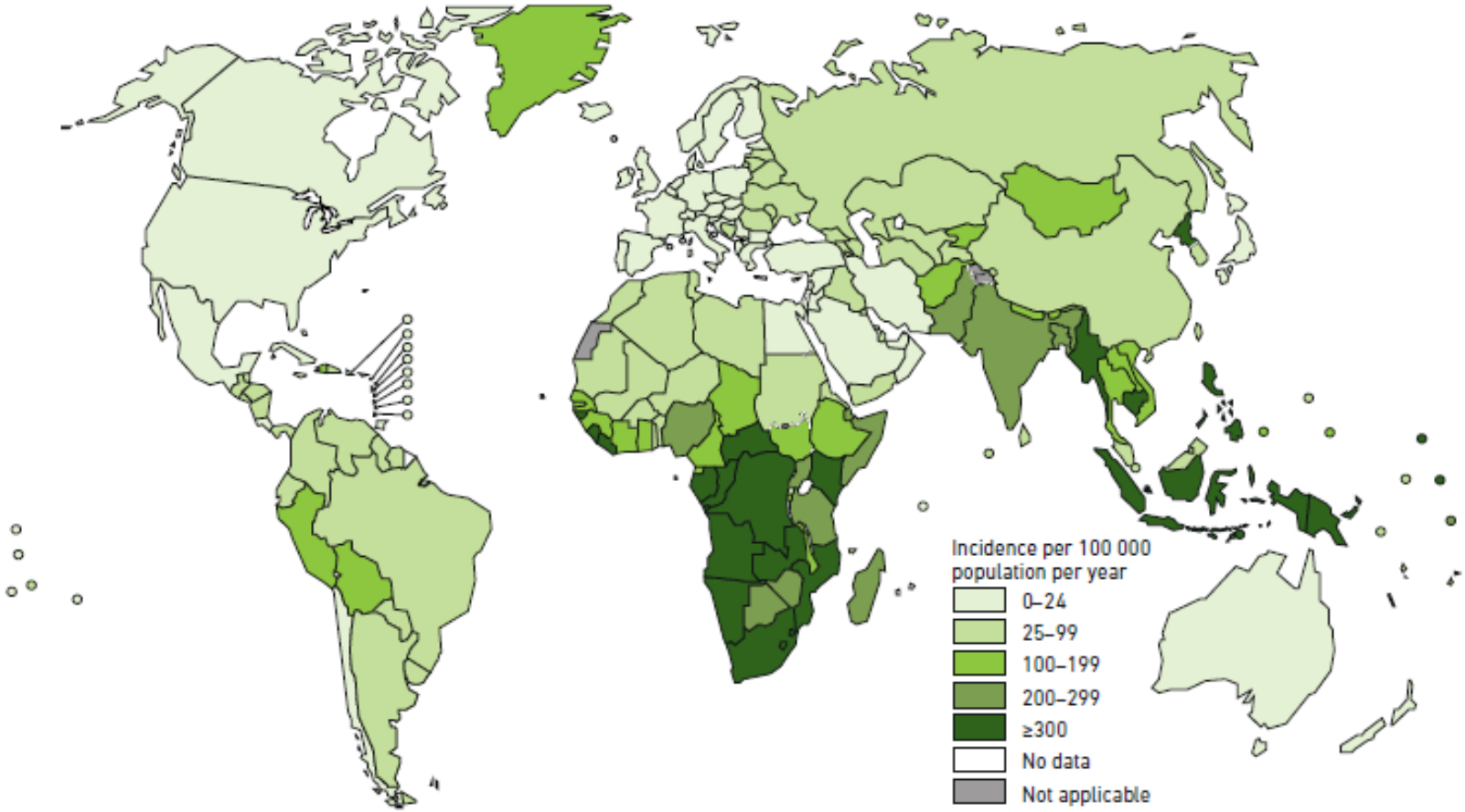


Figure 1.1: Estimated global TB incidence rates, 2017. South Africa is one of the countries with the highest burden of TB. Reproduced with permission from WHO, Global Tuberculosis Report 2018, **(**https://www.who.int/tb/publications/global_report/en/) (cited on July 4, 2019) ***(Appendix IX)***

In 2017, there were 51% cases of TB patients (464 633 out of 920 000 newly identified case of TB) had HIV, of these only 84% received anti-retroviral therapy. Majority of the gap in detection and treatment of HIV/TB coinfection was in WHO African region, where the burden of HIV-associated TB is highest (WHO | Global tuberculosis report 2018).

Another factor fuelling TB pandemic is the global increase of type 2 diabetes mellitus (T2DM). People with T2DM have three times higher risk to develop TB as compared to non-diabetic individuals (Ronacher et al., 2015). The alarming fact is that there are more individuals with T2DM than TB-HIV co-infection (Jeon et al., 2008). WHO has recognized T2DM as important and re-emerging risk factor towards the development of TB (Ottmani et al., 2010). One of the noteworthy factors for the development of active TB in patients with T2DM is undiagnosed latent TB (where *M. tuberculosis* bacteria remain dormant for years) prior to development of T2DM. In these cases, the dwindled host immune system in patients with T2DM resulted opportunistic infection like TB. Though researchers have suggested the effect of T2DM on TB, there is controversy over the directionality (i.e. which comes first?) due to the publications reporting temporary hyperglycaemia which resolves with anti-TB treatment (Başoğlu et al., 1990). It is expected that T2DM/TB comorbidity will be disturbing in coming years, since, 422 million people live with T2DM worldwide in 2016 (and the numbers are growing) (WHO | *Global report on diabetes*).

1.3 TB risk in South Africa

South Africa is among the eight high TB burden countries, contributing 3% of global TB incidence. In 2017, there were 500 new cases out of every 100 000 people in South Africa. This makes TB national health hazard claiming enormous loss of life every year (WHO | Global tuberculosis report 2018). South Africa has the highest number of HIV/TB co-infected cases globally, estimated at 193 000 in 2017. Though, statistics suggest a decrease in TB incidence rates and mortality, currently the rate of decrease in

TB incidence is slow to meet the sustainable development goals or the 2035 end TB strategy targets (WHO | WHO End TB Strategy).

As per the 2015 data, the provinces with highest rates of TB in South Africa are Eastern Cape, KwaZulu-Natal and Western Cape with 692, 685 and 681 cases per 100 000 respectively (TB Statistics for South Africa | National & Provincial). One of the identified reasons for slow decline of incidence cases can be attributed to the fact that only 53% of TB cases complete their treatment. Previous publications also pointed out losses at test access (5%), at diagnosis (13%), initiation of treatment (12%) and completion of treatment (17%) (Naidoo et al., 2017).

Globally, South Africa has an excessively higher number of multidrug resistant (MDR) and extensively drug-resistant (XDR) TB cases. About half-a-million cases of MDR-TB were reported in 2017, out of which about 19 000 were from South Africa (WHO | Global tuberculosis report 2018). Since the increased prevalence of MDR/XDR-TB is an indication of lacuna in the efficacy of existing national TB control programmes, there is an urgent need for new immune-therapies which could help in countering *M. tuberculosis* without generating drug resistance.

1.4 Types and symptoms

TB can be broadly classified into pulmonary (TB of lung) and extra pulmonary TB. Pulmonary TB comprises primary-pulmonary TB and reactivation of TB. Symptoms of primary-pulmonary TB are mild, e.g. low-grade fever, and two-thirds of such cases remain asymptomatic. Radiological evidence includes hilar adenopathy and mid/lower pulmonary infiltrates (Poulsen et al., 1950).

About ~90% of TB cases among the adults can be credited to reactivation (post-primary) TB, with fever, cough, weight loss, fatigue, and night sweats as common symptoms **(Figure 1.2)**. Less common symptoms include dyspnea, chest pain, and haemoptysis. The

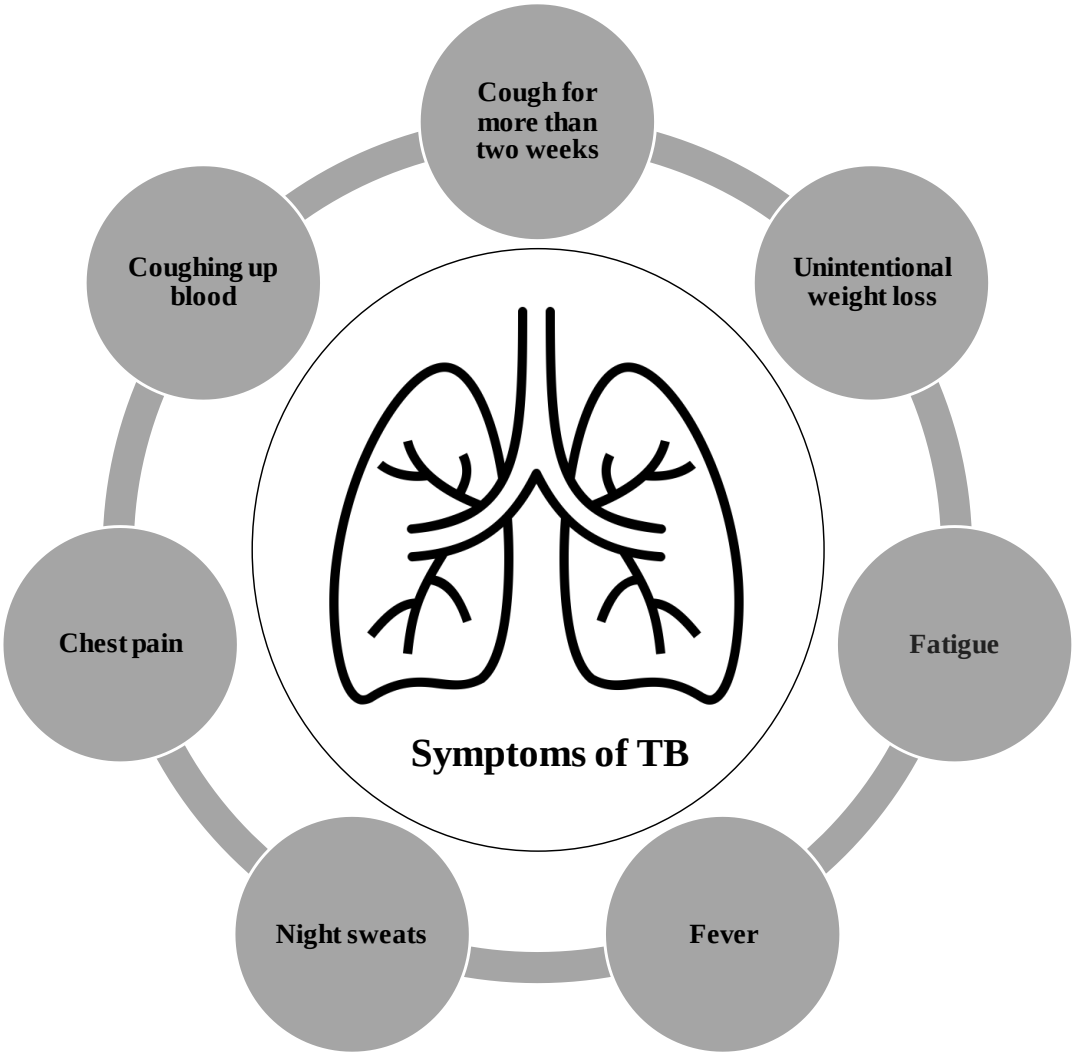


Figure 1.2: Symptoms of pulmonary tuberculosis. Pulmonary tuberculosis is curable with an early diagnosis and antibiotic treatment.

radiological evidence includes infiltrates in apical-posterior section of the upper lobes of the lungs, and about 20% of these infiltrates are accompanied with cavities by air-fluid (Im et al., 1993; Lee et al., 1993).

Extra pulmonary TB (EPTB) accounts for approximately 15% of TB cases among the immunocompetent hosts and ~50-70% cases occur in HIV-TB co-infected individuals (Peto et al., 2009). EPTB type may include lymph node, central nervous system (CNS),

pleural, peritoneal, pericardial, skeletal, genitourinary and miliary TB (Sia & Wieland, 2011). The laboratory diagnosis of EPTB is still substantially inadequate and largely dependent on discretion of the physician.

In the preantibiotic era, TB meningitis and miliary TB were often fatal, but self-healing occurred in ~50% of patients with pulmonary TB, indicating stronger host immune response in half of the patients (Millington et al., 2010). Also, in the first randomised control trial (RCT) of streptomycin vs bed rest published in Lancet (1948), about 1/3 of the patients recovered spontaneously in the bed rest arm ("STREPTOMYCIN treatment of tuberculous meningitis," 1948).

1.5 Enigma of host immune response to *M. tuberculosis*

On encountering *M. tuberculosis,* only 30% of exposed individuals acquire the primary TB infection whereas the remaining 70% stay uninfected, as bacilli are destroyed by the host's innate immune system through a mechanism largely unknown. Of 30% primary infected individuals, 5–10% progress to active TB disease and in remaining 90–95% individuals, infection is contained by the host immune system (Manabe et al., 2000). We need to understand the host response in the healthy individual towards pathogenic and non-pathogenic strains of mycobacteria before we can unravel the molecular mechanisms underpinning mycobacterial killing.

The response of the host immune system can be measured by the change in transcriptomic signatures such as RNAseq and AmpliSeq, and has been used previously before and after infection with *M. tuberculosis*. This can help to identify biomarkers suitable for potential diagnostic, prognostic, and therapeutic use. It is believed that immunotherapies could modulate the immune system in patients with latent TB infection or active disease, enabling better control of *M. tuberculosis* replication (Abate et al., 2016). The emergence of drug-resistant strains, the toxic effect of drugs on the human body and longer treatment regimens demand identification of newer targets for immuno-therapy of

TB. In the present study, we studied transcriptomic signatures using AmpliSeq to compare host response after infection with different mycobacterial strains.

1.6 Transcriptomics

The central dogma of molecular biology involves a two-step process, i.e. transcription and translation. These two are basic steps through which genetic information passes from genes to protein. A particular segment of DNA (gene) is transcribed into RNA by the specific enzyme RNA polymerase. The transcribed RNA includes protein coding (messenger RNA, i.e. mRNA) and non-coding RNA molecule (Venters et al., 2009). Messenger RNA is a short-lived transitional molecule in the information network, whilst noncoding RNAs have other miscellaneous functions. Transcriptomic technology records a snapshot in time of the total RNA present in a cell.

Transcriptomic is the study of transcription, covering the complete set of RNA molecules that are produced by the genome, under specific intervention or in a specific type of cell—using high-throughput methods, such as RNAseq, AmpliSeq and microarray. The term transcriptomic was first used in the 1990s, and scientific advancement since the 1990s have made transcriptomic an extensive discipline (Piétu et al., 1999; Velculescu et al., 1997). These developments helped to generate, and process huge biological data sets which are encouraging and are crucial shift in health sciences studies.

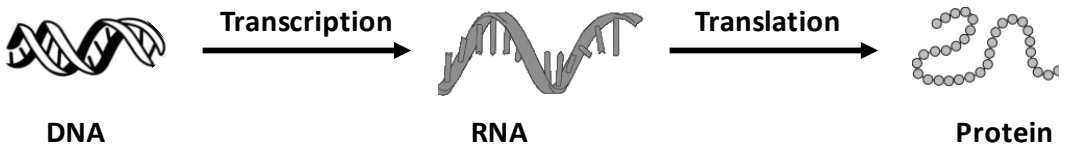


Figure 1.3: Central dogma of molecular biology where DNA transcribes to RNA and the RNA translates to protein

Transcriptomic analysis has empowered researchers to study how gene expression changes in diverse organisms and has been helpful in the understanding of human diseases (Li et al., 2015; Lowe et al., 2017). Transcriptomics have broad spectrum applications across

different diseases, including disease diagnosis and therapeutics. The transcriptome contains the full information of the transcribed RNA of the genome in a cell type or specific tissue, during a certain pathological or physiological condition (Byron et al., 2016; Jacquier et al., 2009).

Transcriptome analysis helps in understanding the transcription of human genome at the developmental level. More importantly, it may reveal the key modifications of biological processes activating human diseases, thus contributing novel tools useful not only for the understanding of their fundamental mechanisms but also for the molecular diagnosis and therapeutics. Numerous ongoing clinical trials employ transcriptome profiling strategies as an important tool in the diagnosis of several human pathologies such as molecular biomarkers and therapeutic targets (Casamassimi et al., 2017).

The major transcriptomic tools widely employed are RNAseq, AmpliSeq and Microarray. AmpliSeq and RNAseq are considered to be sharing similarities in sequencing and data processing with few differences. The major difference between AmpliSeq and RNAseq methodology is that AmpliSeq can profile over 20,000 different human RNA sequences (amplicon) using a highly multiplexed amplification method. Also, it has been shown by previous publications that AmpliSeq is a highly sensitive and cost-effective approach for gene expression analysis and identification of mRNA marker with high accuracy (Li et al., 2015). Each amplicon represents an exclusively targeted gene with an average size of ~150 bp. On the other hand, RNAseq is a powerful tool for the entire transcriptome gene expression analysis. However, it has few limitations, e.g., the prerequisite of a substantial amount of input RNA and complications due to the non-specific mapping of short sequences (Li et al., 2015).

RNAseq being more specific for global transcriptome analysis is used to analyse the changing cellular transcriptome in continuum (McHale et al., 2013). It enables to look at alternative gene spliced transcripts, post-transcriptional modifications, gene fusion,

mutations/small nucleotide polymorphisms (SNPs) and changes in gene expression with time, or differences in gene expression in different conditions (Costa et al., 2013). The limiting factor for RNAseq is that it is a read-out array of comparisons and associations under certain conditions.

On the other hand, AmpliSeq does not have quantification capacity for isoform-level or resolution up to a single base. But targeted and sequencing based quantification provide AmpliSeq technique an advantage in handling off-target amplification. Thus, AmpliSeq can diminish the complexity and could avoid problems related to non-specific mapping. Therefore, AmpliSeq stands as a very useful and cost-effective technique for large-scale high accuracy gene expression analysis (Li et al., 2015).

Table 1.1: Difference between RNAseq and AmpliSeq

RNAseq	**AmpliSeq**
Non-specific mapping of short reads	Specific mapping for the targeted gene mapping
Can profile entire transcriptome gene expression	Can profile over targeted 20,000 amplicons
Require significant amount of input RNA	Require less concentration of RNA
Expensive	Highly sensitive and cost-effective

1.7 Ingenuity pathway analysis (IPA)

Ingenuity pathway analysis permits searching and using information gathered from public databases and from peer researched articles. It categorises its results based on a curated ontology, encompassing biomolecules (proteins and other molecules) and interactions between the biomolecules (inhibits or activates) and functional information. IPA helps in

identifying canonical pathways, functions, gene networks and differentially expressed genes in specific diseases (Yu et al., 2016).

IPA is a web-based application that helps in analysing the role and function of biologically useful entities. It helps in establishing the new targets and candidate biomarkers in the context of biological systems. It provides useful information across different disciplines, helping to understand the role of biomolecules (Cirillo et al., 2017).

1.8 Multiplex ELISA (Luminex)

One of the powerful techniques to measure the biomarkers of biological importance in a specimen and cell culture supernatants is multiplex immunoassay. It utilizes magnetic beads to simultaneously measure the level of multiple analytes in a single specimen (Elshal et al., 2006). Multiplex immunoassay is an improvised alternate of enzyme linked immunoassay (ELISA) using the magnetic beads adhering to the capture antibody. Multiplex assay usually follows the principle of sandwich ELISA, where microspheres of different colours are coated with specific antibodies (capture antibody), that adhere to the complementary biological marker present in the specimen, followed by subsequent binding of the complex to the detection antibody releasing the signal (Schmidt et al., 2012).

The illumination signals from the color-coded microbeads are than read by dual-laser flow-based multimode readers (e.g. Luminex ® 200™). One laser identifies and categories the beads, while the second laser determines the degree of dye-related signal to decipher the amount of analyte.

1.9 Pathophysiology of *Mycobacterium tuberculosis*

Tuberculosis caused by *M. tuberculosis* is one of the oldest known infections to human beings. The pathophysiology of *M. tuberculosis* infection remained intricate to researchers for several decades. Previous research work establishes monocytes, particularly macrophages responsible for the primary host cellular defence against *M.*

tuberculosis (Zhai et al., 2019). Macrophages are present in virtually all tissues. The cellular differentiation of these cells start with progenitor hematopoietic stem cells (HSCs) in the bone marrow, which are precursor cells for distinct cellular lineages. These stem cells are further divided into myeloid and lymphoid progenitors. Peripheral blood mononuclear cells (PBMCs) include both these above said categories. Monocyte derived macrophages (MDMs) are generated from myeloid progenitors which are prime cells involved in phagocytosis of *M. tuberculosis* (McGrath et al., 2015).

Since cellular fate is defined by either differentiation or proliferation, it is important to note the basic difference between the two, which often confounds with each other. Proliferation may be defined as the cellular expansion by the continuous division of single cells into two daughter cells. On the other hand, differentiation involves undifferentiated cell, e.g. a stem cell, acquiring the features of a specialized cell (Trosko et al., 2008).

In the peripheral blood, monocytes are not a homogeneous population, and there is substantial evidence that specific monocytes give rise to tissue specific macrophages (Mosser et al., 2008). Although the enigma about the heterogeneity of macrophages is not fully understood, one school of thought emphasizes that monocytes continue to develop, mature and differentiate to macrophages in the blood (Yang et al., 2014). The monocytes which differentiate to macrophages, polarize to M1 and M2 state under specific stimulations. The M1 macrophages are pro-inflammatory and the M2 macrophages are anti-inflammatory in nature (Liu et al., 2014).

Macrophages are exceptional phagocytic cells that clear up to 2×10^{11} erythrocytes daily; this amounts to 3 kg of iron and haemoglobin each year that is ‘recycled’ for the host. This process is a crucial metabolic contribution, affecting the survival of the host. Macrophages also contribute to the clearance of cellular debris generated during tissue remodelling and apoptosis (Brady et al., 2016).

Some of the previous research work demonstrated that infection of MDMs by *M. tuberculosis* results in early apoptosis of helper T cells. Hence, this could lead to a supressed immune response in the host and survival of *M. tuberculosis* due to suitable niche which promotes survival (Smith et al., 2003). Further, the survival of *M. tuberculosis* within host macrophages is controlled by the phenotypic nature of pathogen and immune response of the host. Experiments using high-virulence strain (*M. tuberculosis*) and studying global transcriptome using RNAseq in mice bone marrow derived macrophages (BMDMs) revealed the role of few early response genes (Leisching et al., 2017).

The maturation of the phagosome after infection with *M. tuberculosis*, is a well-known phenomenon. For its pathogenic activity *M. tuberculosis* needs to evade host immune cell signalling pathways to enhance its intracellular survival. The interruption of cell signalling resulted in impaired maturation of phagosomes to phagolysosomes (the acidic organelles rich in hydrolytic enzymes which digest engulfed bacteria/pathogens), tempers host-cell apoptosis and suppress host-immune response (Koul et al., 2004). Pathogenic *M. tuberculosis* enters into the macrophages using different cell-surface receptors such as mannose, Fcγ and integrin family (CRs 1, 3 and 4) receptors (Ernst et al., 1998). These receptors are important for feat of *M. tuberculosis* against macrophages and, which is evident by the fact that an antibodies against CR3 hinders mycobacterial internalisation by 80% (Schlesinger et al., 1990). Several bacterial pathogens use CR receptors to block the production of harmful oxygen intermediates by inhibiting production of NADPH oxidase to phagosomes (Caron et al., 1998; Hellwig et al., 2001). Although, CR3 is important for internalisation of *M. tuberculosis*, current research suggests that CR3 knockout mice have no alteration in mycobacterial survival (Melo et al., 2000).

Pathogenic *M. tuberculosis* tend to bind to those phagosomes that fail to fuse to lysosomes, and hence, do not undergo further acidification due to absence of proton-

ATPase (H^+ pump) molecules from vacuolar membrane, and reduced level of acidification facilitates the intracellular growth and survival of *M. tuberculosis* (Sturgill et al., 1994). Another important biomolecule is TACO (a tryptophan aspartate-containing coat protein), the stable association of TACO with mycobacterial phagosomes inhibit the fusion with lysosome. However, TACO is involved in uptake of *M. bovis* BCG in human macrophages but not in phagosome (Schüller et al., 2001).

1.10 Intracellular pathways effected by mycobacteria

Mycobacteria is also believed to interfere with intracellular Ca^{2+} and PI3K signalling pathway to impair phagosome maturation, both Ca^{2+} and PI3K are important for maturation of phagosome (Christoforidis et al., 1999; Jaconi et al., 1990; Wurmser et al., 1999). Another mycobacterial evasion mechanism involves interference of MAPK pathway. MAPKs enzymes are crucial in signal transduction. The three main families of MAPKs are, a) JNKs 1, 2 and 3 (c-Jun N-terminal kinases), b) ERK1/2 (the extracellular signal-related kinases) and c) p38 α, β, γ and δ (p38 MAPK). These MAPKs phosphorylate a variety of kinases, including transcription factor activator protein 1 (AP1), thereby controlling the synthesis of pro-inflammatory cytokines like IL-1, TNF-α and IL-12 (Johnson et al., 2002).

Mycobacteria can also evade phagocytosis by suppressing IFN-γ and JAK/STAT signalling pathways (Hussain et al., 1999). Both IFN-γ and JAK/STAT have been shown to be vital for antibacterial response (Decker et al., 2002). Phosphorylation of STAT and JAK1/2 is facilitated by the binding of IFN-γ to its cell surface receptor, which results in strong bactericidal response, due to production of reactive oxygen and nitrogen intermediates and synthesis of cytokines like IL-12 and TNF-α.

It is also known that mycobacteria avert the adaptive immune response by meddling with antigen presenting cells such as macrophages and dendritic cells. Mycobacteria use

various mechanisms for defeating antigen presentation, including sequestering mycobacterial antigens from molecules that are required for T-cell activation (Pancholi et al., 1993). Mycobacteria can also downregulate the expression of MHC class II, and co-stimulatory molecules like CD1 (Mariotti et al., 2002; Wojciechowski, et al., 1999).

Vacuoles comprising pathogenic mycobacteria are accompanied by actin-binding protein - tryptophan aspartate-containing coat protein (TACO) - and a small GTPase - Rab5 - on their outermost membranes. These organelles contain little of the phosphorylated and activated form of calmodulin-dependent protein kinase II (CaMKII) **(Figure 1.4).**

Vacuoles comprising non-pathogenic mycobacteria show initial endosomal markers, such as Rab5 and early endosomal antigen (EEA1). These vacuoles bind with late endosomal vesicles and obtain proteins such as proton-ATPase pump, lysosome-associated membrane glycoprotein 1 (LAMP1), V-ATPase and lysosomal hydrolases like cathepsin D - an aspartyl protease. Though vacuoles comprising pathogenic mycobacteria do not bind to lysosomes/late endosomes (represented by a red cross in Figure 1.4) due to host factors largely unknown, they still obtain immature pro-cathepsin D from the *trans*-Golgi network, which is evident by their dynamic nature and availability to components of the endosomal pathway. Finally, phagosome comprising pathogenic mycobacterial evasion binding to lysosome results in survival and replication inside the phagosomes, whereas non-pathogenic mycobacteria are readily killed by formation of phagolysosomes.

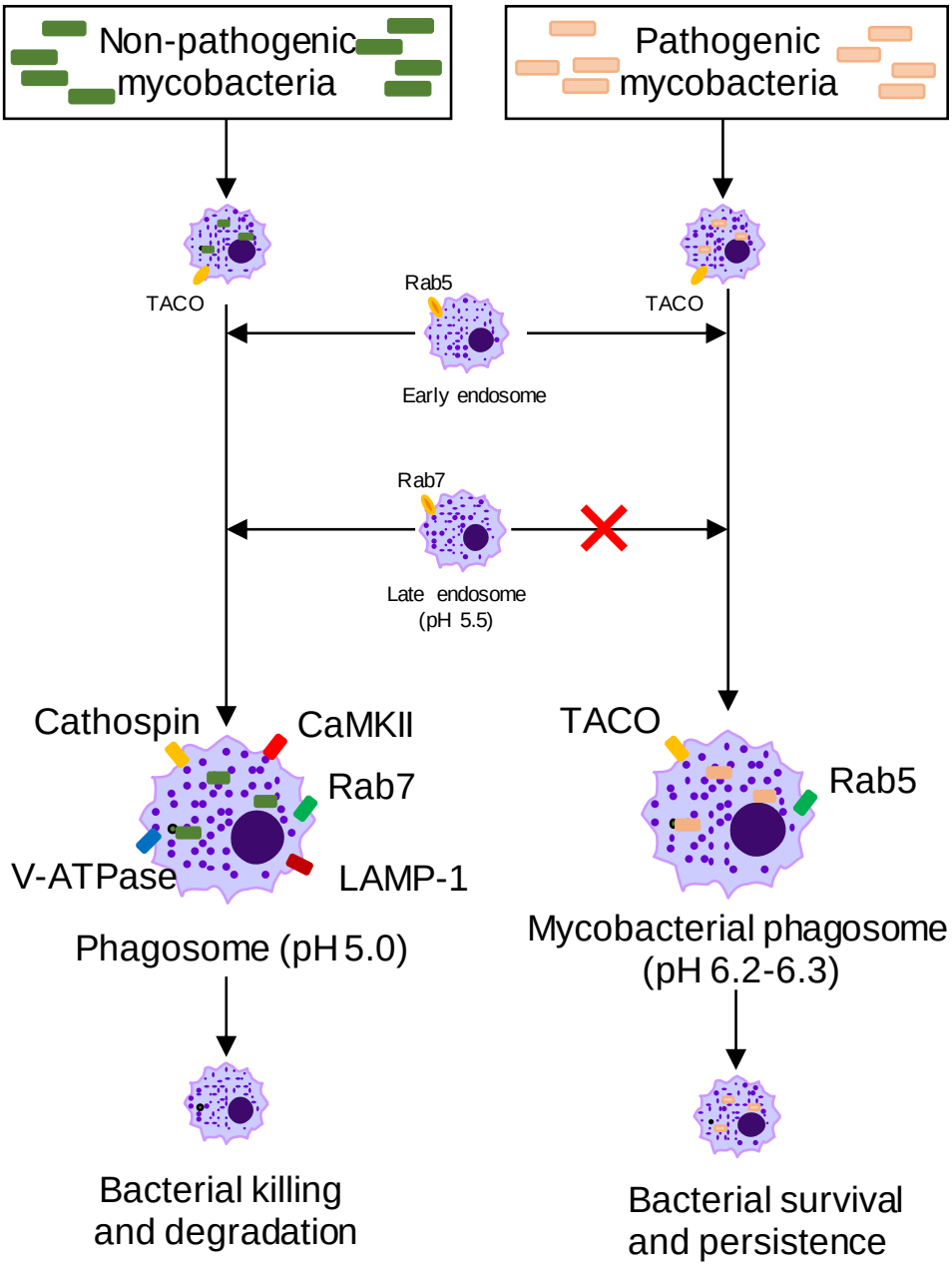


Figure1.4: Comparison of phagosomes harbouring pathogenic or non-pathogenic mycobacteria. This depicts mounting of effective host immune response against non-pathogenic mycobacteria (at enzyme/protein level) as compared to pathogenic mycobacteria.
(Image adapted from Koul *et. al*, 2004, Nature Microbiology)

These phagolysosomes are rich in low pH hydrolytic enzymes, hence indicating successful transcriptomic host response in non-pathogenic mycobacteria as compared to pathogenic mycobacteria.

A recent publication used AmpliSeq for comparing human alveolar macrophages (HAM) and monocyte derived macrophages (MDM) and identified key genes involved in response to *M. tuberculosis* infection. The results indicated that transcriptional response

from MDM is greater than HAM upon *M. tuberculosis* infection, with 2 -10 times more genes up/down-regulated (Papp et al., 2018). The results indicated the advantage and importance of studying *in vitro M. tuberculosis* infection using MDMs.

1.11 Immune response to mycobacteria

Host immune response, tissue loss and subsequent repair processes recognized during studies of cancer immunotherapy share common mechanisms with MDR-TB. T-cell receptors aiming host-derived mutated molecules (major histocompatibility class-dependent/ independent) can be devised for identification by TCR γδ and natural killer (NK) cells. T-cell subsets and NK cells are known to be protective against TB (and MDR-TB). These cells can also be triggered by immune checkpoint inhibitor (ICI) or derived from allogeneic sources and serve as potentially improved clinical outcomes against TB (Rao et al., 2019). Further, it has been indicated that the use of immunotherapy with IL-2, IL-7 and IFN-γ as an adjunct to anti-TB treatment may improve treatment of MDR-TB, reduce treatment time for drug-sensitive TB, and improve the immunity of individuals by enhancing *M. tuberculosis* elimination to prevent recurrence of the disease (Uhlin et al., 2012).These results indicate the effectiveness of immunotherapy in fight against TB.

A recent study used BMDMs stimulated with IFN-γ (M1 macrophages) or IL-4/IL-13 (M2 macrophages) in a time-dependent manner using unique CAGE technology. They showed that *M. tuberculosis* infection broadly and considerably alters macrophage gene expression with induction of inflammatory response, which is far larger than that of the activation of M1 or M2 macrophages. This immense gene expression change was facilitated by expressional modification of various known and novel *M. tuberculosis*-infection related genes including both M1 and M2 activation. The pre-activation brings global effect on transcriptional landscape of *M. tuberculosis*-infected macrophages by augmenting M1 or M2 genes along with different features of lncRNA expression in *M. tuberculosis*-infection (Roy et al., 2018).

In order to recognize the factors of *M. tuberculosis* that may be responsible for effective survival of intracellular bacteria, many distinct genes whose expression levels are up-regulated inside the phagosome have been examined in the past (Dubnau et al., 2002). We now need to evaluate host immune response against different strains of mycobacteria.

As mentioned previously, host immune response is believed to be different against pathogenic (e.g. R179) and non-pathogenic mycobacteria (*M. smegmatis*). R179, a known multi drug-resistant clinical isolate (Beijing genotype strain R220) prevalent in the western cape of South Africa was used as pathogenic strain in the present study (Ioerger et al., 2010). *M. bovis* BCG is known to cause disease in immunocompromised individual (Bohsali et al., 2010). On the other hand, *M. smegmatis* being a non-pathogenic mycobacterial strain is known to be killed within 48 hours of *in vitro* infection (Anes et al., 2003; Kuehnel et al., 2001). It is therefore important to study host response against various mycobacterial strains based on their pathogenicity at different time points of infection.

1.12 Rationale of the study

In the present study, we compared broad spectrum *in vitro* host response in human monocyte derived macrophages (hMDMs) against pathogenic (R179), non-pathogenic (*M. smegmatis* and *M. bovis* BCG) mycobacterial strains. All mycobacterial strains were grown in the absence of detergent, as tween-80 is known to alter macrophage uptake and immune response to *M. tuberculosis* (Leisching et al., 2016). Some of the previous publications demonstrated that Tween induces alteration on the mycobacterial cell wall, and thus, affects uptake of *M. tuberculosis* by macrophages and the host immune response to *M. tuberculosis* (Sani et al., 2010; Wang et al., 2011). Tween was introduced to mycobacterial culture almost 73 years ago and is found to be effective in reducing cell clumping and obtaining homogenous culture (Dubos et al., 1946). The cell clumping of

mycobacteria can be attributed to many factors, mainly associated with cell wall structure, including lipid cell wall component such as trehalose dimycolate (TDM), HbhA and PE-PPGRS proteins (Brennan et al., 2001; Menozzi et al., 1996). TDMs are known to have immunostimulatory properties and are important for broad-spectrum resistance to infectious agents (Madonna et al., 1989; Yarkoni & Bekierkunst, 1976).

Previous studies have demonstrated the differential regulation of genes encoding PE/PPE family proteins within the macrophages associated with the rearrangement of the bacterial cell envelope (Delogu et al., 2006; Dheenadhayalan et al., 2006). Also, the host transcriptional response of *M. tuberculosis* inside THP-1-like macrophages was compared to global *in vitro* gene expression in stress conditions like low pH (Fisher et al., 2002), starvation (Betts et al., 2002), heat-shock (Steyn et al., 2002), hypoxia (Voskuil et al., 2004), low iron (Rodriguez et al., 2002), and transcriptomic response of *M. tuberculosis* in resting murine bone marrow macrophages (BMM) (Schnappinger et al., 2003). It was discovered that a significant number of DEGs were regulated in THP-1 cells under different stresses indicating that these *in vitro* conditions can mimic some of the expected properties of the intra-phagosome environment.

There is a need for intervention experiments using knock-down and knock-up (vector-based over-expression) of differentially expressed transcriptomes to decipher their functions and comprehensive roles. Small interfering RNA (siRNA) is one of the most commonly used RNA interference (RNAi) technique for short-term silencing of protein coding genes.

siRNA is a synthetic RNA duplex designed to specifically target a particular mRNA for degradation (Vickers et al., 2009). This can provide a powerful tool as a follow-up experiment to knock-down differentially expressed transcriptomes establishing their potential functions and roles in the pathophysiology of the disease. In the present study,

we used vector-based *in vitro* transfection for knocking-up differentially expressed transcripts.

Some of the previous publications encompassing the study of the transcriptome in mice BMDMs (bone marrow derived macrophages) infected with *M. tuberculosis* using RNAseq, showed upregulation of high virulence gene (Jang et al., 2013). They lacked comparison of the transcriptome, during infection of human MDMs with pathogenic and non-pathogenic *M. tuberculosis* strains and also, there was a need for carrying out the downstream processing [knock-up (vector-based over-expression) and knock-down based experiments] for differentially expressed transcriptomes. This could establish their functional role and could lead to host based therapeutics.

In the present sutdy, we analysed the early transcription profiles of the interplay between the host and pathogenic/non-pathogenic mycobacterial strains (with vastly distinctive characteristics). **Figure** 1.5 & 1.6 depicts the systematic representation of the study hypothesis. As we know that pathogenic bacteria survives inside the phagosome evading formation of phagolysosome and non-pathogenic mycobacteria fails to do so (left hand side **Figure** 1.5). In this study the non-pathogenic *M. smegmatis* was employed as the only known mycobacterial species that is killed in primary human macrophages (Jordao et al., 2008) The non-pathogenic *M. bovis* BCG and the pathogenic *M. tuberculosis* species have been demonstrated to survive in primary human macrophages . We therefore endeavoured to identify genes that were differentially expressed in hMDMs between *M. smegmatis*, *M. bovis* BCG and *M. tuberculosis*. We hypothesised that the differentially expressed genes (DEGs) may potentially be involved in the killing of *M. smegmatis* in human macrophages. We therefore speculate that comparing *in vitro* infection of human monocyte derived macrophages (hMDMs) by pathogenic and non-pathogenic mycobacteria followed by their culture at different time points may reveal the enigma behind host-immune response against mycobacteria.

Using Ion-torrent AmpliSeq we were able to identify differentially expressed genes (DEGs) as host response from three distinct mycobacterial strains at various time points. The key DEGs, were choosen for subsequent knock up/down using vector-based transfection/siRNA approach respectively, for the potential *in vitro* intervention where intracellular mycobacterial survival was studied using colony-forming units (CFUs). These results were validated using qRT-PCR and supported by cell cytotoxicity assay (to assess host cell survival post knock-up (vector-based over-expression)/knock-down approach), and Western blotting. We performed Multiplex ELISA assay (Luminex) to study the cytokine profiles. This knowledge will broaden the scope of host drug targets for resistance free bacteriostatic immuno-therapy adjunct to current chemotherapy.

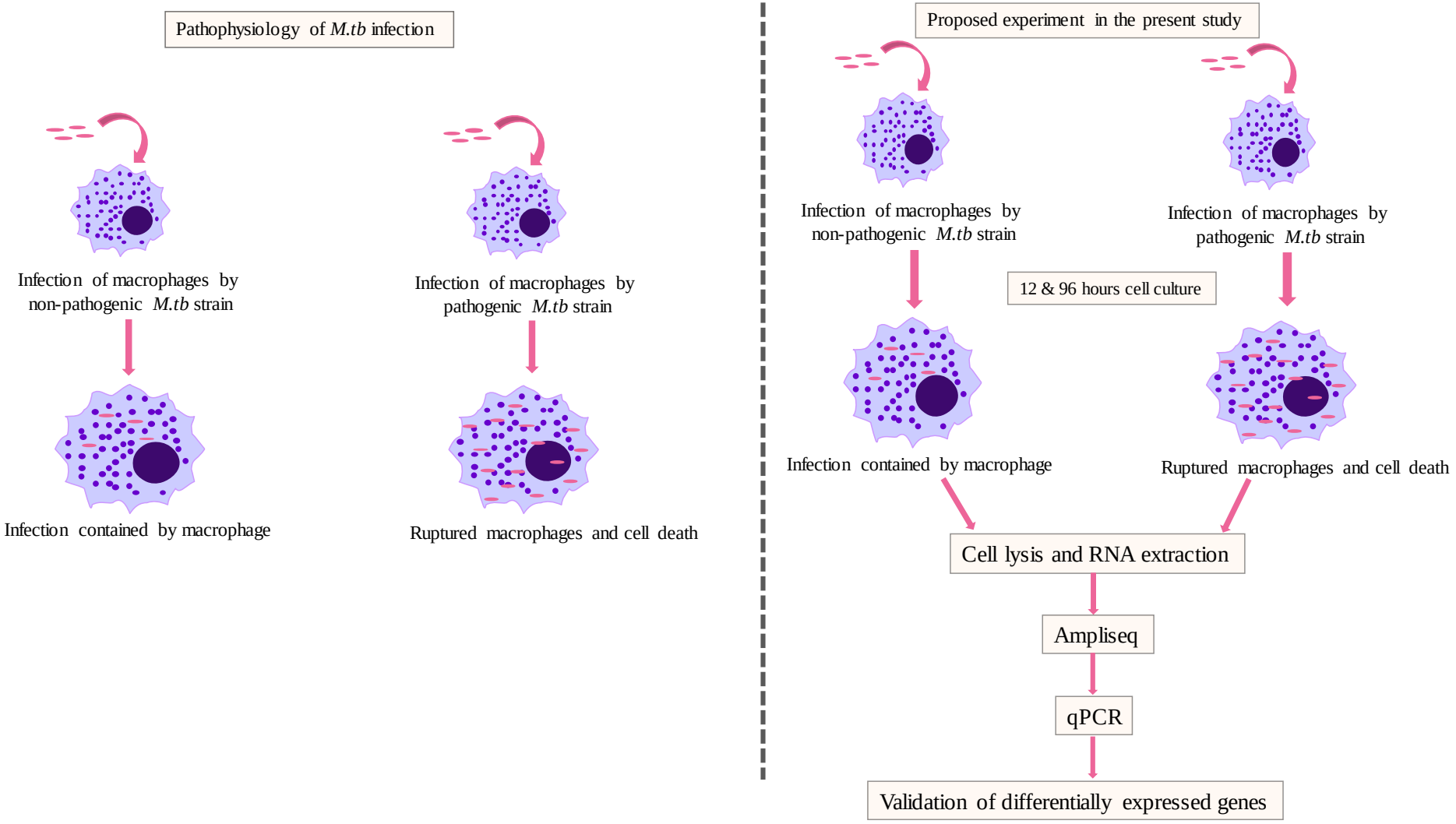


Figure 1.5: Pathophysiology of macrophage infection with mycobacteria and outline of the study
Abbreviations: M. tuberculosis=Mycobacterium tuberculosis, RNAseq=RNA sequencing, qRT-PCR=Quantitative PCR

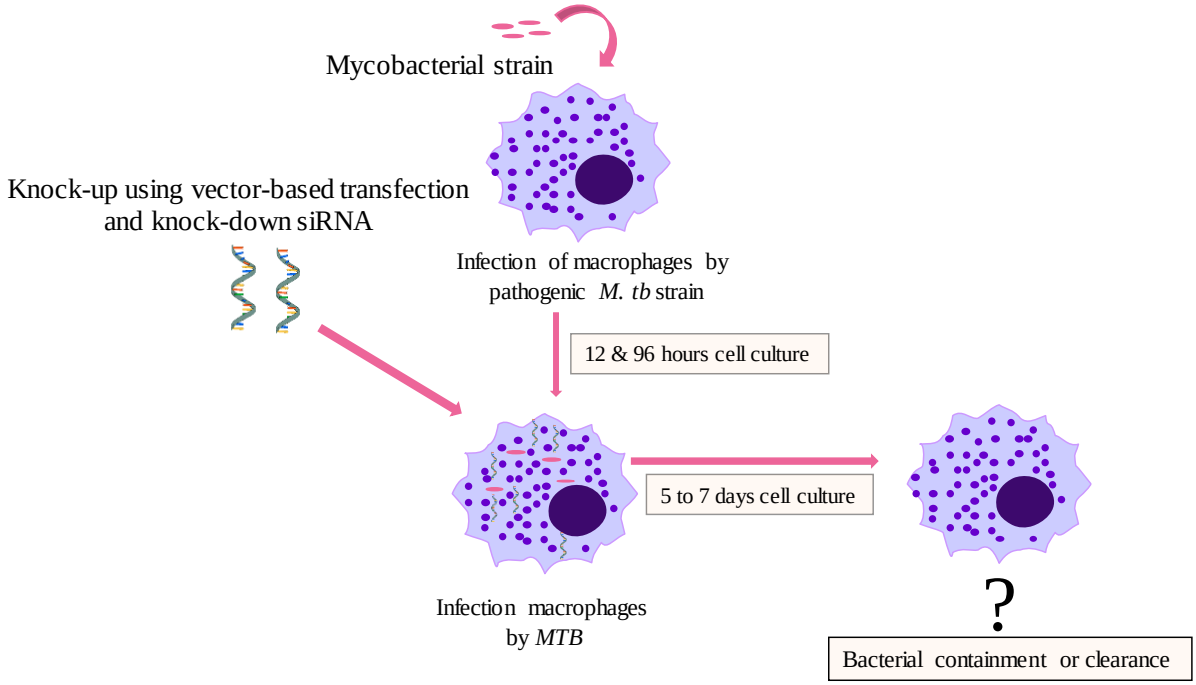


Figure 1.6: Proposed knock-up (vector-based over-expression)/knock-down experiment to establish functional role of differentially expressed gene(s). This can help in identifying markers of therapeutic potential against tuberculosis.
Abbreviations: M. tuberculosis=Mycobacterium tuberculosis, siRNA=Small interfering RNA, saRNA=Small activating

CHAPTER 2

CHAPTER 2: Transcriptomic investigation of the human macrophage response upon infection with mycobacteria

2.1 Study Design:

In the present study, we measured host-immune response from human monocyte derived macrophages against three strains of mycobacteria with varying pathogenicity. We chose to study host-immune response against non-pathogenic and pathogenic drug-resistant mycobacteria. Following are the aim and objectives of the study:

Aim: To investigate human macrophage response to infection with pathogenic and non-pathogenic mycobacteria

Objectives:

1. To enumerate and compare (AmpliSeq) differentially expressed transcriptomes from human monocyte derived macrophages (hMDMs) post infection with various mycobacterial strains (*M. smegmatis*, *M. bovis* BCG, *M. tuberculosis* R179 (clinical isolate))
2. To validate (qRT-PCR) differentially expressed transcriptomes from human monocyte derived macrophages (hMDMs) post infection with various mycobacterial strains
3. To study the cytokines expression (Multiplex ELISA) of the differentially expressed transcriptomes from human monocyte derived macrophages (hMDMs) post infection with various mycobacterial strains
4. To study the Biological network analysis (IPA) of the differentially expressed transcriptomes from human monocyte derived macrophages (hMDMs) post infection with various mycobacterial strains

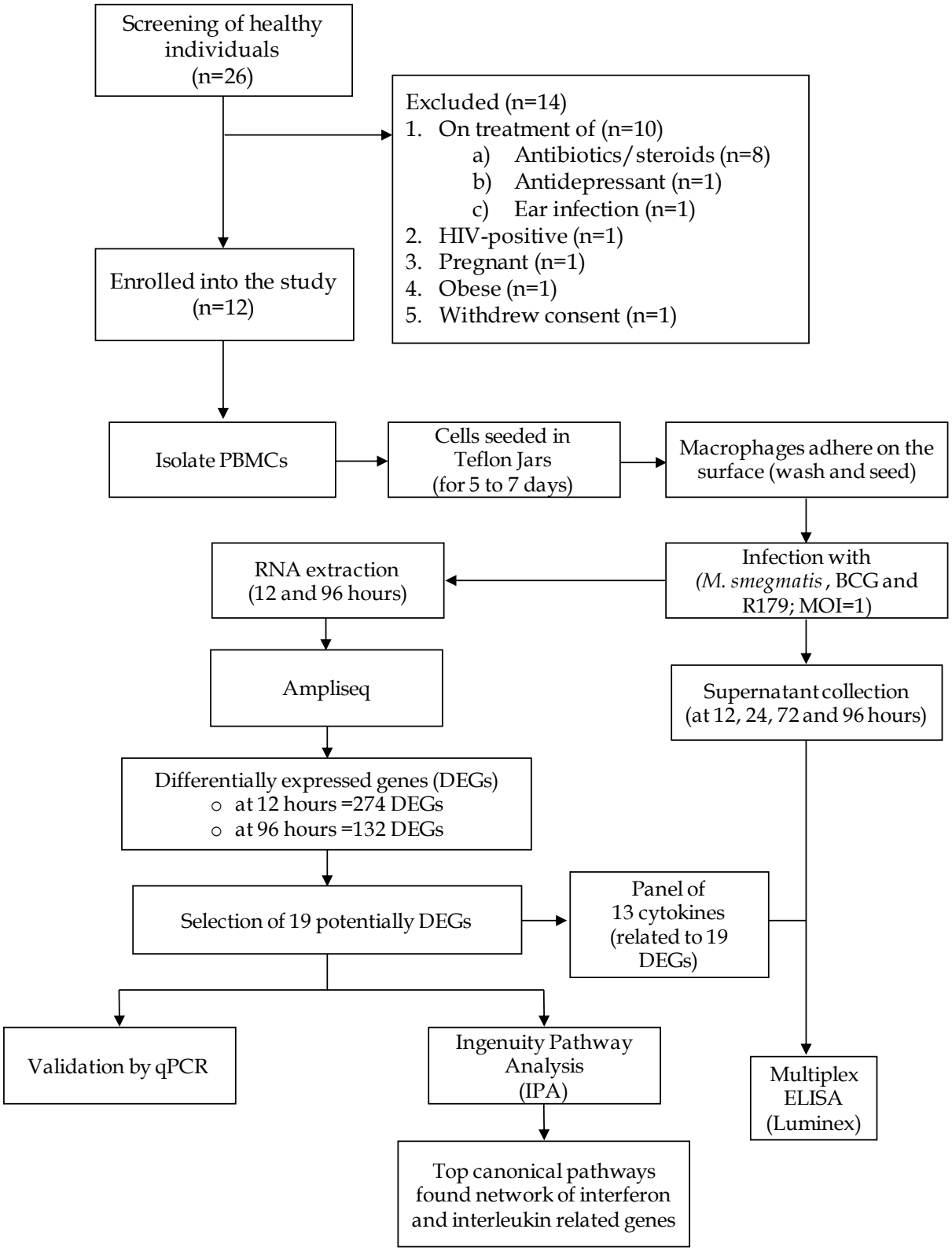


Figure 2.1: Flow of study and methodology.
Abbreviations: n = Sample number, PBMCs = Peripheral Blood Mononuclear Cells, DEGs = differentially expressed genes, MOI = Multiplicity of infection, qRT-PCR = Quantitative Polymerase Chain Reaction.

Materials and Methods

We conducted a detailed investigation of *in vitro* host response (from hMDMs of healthy individuals) towards different strains of mycobacteria (grown in detergent-free media), i.e. pathogenic (*M. tb* R179) and non-pathogenic (*M. smegmatis* and *M. bovis* BCG). The host response was measured post-infection using AmpliSeq which was validated through quantitative real time PCR. Multiplex ELISA (Luminex) was performed to study the cytokines expression, intracellular biological network was analysed through IPA and intracellular mycobacterial survival was measured through CFUs.

2.2 Recruitment of healthy controls

Study participants were enrolled in the study by a Good Clinical Practice (GCP) trained research nurse. Each participant was enrolled after written informed consent was obtained. For attracting the potential volunteers, advertisements were floated in the Faculty of Medicine and Health Sciences (FMHS), Stellenbosch University (Appendix I). As per the recommendations by the Institutional Ethics Committee, each participant was compensated for their time for participation in the study.

We screened 26 individuals, out of which 54% (14/26) were excluded based on the exclusion criteria of the study. Volunteers on antibiotics/steroid (n=8), antidepressant (n=1), with ear infection (n=1), HIV (n=1), pregnancy (n=1), and obesity (n=1) were excluded. One volunteer (n=1) withdrew consent.

Enrolled participants with no indication of diseases based on medical record and clear chest X-ray (if not, Chest X-ray performed and paid by the Department) were included. Equal number of males and females were recruited (six males and six females). According to the self-reported ethnicity, six of the participants were black and six were white. The

twelve healthy participants, meeting the inclusion criteria were finally enrolled for phlebotomy. A total of 80 ml (ethically permitted) of blood was drawn from each participant. **Figure 2.1** provides a detailed summary of the enrolment of study participants and flow of methodology.

Inclusion criteria:

1. Age between 21-40 years
2. BMI (body mass index) between 18-25
3. Clear chest X-ray
4. IGRA test negative (Pai et al., 2003)
5. Provide written informed consent **(Appendix IV)**
6. Healthy life style (non-smoker, good sleeping habits, low alcohol consumption)

Exclusion criteria:

1. On any medication (antibiotics or antipyretics)
2. On steroids
3. Suffering from any chronic disease or history of HIV infection
4. Surgery in the recent past
5. Pregnant or anaemic
6. Poor sleep habits

2.3 Ethics approval

Ethical permission for the study was obtained from the Health Research Ethics Committee (HREC), Stellenbosch University, Tygerberg campus, Cape Town (HREC Reference #S17/10/211). Ethical permission was renewed annually (2018-2019).

2.4 Cell isolation and culture of hMDMs

Eighty millilitres of intravenous blood was collected from each study participant in Na-Heparin vacutainers (VGRV455051R, Lasec). Whole blood was mixed with Phosphate Buffer Saline (PBS) in 1:1 ratio and the diluted mixture was gently poured over the Histopaque (10771, Sigma Aldrich) layer and subjected to centrifugation at 805 g for 20 min. The white buffy coat that appears between the blood plasma and the RBCs, containing PBMCs was carefully aspirated and collected in clean 50mL falcon tubes and washed twice with PBS.

The number of total cells (PBMCs) was counted by an automated cell counter (Countess, C10281, Invitrogen) and validated through manual counting. Human macrophages were cultured from PBMCs in Teflon jars (De Bruyn Spectroscopic solutions, Cat. No. 100-0120-01-Savillex) using RPMI-1640 medium supplemented with 20% heparinized plasma and incubated at 37^0C, 5% CO_2 for 5 days. The Teflon jars were loosely capped and kept inside the incubator to ensure proper aeration and optimal growth of cells. This allowed the differentiation of monocytes into macrophages.

After 5 days, the lids of the Teflon jars were tightened and placed on ice for 30 minutes. Cells were collected from the Teflon jars using a Pasteur pipette. Each Teflon jar was washed and pooled into the same tube with cold RPMI-HEPES (4 ml per wash). The tubes were centrifuged at 130 × g for 10 min at 4^0C without a brake. The pellet was re-suspended in RPMI-glutamine and cell counting was performed.

Small cells as lymphocytes and large cells as hMDMs, were counted separately. The required volume of heparinized plasma (20% final) and RPMI-glutamine was added to the cell suspension and plated in 12-well culture plates. The cells were incubated for 2 hours at 37^0C with 5% CO_2 for adherence of hMDMs (for 2 hours, otherwise lymphocytes will be difficult to remove) (Schlesinger et al., 1990). Cells were transferred to a CO_2 incubator in a biosafety

level 3 (BSL3) laboratory and proceeded with infection. **Figure 2.2** provides the detail flow of PBMC isolation, macrophage differentiation and maturation.

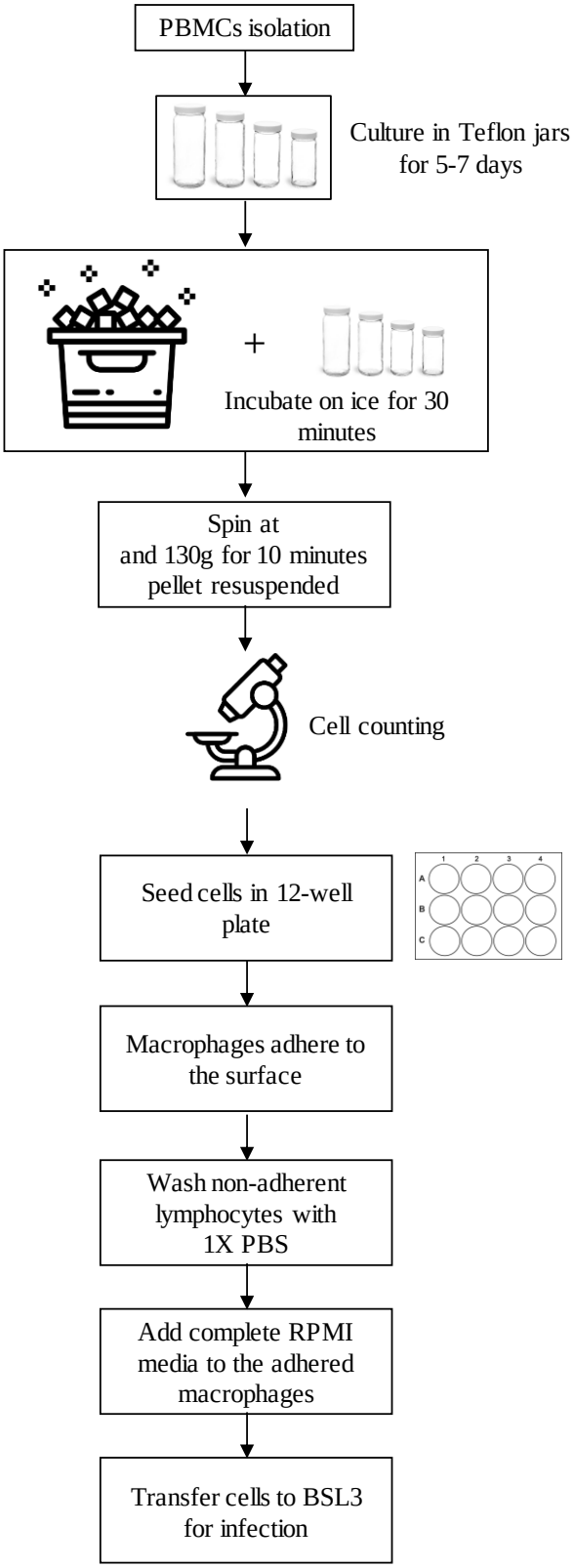


Figure 2.2: Isolation of PBMCs and differentiation into macrophages.

2.5 Detergent-free mycobacteria preparation for infection

The three different strains of mycobacteria (*M. smegmatis*, *M. bovis* BCG and *M. tuberculosis* R179) were cultured separately in T25 flasks with 10 ml volume in each flask (to have an appropriate air space). These were incubated in a 37^0C incubator for 2-3 weeks. The subcultures were grown up to an optical density of 0.4 as maximum. Agitation was required` for *M. smegmatis* but not for BCG and R179 strains. The cultures were finally stocked at -80^0C for future use. Stock cultures of mycobacteria were thawed and clumps were disrupted by pipetting 10 times with 1ml tip. This was then syringed 10 times (20 passes) through a G25 needle (Stokes et al., 2004), followed by allowing the major clumps to settle down for their respective settling time (Cywes, Hoppe, Daffé, & Ehlers, 1997). The settling time was different for each strain (*M. smegmatis*: 30 seconds, BCG: 1 minute, R179: 1 minute). The top 750 µl was collected and added to 4.25 ml of RPMI 1640 media. This 5 ml bacterial suspension was then immediately filtered through a 5.0 µm pore size filter (Merck Millipore, Germany) to which 10% Human Serum was added. The required volume (according to the titration and MOI calculation) was then added to human monocyte derived macrophages (hMDMs) in complete medium (RPMI1640 + 10% human serum). *M. tuberculosis* stock titration was also done by this procedure (no human serum was added in this case), where an average CFU was obtained by processing 3 stock vials.

Table 2.1: Mycobacterial strains used in the study

Strains	**Description**	**Source/Reference**
M. smegmatis	*M. smegmatis* MC155	Laboratory collection (Harper et al., 2010)
BCG	*M.bovis* BCG strain Pasteur 1743P2	Laboratory collection (Viljoen et al., 2013)
R179	Beijing genotype strain R220	Clinical isolate, multi drug resistant strain (Johnson et al., 2006)

Abbreviations: BCG, Bacille Calmette Guerin

2.6 Infection of hMDMs with mycobacteria

For infection experiments, human macrophages were seeded in 12-well plates (Greiner Bio One Cat. No. 665180) with $0.7x10^6$ cells per well. Pathogenic (R179) and non-pathogenic (*M. smegmatis* and *M. bovis* BCG) strains of mycobacteria were used for infection. Mycobacteria were cultured in 7H9 (Sigma-Aldrich, cat. No.M0178) with 10% Oleate-Albumin-Dextrose-Catalase (OADC) (Sigma-Aldrich, Cat. No. M0553) and 0.5% glycerol (Sigma-Aldrich, Cat. No. 49767) without Tween 80. We avoided the use of Tween, as Tween is known to affect macrophage uptake and immune response to *M. tuberculosis* (Leisching et al., 2016). The stock concentration of all three strains were used to calculate a dilution series to achieve a working concentration as Multiplicity of Infection (MOI) of 1 before proceeding for infection.

The stock concentrations were different for different strains (*M. smegmatis*: 7.54×10^6 bacteria/ml, BCG: 2.71×10^6 bacteria/ml, R179: 10.2×10^6 bacteria/ml). Human macrophages were infected with each mycobacterial strain at a multiplicity of infection of 1 (MOI=1) and uptake permitted for four hours. The cells were washed three times with PBS to remove any extracellular mycobacteria. For each infection, the MOIs were confirmed through CFU counts. We setup two separate *in vitro* infection experiment at 12 hours (early response) and 96 hours (late response) post-infection. Here we emphasized to study the early and late host response towards the slow and the fast growing mycobacteria (although it is evident that the doubling time is different for the three stains). In the first experiment, cells were infected with respective mycobacterial strains and then washed after 4 hours of infection. Cells were then incubated for another 8 hours (total duration 12 hours) in an incubator at 5% CO_2 and 37^0C. Similarly, for second experiment, infected cells were washed after four hours of infection and then incubated for another 92 hours (total duration 96 hours) in 5% CO_2 incubator at 37^0C.

Uninfected hMDMs served as control/uninfected samples. **Figure 2.3**, depicts the images of the isolated PBMC's and subsequent maturation of hMDMs.

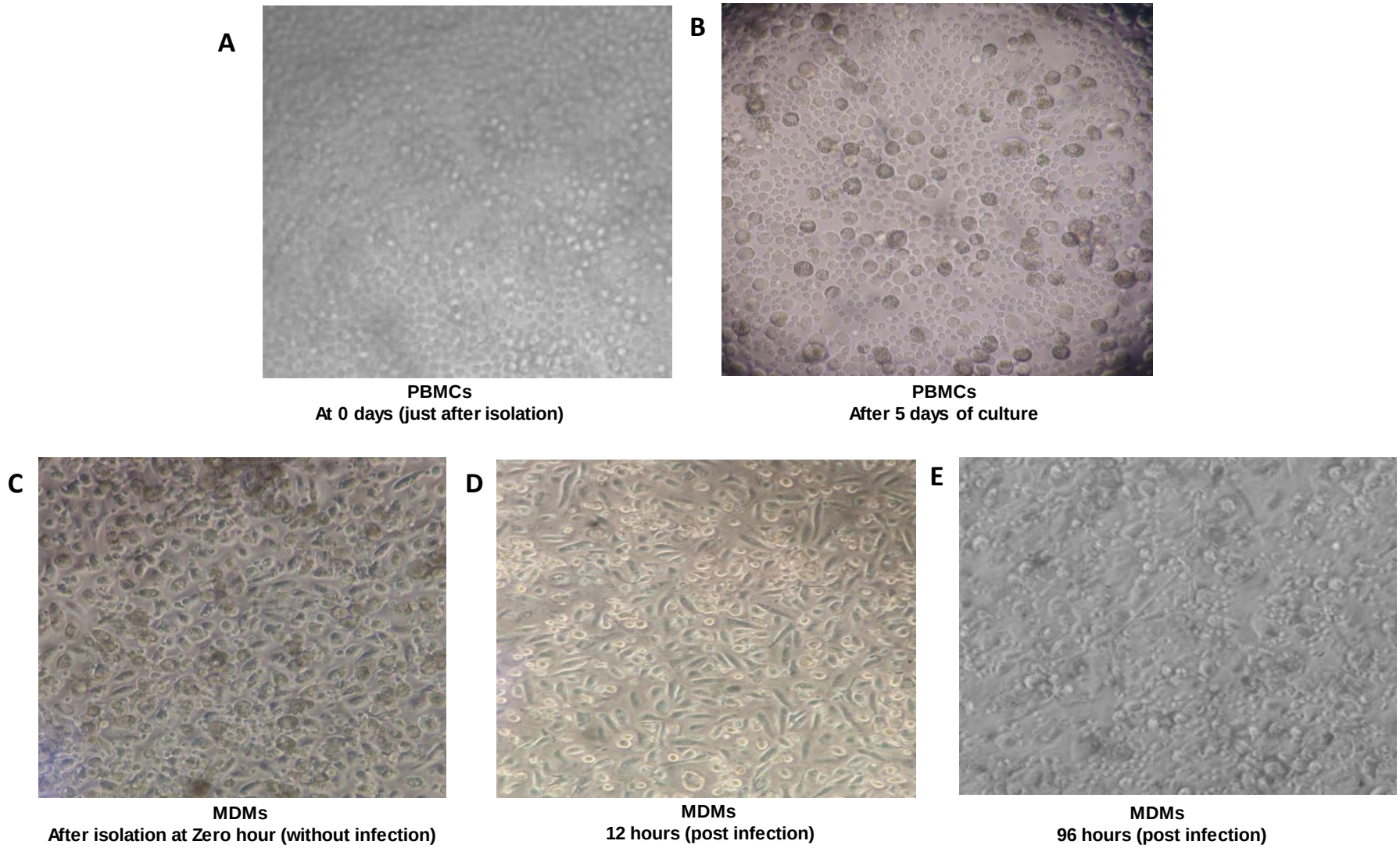


Figure 2.3: (A) PBMCs at day zero and (B) at day five of culture, with the larger cells as enlarged monocytes. Panels (C), (D) and (E) show hMDMs before and after infection with mycobacteria observed at 10x Objective. Different sizes of monocytes after isolation, maturation and pre/post-infection can be observed.

2.7 Determination of bacterial uptake

Infected cells were lysed using 0.1% Triton X-100 (Sigma-Aldrich Cat. No. 93443) at 4, 12, 24 and 96-hours post-infection respectively (with *M. smegmatis* not measured at 96 hours). At each time point, bacterial uptake was determined by serial dilution (10^{-1} – 10^{-4}) and plated out on 7H11 agar plates. These agar plates were incubated at 37°C and CFUs/ml readouts were recorded weekly for 6 weeks. Intracellular colony forming units (CFUs) were determined for all three strains, i.e. *M. smegmatis*, BCG and R179.

CFUs for each mycobacterial strain and their respective MOI were recorded meticulously for every time point **(Table 2.2)**.

Table 2.2: An example of CFU counts performed, bacterial uptake and MOI calculation:

Colony counting	
Serial Dilutions	**Counts (Colonies)**
10^{-3} (selected dilution for colony counts)	29
	29 x 10^{-3} x 20 (as we used 50 µl for plating out on 7H11 plates, hence we need to multiply by 20 for per ml calculation) = 0.58 x 10^{6} bacteria/ml (taken up)
Harvesting the bacteria	
Bacteria taken up by hMDMs	0.58 x 10^{6} bacteria/ml
hMDMs/well (of 12-well plate)	0.7 x 10^{6} cells/well
Multiplicity of Infection (MOI)	Taken up / No. of macrophages in each well 0.58 x 10^{6} / 0.7 x 10^{6} = 0.82

Abbreviations: hMDMs, human monocyte derived macrophages; MOI, multiplicity of infection

2.8 Cytotoxicity

Cell cytotoxicity was tested with Roche Water Soluble Tetrazolium (WST-1) Cell Cytotoxicity Reagent (Roche, USA) in 1:10 dilution of WST-1 reagent to RPMI complete media (RPMI +

10% Human Serum). Cells post-infection and at different time points (12 and 96 hours) were processed for cell cytotoxicity. A total of 300 µl of 1:10 dilution of WST-1 reagent to complete media was added to the wells of a 12-well plate.

The culture plates were covered properly with aluminium foil as the cytotoxicity reagent is light sensitive. Cells were incubated for 1 hour at 37^0 C and 5% CO_2. Cells were then transferred to a multi-mode reader placed in the dark room. Absorbance was measured at 450 and 630 nm (wavelength correction). The difference between the two absorbance readings was taken and plotted in Microsoft Excel as percentage values. **Figure 2.4** provides the flow of cell cytotoxicity analysis performed in hMDMs post-infection.

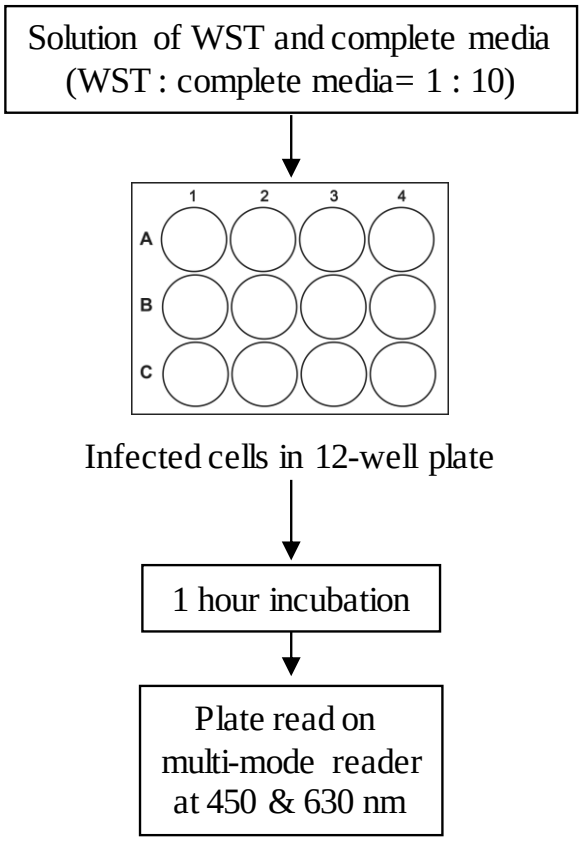


Figure 2.4: Flow of **c**ytotoxicity experiment
Abbreviations: WST, Water soluble tetrazolium; nm, nano-meter.

2.9 RNA extraction

Total RNA from human macrophages was extracted with the help of a kit RNeasy Plus Mini Kit (Cat. No. 74134, Qiagen, Limburg, Netherlands). The cell culture medium was completely aspirated from the culture plates. Cells were washed three times with ice-cold 1x PBS. A total of 350 µl of RLT Plus Buffer (with 10µl/ml β-mercaptoethanol) was added to the wells of a 12-well plate and scrapped with a pipette tip to disrupt the cells. The lysate was then pipetted into a micro centrifuge tube and vortexed to ensure that no cell clumps are visible. The lysate was then loaded directly to a QIAshredder spin column and centrifuged for 2 minutes at maximum speed.

The homogenised lysate was then transferred to a gDNA eliminator spin column and centrifuged for 1 minute at 8000 x g. The gDNA eliminator column ensures removal of any genomic DNA from all the samples. A total of 350 µl of 70% ethanol was added to the flow-through and mixed well by pipetting. Up to 700 µl of the sample (including any precipitate) was transferred to RNeasy spin column placed in a 2 ml collection tube and the lid was closed gently. The tube was centrifuged for 15 seconds at 8000 x g and the flow-through was discarded.

A total of 700 µl of 'RW1' Buffer was then added to the RNeasy spin columns and the lid was gently closed. This was then centrifuged for 15 seconds at 8000 x g to wash the spin column membrane and the flow-through was discarded. A total of 500 µl of 'RPE' Buffer was then added to RNeasy spin column and the lid was gently closed. This was then centrifuged for 15 seconds at 8000 x g to wash the spin column membrane and the flow-through was discarded. A total of 500 µl of RPE Buffer was then added to RNeasy spin column and the lid was gently closed. This was then centrifuged for 2 minutes at 8000 x g to wash the spin column membrane and the flow-through was discarded. The RNeasy spin

column was then placed in a 2 ml collection tube and centrifuged at full speed for 1 minute. The RNeasy spin column was then placed in a 1.5 ml collection tube. A total of 30 µl of RNase-free water was then added directly to the spin column membrane and then centrifuged for 1 minute at 8000 x g to elute RNA.

For each experiment, RNA quantity and quality were evaluated using Agilent 2100 Bioanalyzer. The RNA with a high RNA integrity Number (RIN) (≥ 9) was used for AmpliSeq and quantitative real time qRT-PCR experiments. Total RNA was aliquoted in three different aliqoutes (for AmpliSeq analysis, for cDNA preparation leading to qRT-PCR and one aliquote was saved as a back-up stock) and frozen immediately at -80^{0}C until AmpliSeq was performed. **Figure 2.5** provides the representative snapshot of Bioanalyzer results for quantification of RNA samples and corresponding RIN score.

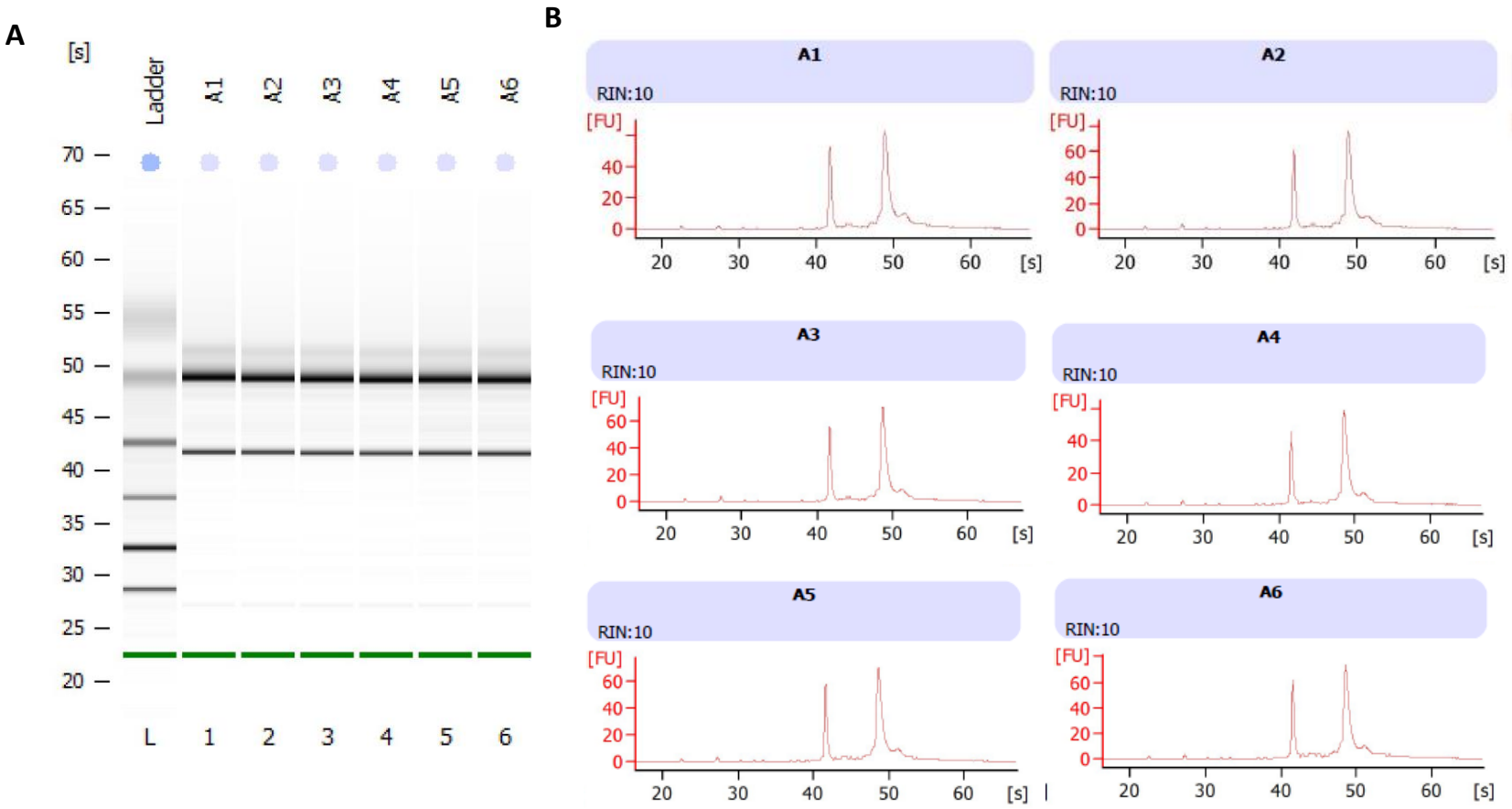


Figure 2.5: Quantification of RNA, **A)** virtual gel, RNA quantification using Bioanalyzer and **B)** first peak indicates 18S and second 28S rRNA, Samples depicting RNA quality with RIN: 10
Abbreviations: L, ladder; FU, fluorescent unit; RIN, RNA integrity score; s, second (time)

2.10 IFN-γ activation

In a separate *in vitro* infection experiment, hMDMs were seeded ($0.07x10^6$ cells per well) in a 48-well plate. Cells were then infected with *M. smegmatis*, BCG, and R179 (MOI=1). After 4 hours of uptake post-infection, cells were washed carefully three times with PBS (1x). Cells were then treated separately with different concentrations (5, 10, 20, 25, 50, 75, 100 ng/ml) of IFN-γ recombinant protein (Sigma Aldrich Cat no. I9016) in RPMI with 10% FBS in order to attain a titrated working concentration. Upon titration, EC_{50} (effective concentration of a drug that gives half-maximal response) was obtained at cells activated with 20 ng/ml of the recombinant protein (working concentration of IFN-γ). EC_{50} was calculated through an optimized titration curve in Microsoft Excel. Cells were incubated at 37^0C with 5% CO_2 for 12, 24 and 96 hours respectively, and then processed for RNA extraction and CFU evaluation.

2.11 AmpliSeq and gene filtering measures

Targeted transcriptome sequencing was performed at the Central Analytical Facilities (CAF) at Stellenbosch University, South Africa using the Ion AmpliSeq™ Transcriptome Human Gene Expression Kit (Thermo Fisher Scientific) according to the manufacturer's protocol (protocol MAN0010742 Rv B.0). Total RNA (100 ng) was reversed transcribed using the SuperScript® VILO™ cDNA Kit to generate cDNA. The target regions were amplified for 10 reaction cycles using the Human Gene Expression Core Panel.

After partial digestion, barcode adaptors (from the IonCode Adapters Kit) were ligated and the libraries were purified with Agencourt ™ AMPure™ XP reagent and eluted in low TE buffer. The concentrations of the RNA library determined using the Ion Library TaqMan Quantitation Kit according to the protocol, (MAN0015802 Rev A.0) and all libraries were diluted to a target concentration of 60 pM.

To avert potential bias during sequencing or emulsion PCR, the 84 libraries were mixed before emulsion PCR, enrichment and chip loading on the Ion CHEF using the Ion PI™ Hi‑Q™ Chef Reagents, Solutions and Supplies according to the protocol, (MAN0010967 Rev B.0). To limit the effect of the run to run variation, all libraries were pooled and sequenced on multiple Ion P1™ v3 Chips on the Ion Proton™ according to the manufacturer's protocol (MAN0010967 RevB.0).

Analysis of sequence data was performed with the help of Torrent Suite Version 5.4.0 Software Partek Flow. All the reads were mapped by the TMAP module (as implemented in Torrent Suit). These mapped reads were exported as (.bam extension) specific files into the Partek Flow Software tool where the various data files for each sample were combined to create one dataset for each sample. After alignment, the QC module of Partek Flow was used to visualize the average base quality score per-position and per-alignment mapping.

These mapped reads were quantitated using a separate set of software (RefSeq transcripts) annotation for quantification using the Partek E/M method. Differential expression analyses were performed using “edgeR” version 3.4.1. Statistical analyses of results were carried out by the Division of Statistics at Stellenbosch University. We used edgeR to model a negative binomial distribution of count data and estimated dispersion parameters. Subsequently, we determined differential expression using a One-way repeated measures ANOVA “Limma”. We used Benjamini-Hochberg false discovery rate to correct for multiple testing.

The edgeR statistical method was used to select DE genes. The differentially expressed gene list was filtered using a combination of stringent gene filtering measures. These include, strong False Discovery rate (FDR) (<0.001), strong p-value (<0.001), log counts per million (CPM), Fold changes (>1.5), Ingenuity Pathway Analysis (positive

z scores), and Biological functions as the basic and essential measures which were used to filter the AmpliSeq data to obtain potential DEGs as important host response towards mycobacteria.

2.12 Quantitative real time PCR (qRT-PCR)

Good quality RNA (RIN>9, 0.8 µg) was used for cDNA preparation using the Quantitect^{R} Reverse Transcription Kit (Cat No. 205311, Qiagen, Limburg, Netherlands). To ensure the removal of genomic DNA, 'gDNA wipe-out buffer' was added to RNA (included in the kit) prior to the RNA conversion step. qRT-PCR amplification was run on a LightCycler ^{R} 96 system (Roche, Germany). LightCycler^{R} 480 SYBR Green I Master (Roche Light Cycler, FastStart Essential DNA Green Master, Cat. No. 06402712001) was used for various differentially expressed genes using QuantiTect^{R} primer assays (Qiagen, Limburg, Netherlands) with 20 µl of reaction volume (list of primers used provided in **Table 2.3**).

Hs-GAPDH and Hs-UBC were selected as reference genes conferring to stable expression levels from AmpliSeq data and validated through qRT-PCR. The amplification process involved 45 cycles of 95^{0}C for 10 s (denaturation) followed by 60^{0}C for 10s (annealing) and finally 72^{0}C for 10s (extension). Gene expression fold-changes was computed for pathogenic infected and non-pathogenic infected macrophages using calibrated normalized relative quantities using the equation $N = N_0 \times 2^{Cp}$. All qRT-PCRs were performed on RNA extracted from 4 separate experiments. All biological replicates having a positive control and a non-reverse transcription control was run in triplicate (along with calibrator) as per the MIQE Guidelines (Bustin et al., 2009). Table 2.3 describe commercially bought primers that were used for setting up the qRT-PCR assay.

Table 2.3: Description of primer sequences used for quantitative real time PCR

Primer	**Catalogue Number**
Hs IFIT1-1-SG	QT00201012
Hs IFIT2-1-SG	QT00219345
Hs IFIT3-1-SG	QT00100030
Hs MX1-1-SG	QT00090895
Hs MX2-1-SG	QT00000581
Hs IFI44-1-SG	QT00014399
Hs IFI44L-1-SG	QT00051457
Hs ISG15-1-SG	QT00072814
Hs IL12B-1-SG	QT00000364
Hs IL23A-1-SG	QT00204078
Hs IL6-1-SG	QT00083720
Hs IL8-1-SG	QT00000518
Hs RSAD2-1-SG	QT00005271
Hs TRIB3-1-SG	QT00088543
Hs MT1A-1-SG	QT01004591
Hs IDO1-1-SG	QT00000504
Hs EIF2AK2-1-SG	QT00022960
Hs IFNγ-1-SG	QT00000525
Hs IL1β-1-SG	QT00021385
Hs UBC-1-SG	QT01326752
Hs GAPDH-1-SG	QT00079247

Abbreviations: bp, base pair; Hs, homosapeins; EIF2AK2, Eukaryotic translation initiation factor 2 alpha kinase 2; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; IDO, Indoleamine 2,3-dioxygenase; IFI, Interferon induce protein; IFIT, Interferon induced protein with tetratricopeptide; IFN, Interferon gamma; IL, Interleukin; ISG, Interferon stimulated gene; MTA, Metastasis-associated protein; MX, Interferon induced GTP binding protein; RSAD Radical S-Adenosyl Methionine Domain-Containing protein 2; TRIB, Trible homolog; UBC, Polyubiquitin-C

2.13 Ingenuity Pathway Analysis (IPA)

Ingenuity Pathway Analysis (IPA) was performed using IPA Gene View Software version 01-13. To identify potential target from the AmpliSeq data, it was essential to filter the list of DEGs obtained after analysing the AmpliSeq data. IPA was one among the stringent filtering measures applied on the DEGs list. Analysed AmpliSeq data with strong FDR, p-values and fold changes was used for IPA analysis. The baseline data was uploaded on IPA. Statistical measures reflecting dataset genes interacting with each other (overlap p-value) and activation (z-score) were based on the known direction of up and down regulated genes. For detailed pathway evaluation differentially expressed genes (DEGs) with fold changes > 1.5 and adjusted p-value ≤ 0.05 was used in the analysis. The data was also used to generate top canonical pathways, upstream regulators and regulator effect networks in hMDMs after infection with mycobacteria with respect to potential DEGs which were selected based on the stringent gene filtering parameters.

2.14 Multiplex ELISA

A panel of 13 cytokines including IDO-1, TNF-α, eight interleukins (IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-12p40, IL-12p70 and IL-23), type-I interferon (IFN-α and IFN-β) and type-II interferon (IFN-γ) was designed for studying the host response upon infection with mycobacteria at different time points using a multiplex ELISA as implemented on Luminex platform. This panel was strictly designed based on the 19 DEGs obtained after AmpliSeq analysis and carefully filtered through the above mentioned gene filtering measures **(Table 2.4)**. Cytokine concentrations were evaluated using Procarta Luminex kits - ThermoFisher on the Bioplex platform (BioRad Laboratories, Hercules, CA, USA) according to the instructions of the kit manufacturer (ThermoFisher).

Samples were assayed in duplicate. The coefficient of variation for duplicate runs was <20% for all samples (range, 5.2% - 19.6%) and the levels of all analytes in the quality control reagents were within their expected ranges. The standard curve for all samples ranged from

3.6-10000 pg/ml. Bioplex Manager Software version 1 was used for analysis of median fluorescent intensities.

Table: 2.4: Description of Luminex Kit and the Cytokine panel assessed by Luminex

Multiplex Kit	Procarta ThermoFisher Luminex Kits
Catalogue Number	PPX-12
Manufacturer	Bio Rad Laboratories, Hercules, CA, USA
Cytokines Assessed	
12-Plex	**1-Plex**
IDO-1	IL-12p40
TNF-α	
IL-1α	
IL-1β	
IL-4	
IL-6	
IL-8	
IL-12p70	
IL-23	
IFN-α	
IFN-β	
IFN-γ	

Abbreviations: IDO, Indoleamine 2,3-dioxygenase; IFN, Interferon; IL, Interleukin; TNF, Tumour necrosis factor

The Procarta ThermoFisher Luminex kit is recommended for use with serum, plasma and culture supernatants. Here, we used the culture supernatants collected at different time points post-infection and stored at -80^{0}C. Samples were brought out of -80^{0}C and thawed 30 minutes before setting up the experiment.

Preparation of standards:

Each antigen standard set vials was centrifuged at 2000 x g for 10 seconds. A total of 50 µl of sample type specific buffer was added into each standard vial. Vials were vortexed for 10 seconds and centrifuged at 2000xg for 10 seconds to collect contents at the bottom of the vial. Vials were then incubated on ice for 10 minutes to ensure complete reconstitution. The entire content of each vial was pooled into one of the vials and filled up with sample type specific

buffer to a total volume of 250 µl. The vial was gently vortexed for 10 seconds and centrifuged at 2000 x g for 10 seconds to collect the contents on the bottom of the vial, then a 4-fold serial dilution was prepared.

Luminex assay protocol:

Magnetic capture beads were vortexed for 30 seconds. A total of 50 µl of the magnetic beads was added to each well of the 96-well plate. The magnetic beads were washed by securely placing the 96-well flat bottom plate into the magnetic plate washer for 2 minutes. The liquid from the wells was removed by quickly inverting the 96-well plate assembly over a sink. For cell culture supernatants, 50 µl of prepared standards and the samples were added into each of the respective wells. For the wells designated as blanks, 50 µl of cell culture medium was added. The plate was sealed and incubated on a shaker at room temperature for 2 hours. Plates were washed twice and then 25 µl of detection antibody was added to each well. The plates were sealed and incubated on a shaker at room temperature at 500 rpm for 30 minutes. The plate was washed twice. A total of 50 µl Streptavidin PE (EPX-SAPE) solution was added to each well. The plates were sealed and incubated on a shaker at room temperature at 500 rpm for 30 minutes. The plate was washed twice. A total of 100 µl of reading buffer was added to each well. The plates were sealed and incubated on a shaker at room temperature at 500 rpm for 5 minutes. The plate seal was removed and placed in the Luminex instrument for plate reading. **Figure 2.6 (A-D)** provides the standard curve generated for each of the thirteen cytokines used in the Luminex panel.

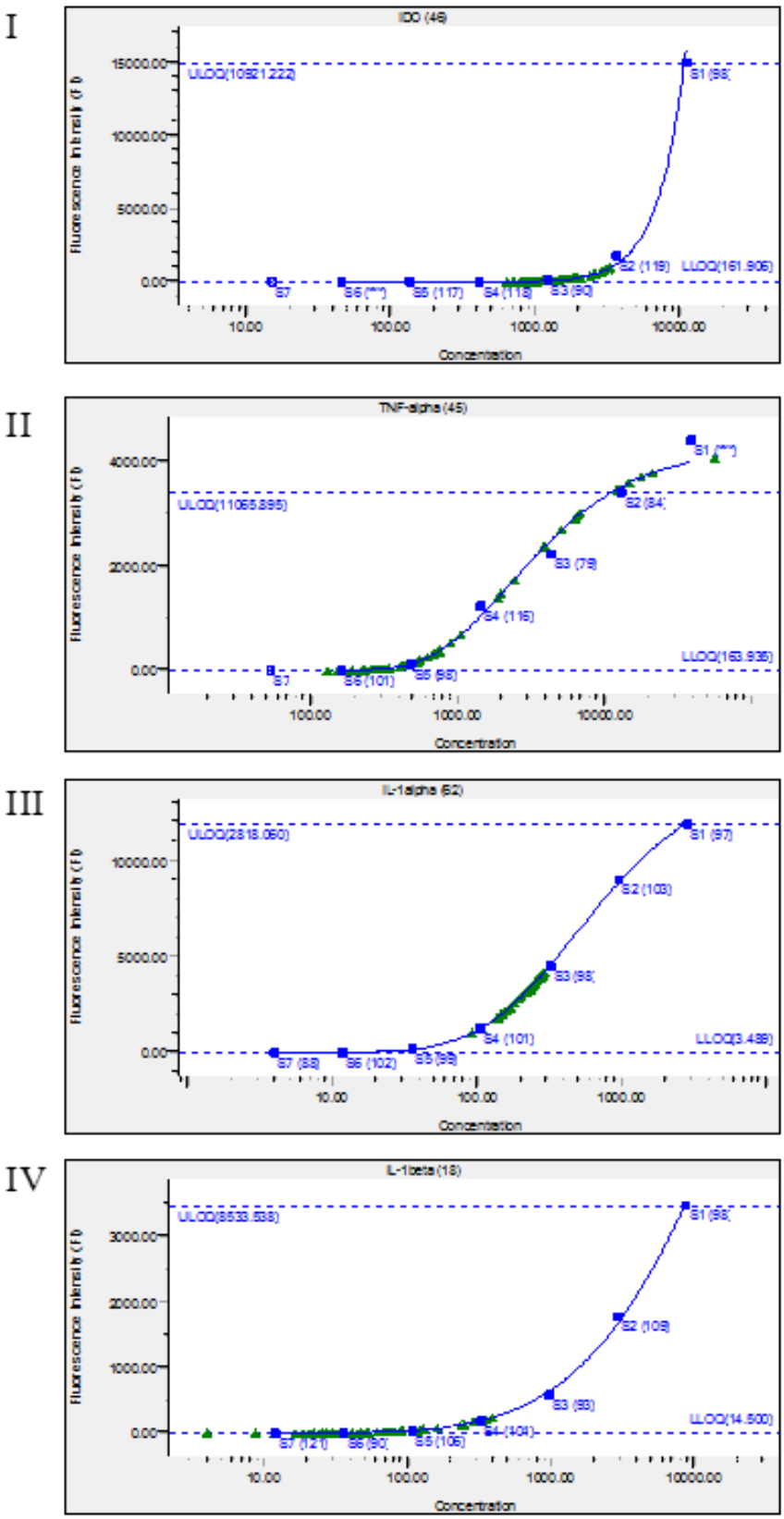


Figure 2.6 A: Multiplex ELISA standard curve for I) IDO-1, II) TNF-α, III) IL-1α and IV) IL-1β Abbreviations: IDO, indoleamine 2,3-dioxygenase; TNF, tumour necrosis factor; IL, interleukin; S, standard; LLOQ, lower limit of quantification; ULOQ; upper limit of quantification.

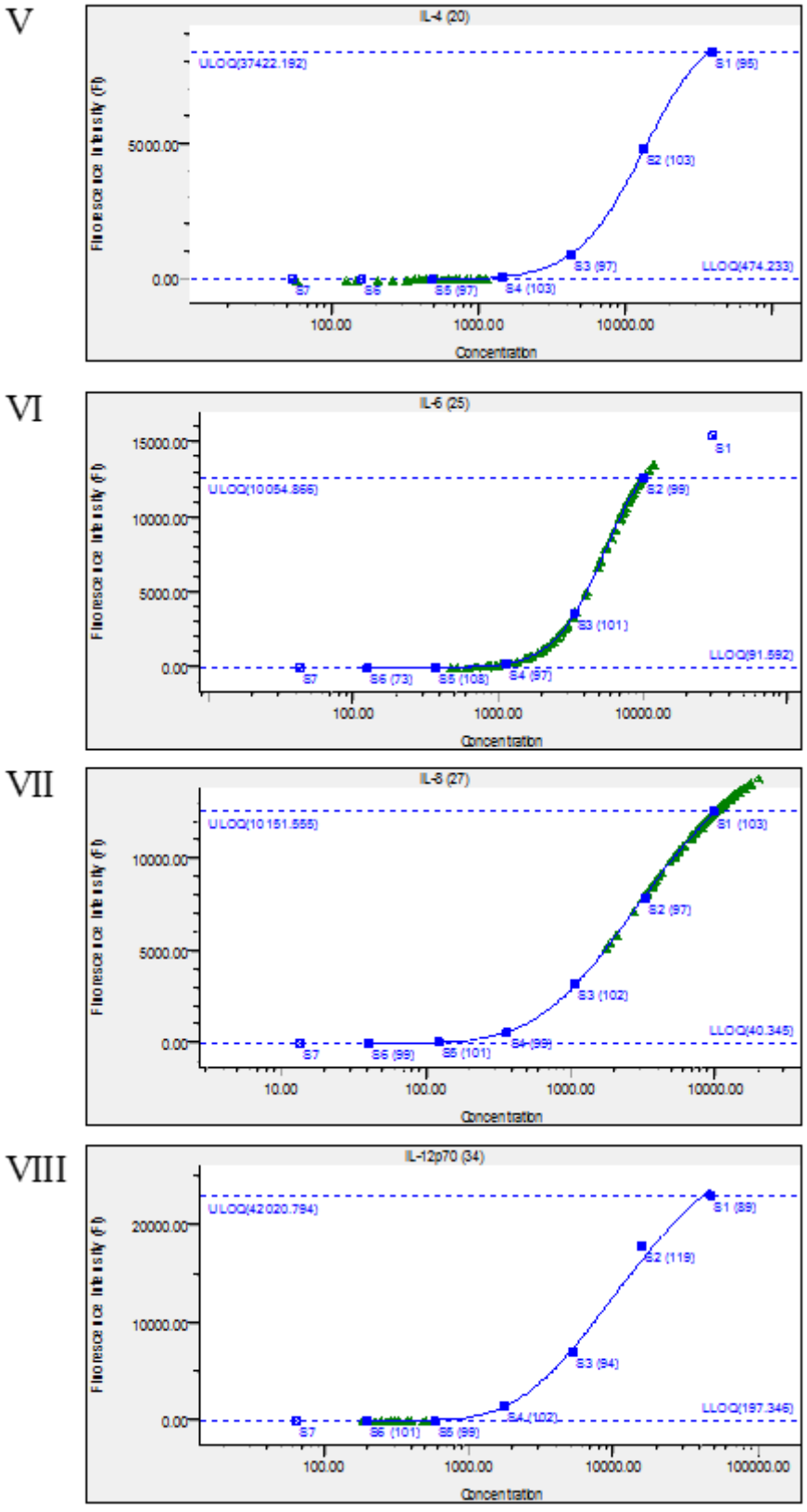
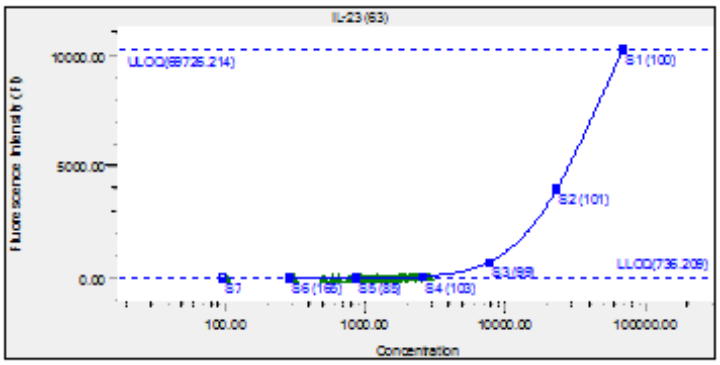


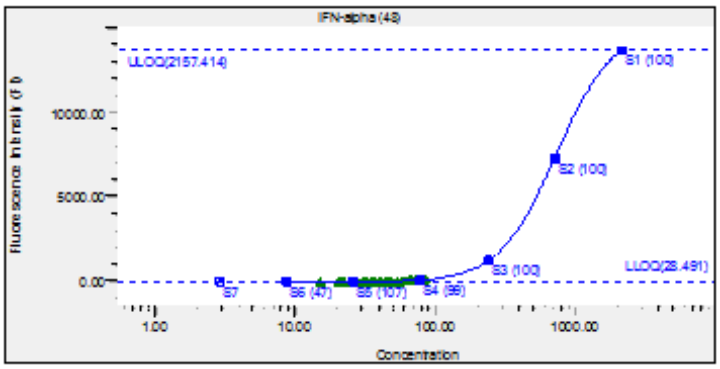
Figure 2.6 B: Multiplex ELISA standard curve for I) IL-4, II) IL-6, III) IL-8 and IV) IL-12p70 Abbreviations: IL, interleukin; S, standard; LLOQ, lower limit of quantification; ULOQ; upper limit of quantification.

.

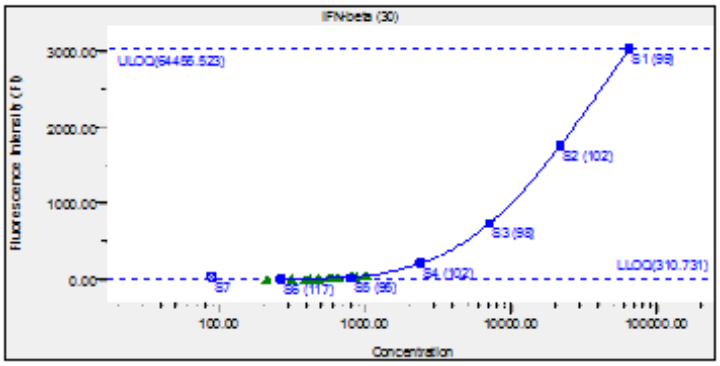
IX



X



XI



XII

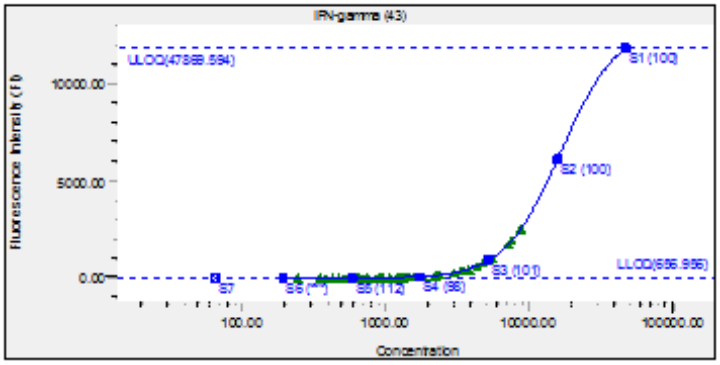


Figure 2.6 C: Luminex assay standard curve for I) IL-23, II) IFN-α, III) IFN-β and IV) IFN-γ Abbreviations: IL, interleukin; IFN, interferon; S, standard; LLOQ, lower limit of quantification; ULOQ; upper limit of quantification.

XIII

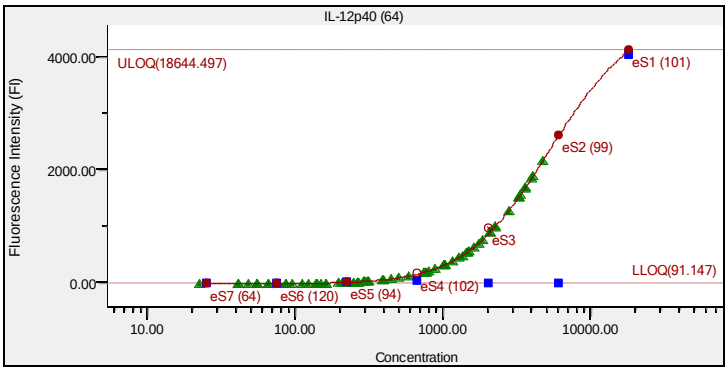
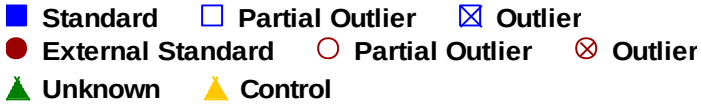


Figure 2.6 D: Luminex assay standard curve for XIII) IL-12p40
IL, interleukin; S, standard; LLOQ, lower limit of quantification; ULOQ; upper limit of quantification.

Figure: 2.6: Standard Curves generated for each respective cytokine.



2.15 Statistical analysis

Real time qRT-PCR data were analysed using Light Cycler 96 SW 1.1 Software and Graph-pad Prism V7. Relative Expression which measures target transcript in a treatment group to that of the untreated group was measured through the software in relation to the Calibrator and non-transcription control. The relative expression data of the cytokines was further analysed through Graph-pad prism to generate the p-values through One-Way ANOVA. The p-values were finally generated through Multiple Testing using Tukey corrections. The data (in technical triplicate) was finally plotted in histograms with respective mean and standard deviations.

Cytotoxicity graphs and CFUs were plotted with an average of the technical triplicates leading to the mean of all the biological replicates. Statistical analysis was performed through Graph-pad Prism V7 software where the percentage of every expressing cell was generated and p-value was calculated using Two-Way ANOVA with Tukey's correction. Luminex

data was analysed by Two-Way ANOVA with Tukey's correction using Graph-pad Prism V7 for Windows (Graph-pad Software, San Diego California, USA).

Results

2.16 Study participant demographics:

We selected twelve healthy participants with an average age of 27 (26-28) years, and an equal proportion of both genders (male/female) and ethnicity (Black/White). The average body mass index of the study participants was found to be within the normal range **(Table 2.5)**.

Table 2.5: Demographic characteristics of study participants

Variables	**Study participants (n=12)**
Age (Years) Median (IQR)	27 (26-28.5)
Gender (%) **Female** **Male**	 6 (50%) 6 (50%)
BMI (kg/m^2) (Mean ± SD)	23.59 ± 2.32
Ethnicity (%) **Black** **White**	 6 (50%) 6 (50%)

Abbreviations: n, sample number; IQR, inter quartile range; BMI, body mass index; SD, standard deviation.

2.17 Intracellular mycobacterial survival and cell cytotoxicity:

Human macrophages were infected with various mycobacterial species at a calculated MOI of 1. The bacterial uptake by macrophages for all three strains were confirmed to be similar [**Figure 2.7 (a)]**. The CFUs were measured at various time points. In *M. smegmatis* infected hMDMs, CFUs significantly reduced ($p<0.001$) at 24 hours post -infection.

In BCG infected hMDMs, CFUs increased significantly at all-time points up to 96 hours ($p<0.001$). We found BCG infected CFUs to be higher at 12 and 24 hours than R179 and *M. smegmatis* (overall $p<0.001$).

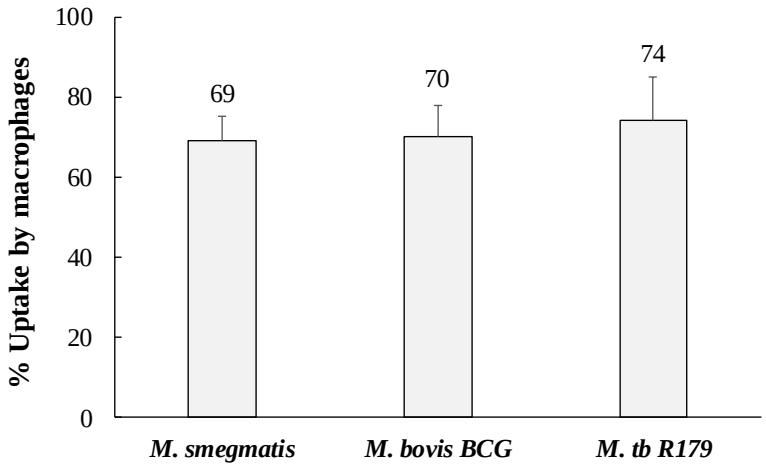


Figure 2.7 (a): Percent uptake of mycobacteria by the macrophages at 4 hours post-infection.

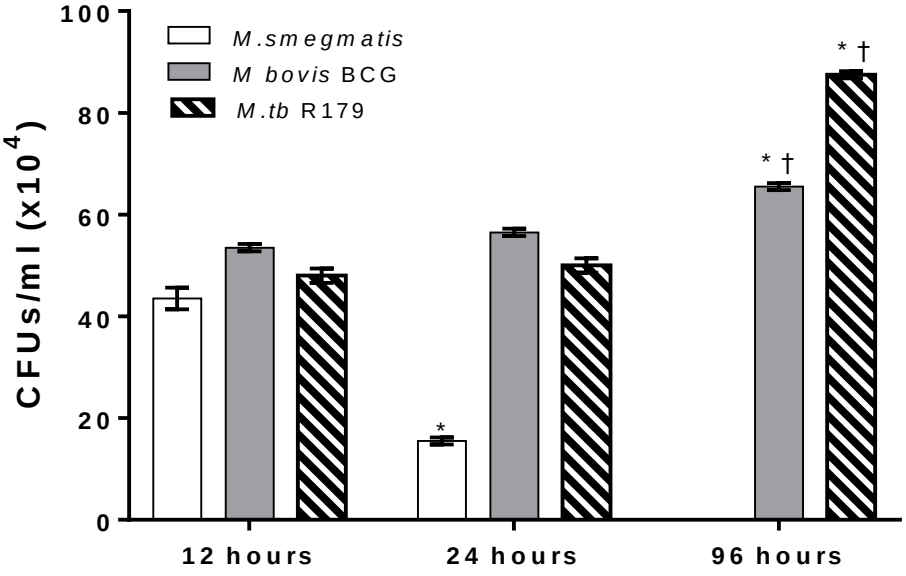


Figure 2.7 (b): Mycobacterial Colony Forming Units (CFUs) at different time points after infection with *M. smegmatis*, BCG and R179. CFUs of mycobacteria with 1 Multiplicity of Infection (MOI) at 12, 24 and 96 hours post-infection (*M. smegmatis* was not done at 96h). Infected hMDMs with mycobacterial strains are depicted with different markers. Data represents mean of twelve infection experiments (biological replicate) performed with three technical replicates in hMDMs of healthy individuals.
$p<0.05$; *vs. 12 hours, †vs. 24 hours
Abbreviations: BCG, Bacille Calmette Guerin; CFUs, colony forming units.

Upon R179 infection, CFUs gradually increased from 12 to 24 hours post-infection, this increase was found to be higher than in experiments carried out with *M. smegmatis*, but lower than with BCG. Interestingly, at 96 hours post-infection with R179, CFUs were found to be higher than both BCG and *M. smegmatis* ($p<0.001$) **[Figure 2.7 (b)]**.

The viability of hMDMs before and after infection with all three strains of mycobacteria were found to be similar at all-time points. The percentage of viable cells were found to be >85% at all-time points throughout the infection **(Figure 2.8)**.

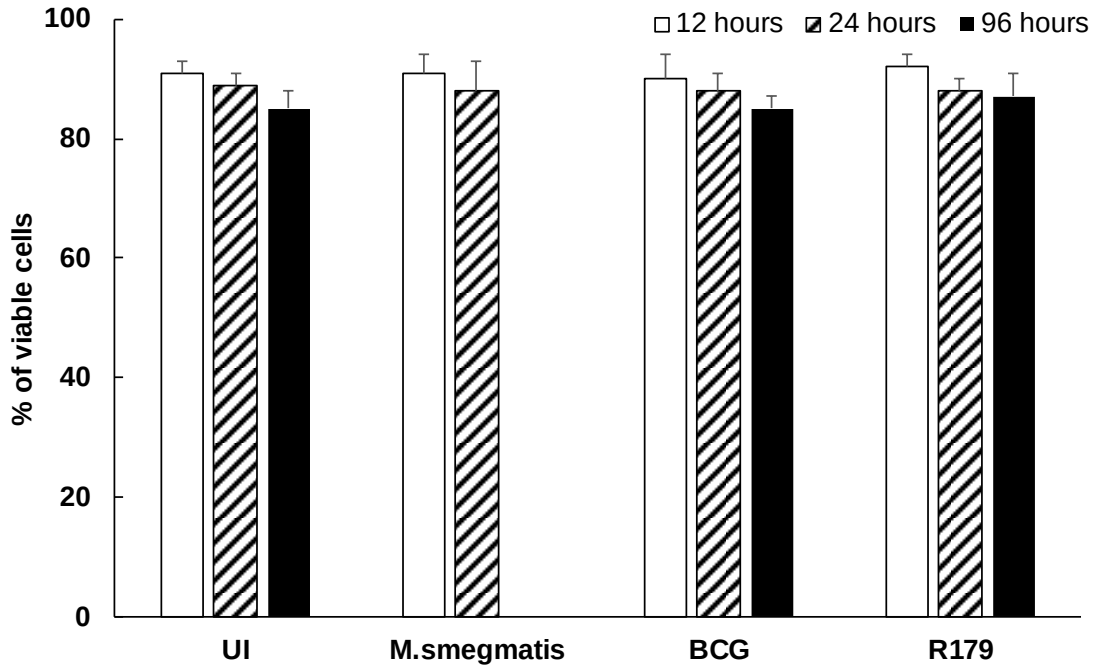


Figure 2.8: Host cell viability upon infection with mycobacterial strains was measured through cytotoxicity at 12, 24 and 96 hours post-infection (*M. smegmatis* measured at 12 and 24 hours post-infection). Data represent mean of twelve individual experiments with standard deviation. Cells at different time points (12, 24 and 96 hours post-infection) were processed with dilution 1:10 of WST-1: complete RPMI.
Abbreviations: %, percentage; BCG, Bacillus Calmette–Guérin; CFUs, colony forming units; hMDMS, human monocyte derived macrophages; WST, water soluble tetrazolium.

We also measured intracellular bacterial survival in mycobacteria-infected hMDMs after activation with IFN-γ recombinant protein. All three strains showed a significant decrease ($p<0.001$) in CFUs upon IFN-γ stimulation at 12 and 96-hours post-infection (*M.*

smegmatis was studied at 12 and 24 hours post-infection). We observed hMDMs infected with *M. smegmatis* at 12 and 24 hours post-infection depicting a highest reduction in CFUs

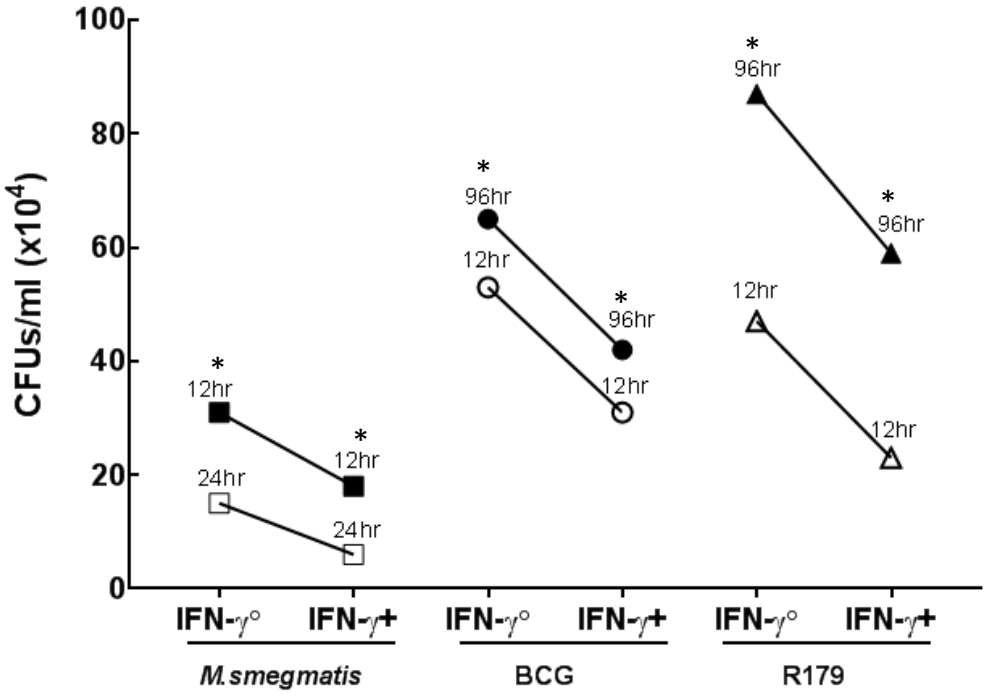


Figure 2.9: Mycobacterial CFUs before and after activation with IFN-γ. CFUs tend to reduce upon activation across the strains at different time points. *M. smegmatis* measured at 12 and 24 hours while BCG and R179 measured at 12 and 96 hours post-infection. Infected hMDMs with mycobacterial strains are depicted with different markers. IFN-γ^{o}, No IFN-γ activation, IFN-γ^{+}, activation by IFN-γ. Data represent mean of twelve infection experiments in hMDMs from healthy individuals. * indicates $p<0.001$ as compared to no IFN-γ activation and activation by IFN-γ .
Abbreviations: CFUs, colony forming units; BCG, Bacille Calmette Guerin.

with 58% (at 12 hours) and 25% (at 24 hours) in IFN-γ activated cells as compared to un-activated hMDMs. IFN-γ activated cells infected with BCG showed 17% and 15.4% reduction at 12 and 96 hours, respectively. Upon R179 infection, there was a fall of 20.4% and 14.7% in CFUs at 12 and 96 hours, respectively **(Figure 2.9).**

2.18 AmpliSeq and qRT-PCR

AmpliSeq analysis of RNA derived from hMDMs before and after infection with *M. smegmatis*, BCG, and R179 identified 274 DEGs (229 upregulated and 45 downregulated)

at 12 hours and 132 DEGs (124 upregulated and 8 downregulated) at 96 hours. The heat map clearly demarcates a strong host response against *M. smegmatis* infection as compared to other two strains of mycobacteria. Differentially expressed genes at 12 hours post-infection were not observed in the 96-hours post-infection data, hence we chose to study the early response here. We selected 19 DEGs (out of 274 DEGs at 12 hours**, Figure 2.10**) of potentially higher importance after applying stringent filtering parameters using edgeR statistical method. Interestingly, none of these 19 DEGs were essentially present in the gene list of 132 DEGs obtained at 96 hours. It was, therefore, interesting to study them in detail to understand their differential expression.

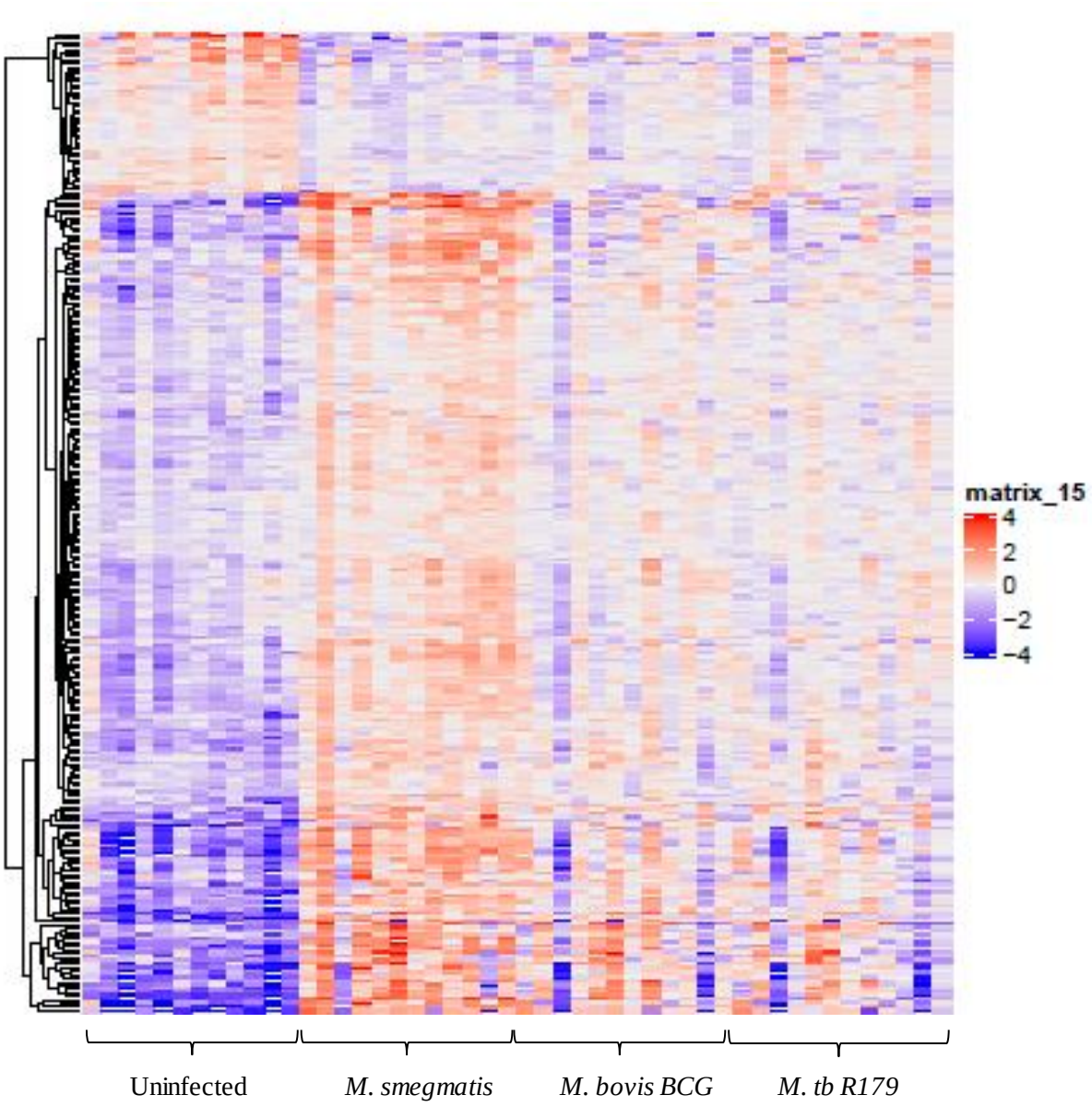


Figure 2.10: Heat map visualization and clustering of differentially expressed transcripts analysed by AmpliSeq where, hMDMs infected with *M. smegmatis*, *M. bovis* BCG and *M. tb* R179 were compared to uninfected hMDMs. Differential expression of host gene transcripts in infected hMDMs at 12 hours with significant p-values (<0.05), FDRs and fold changes are shown in the heat map. The level of expression of each gene in each sample in comparison to the level of expression in an uninfected sample is depicted with a colour scale with red as maximum and blue as the minimum significance. *M. smegmatis* having the maximum red colour indicates stronger host response as compared to other two strains. Dendrogram indicates gene and its clustering. Differentially expressed genes at 12 hours post-infection were not observed in the 96-hours post-infection data, hence we chose to study the early response here.
Abbreviations: hMDMs, human monocyte derived macrophages; BCG, Bacille Calmette Guerin.

Table 2.6 gives a summary of the results on the selected 19 DEGs obtained after gene filtering measures, with p-values, FDRs and fold Changes. We then validated the expression of all these 19 DEGs by qRT-PCR at 12 hours as depicted in a panel figure **(Figure 2.11 A-C). Figure 2.11 D,** is the heat-map depiction of AmpliSeq data of the selected 19 DEGs, demonstrating the color-coded trend confirmed by the qRT-PCR validation. We demonstrate the higher upregulation of 17 DEGs after infection with *M. smegmatis* as compared to BCG and R179. Moreover, we can also notice two highly downregulated DEGs (*TRIB3* and *MT1A*) after infection with *M. smegmatis* as compared to the other two strains.

The validation of 19 DEGs by qRT-PCR, demonstrated a similar trend across the strains confirming the results obtained by AmpliSeq results. Like AmpliSeq results, qRT-PCR also found 17 DEGs (out of 19) with higher expression in the hMDMs post-infection (12 hours) as compared to uninfected hMDMs. Also, two DEGs (*TRIB3* and *MT1A*) were found to have lower expression in hMDMs post-infection as compared with uninfected hMDMs. It is noteworthy, that the expression of all these 19 DEGs was found to be significantly higher ($p<0.001$) in hMDMs infected with *M. smegmatis* as compared to cells infected with BCG or R179.

Table 2.6: Differentially expressed genes selected from AmpliSeq of hMDMs 12 hours post-infection with pathogenic (BCG, R179) and non-pathogenic (*M. smegmatis*) mycobacteria

Gene name	*M. Smegmatis*			BCG			R179		
	Fold change	p value	FDR	Fold change	p value	FDR	Fold change	p value	FDR
IFIT1	6.3	3.36E-19	2.02E-15	1.6	1.16E-18	4.64E-15	1.8	6.09E-18	1.83E-14
IFIT2	4	2.27E-11	1.01E-08	1.6	2.5E-11	1.07E-08	1.8	3.76E-11	1.56E-08
IFIT3	6.9	2.53E-09	4.75E-07	2.8	2.95E-09	5.46E-07	2.5	3.87E-07	6.99E-07
MX1	4.6	6.05E-11	2.27E-08	1.6	6.51E-11	2.37E-08	1.7	6.7E-11	2.39E-08
MX2	3.4	1.85E-17	3.18E-14	1.9	1.45E-16	2.06E-13	1.8	1.54E-16	2.06E-12
IFI44	4	2.19E-08	3.29E-06	1.6	2.42E-08	3.59E-06	1.6	2.58E-08	3.78E-06
IFI44L	9	7.49E-11	2.58E-08	2.3	1.1E-10	3.69E-08	1.7	1.49E-10	4.7E-08
ISG15	3.5	7.23E-10	1.64E-07	1.7	8.71E-10	1.94E-07	1.9	1.08E-09	2.36E-07
IL12B	178.1	1.11E-13	8.38E-11	57.3	1.55E-13	1.1E-10	29.7	2.58E-13	1.73E-10
IL23A	9.1	6.86E-10	1.62E-07	3.2	7.03E-10	1.63E-07	1.7	5.92E-10	1.43E-07
IL6	12.4	5.64E-05	0.00029	6.5	5.89E-05	0.00023	4.6	6.07E-05	0.00023
IL8	8.7	2.45E-06	0.00021	7.2	2.46E-06	0.00021	5.9	2.83E-06	0.000238
RSAD2	7.9	5.62E-07	5.64E-05	3.1	6.75E-07	6.71E-05	2.6	6.8E-08	6.71E-05
TRIB3	-7.5	3.12E-07	0.00031	-7	4.4E-07	0.00041	-1.9	5.23E-07	0.00051
MT1A	-32	1.42E-05	0.00053	-41	1.42E-05	0.00041	-1.8	1.52E-05	0.00053
IDO1	12.6	1.79E-05	0.00015	6.6	1.8E-05	0.00011	5	1.81E-05	0.00014
EIF2AK2	2.3	4.11E-34	4.94E-30	1.6	8.47E-18	1.91E-14	1.6	9.51E-18	1.91E-14
IFN-γ	14.4	7.08E-07	6.93E-05	3.9	7.29E-07	7.08E-05	3.4	7.53E-07	7.25E-05
IL1β	22	4.84E-09	8.32E-07	16	5.04E-09	8.54E-07	10	5.98E-09	1.02E-06

Abbreviations: BCG, Bacillus calmette-guerin; EIF2AK2, Eukaryotic translation initiation factor 2 alpha kinase 2; FDR, False discovery rate; IDO, Indoleamine 2,3-dioxygenase; IFI, Interferon induce protein; IFIT, Interferon induced protein with tetratricopeptide; IFN, Interferon gamma; IL, Interleukin; ISG, Interferon stimulated gene; MTA, Metastasis-associated protein; MX, Interferon induced GTP binding protein; RSAD Radical S-Adenosyl Methionine Domain-Containing protein 2; TRIB, Trible homolog; UBC, Polyubiquitin-C.

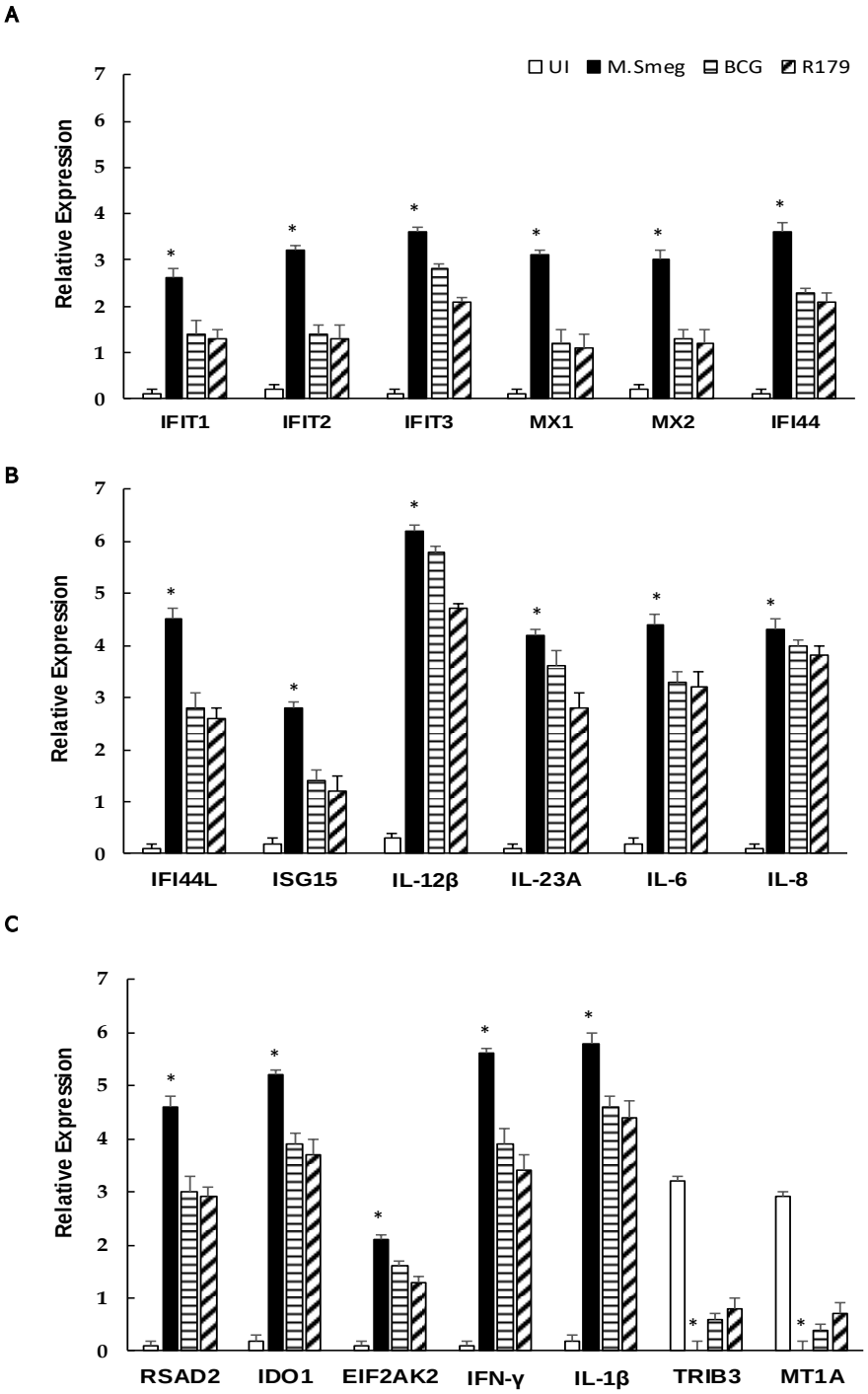


Figure 2.11 A, B & C: qRT-PCR validation (A, B and C) results of 19 selected DEGs. (a), (b) and (c) represent the expression of cytokines in hMDMs infected with different strains of mycobacteria at 12 hours post-infection relative to uninfected hMDMs. Expression of all 19 DEGs were found to be significantly higher ($p<0.001$) in hMDMs infected with *M. smegmatis* as compared to uninfected hMDMs. This was not observed in hMDMs infected with BCG or R179. GAPDH and UBC were used as two house-keeping genes. Data

represent mean of twelve infection experiments in hMDMs of healthy individuals. * indicates p<0.001.

The comparison of DEGs post-infection (12 hours) among BCG and R179-treated cells, showed six DEGs (*IFIT3, IL-12 β, IL-23A, RSAD2, EIF2AK2* and *IFN-γ*) to be significantly higher after BCG infection as compared to R179 **(Figure 2.11 A, B and C).** Whereas, one DEG (MT1A) was found to be significantly higher (p<0.001) after infection with R179 as compared to BCG.

2.19 Ingenuity pathway analysis

We performed a gene network analysis based on molecular interactions (IPA) of the selected 19 DEGs. Top canonical pathways indicated the highest role for interferon signalling (p= 9.51E-08) followed by the role of cytokine mediating communicators (p=8.03E-08) and pattern recognition receptors for bacteria and viruses (p=3.43E-07) **(Table 2.7)**.

IPA found three TLRs (TLR3, TLR7, and TLR9) along with IFN-L1 and IFN-β as the top upstream regulators activated in hMDMs. Interestingly, all five upstream regulators were found to be activated after infection. IFN-L1 was found to be most activated among the five upstream regulators with the strongest overlap (p=4.33E-26) **(Table 2.8)**. Further, IPA analysis revealed four different regulator effect networks, among this highest consistency score (consistency score =119.05), was found for a network with APP, CHUK, CSF2, E1F2AK2, IFNAR, IL-10, IL1β, MYD88, and REL. The main function of this regulator effect network followed by another IFN family-based network (consistency score= 8.05) indicates the role of these 19 DEGs in activation of cells, binding of DNA and viral replication **(Table 2.9)**.

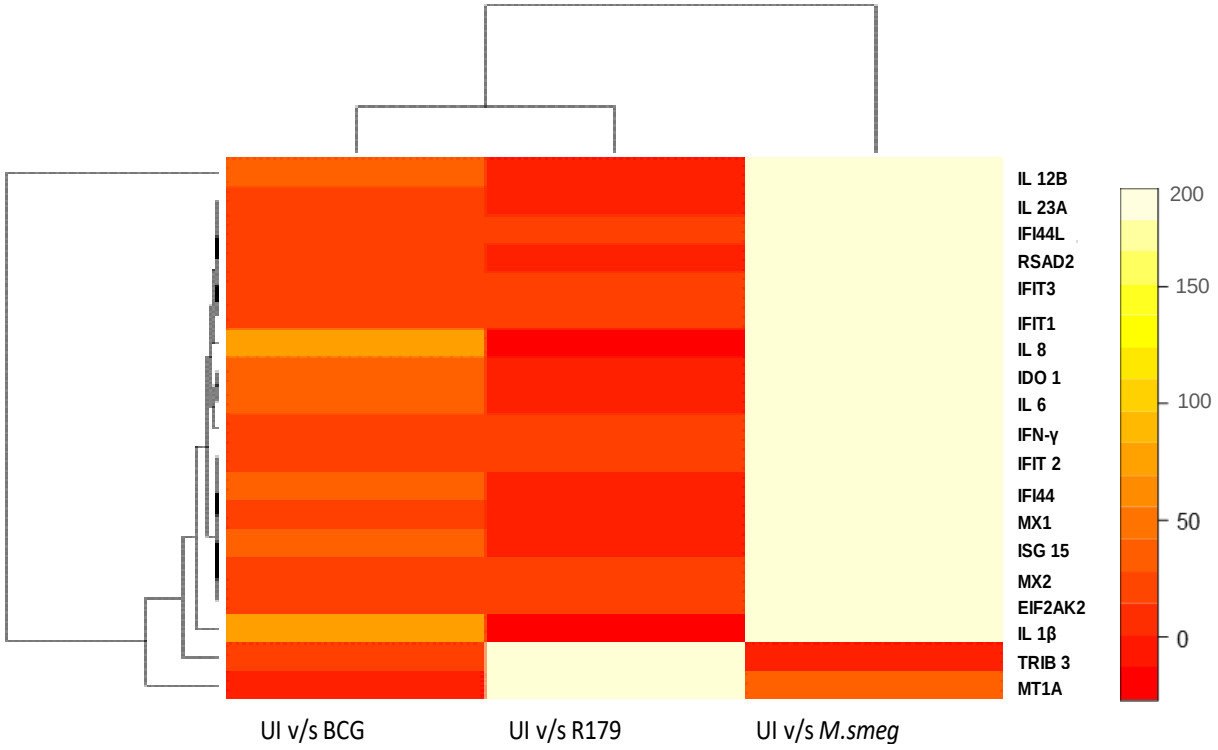


Figure 2.11 D: Heat map visualization of the 19 selected DEGs analysed by AmpliSeq. Transcripts with significant p-values, FDRs and good fold changes are shown in the heat map. The level of expression of each gene in each sample in comparison to the level of expression of uninfected sample is depicted with a colour scale with red as minimum and white as the maximum significance. Dendrogram indicates sample clustering.
Abbreviations: BCG, Bacillus Calmette–Guérin; DEGs, differentially expressed genes; FDRs, false discovery rates; UI, uninfected.

Figure 2.12 demonstrates network analysis of the 19 genes. The complexity of the networks demonstrates inter-relation and the intensity of the genes. The selected 19 genes also play a major role in mediating communication between immune cells **(Appendix VI).**

IPA analysis also suggested that the 19 DEGs have a potential role in Pattern Recognition Receptors (PRRs) in recognition of bacteria and viruses **(Appendix V1a and V1b).** The major receptors include extracellular receptors (Complement C3a, C5a), membrane bound receptors (TLRs 1-9 and 11) and cytoplasmic receptors (RIG-1 and MDA-5).

Table 2.7: Top canonical pathways activated in hMDMs after infection with mycobacteria with respect to 19 potential DEGs

Pathway	p-value	Overlap (%)
Interferon signalling	9.51E-08	11.1%
Cytokines mediated communication between immune cells	8.03E-08	7.4%
Pattern Recognition Receptors in recognition of bacteria and viruses	3.43E-07	2.9%
Hypercytokinemia/hyperchemokinemia in the Pathogenesis	4.87E-06	7%
Hematopoiesis from pluripotent stem cells	6.08E-05	6.2%

Table 2.8: Top upstream regulators activated in hMDMs after infection with mycobacteria with respect to 19 potential DEGs

Upstream Regulator	p-value of overlap	Predicted Activation
IFNL1	4.33E-26	Activated
TLR3	5.86E-25	Activated
IFN-β	1.56E-23	Activated
TLR9	4.68E-23	Activated
TLR7	4.81E-23	Activated

Abbreviations: IFNL, Interferon ligand; TLR, Toll-like receptor; IFN, Interferon;

Table 2.9: Top regulator effect networks in hMDMs after infection with mycobacteria with respect to 19 potential DEGs

Regulators	Disease and Functions	Consistency Score
APP, CHUK, CSF2, EIF2AK2, IFNAR, IL-10, IL1β, MYD88, REL	Activation of cell, binding of DNA	119.05
IFN, IFNA/IFNA13, IFNA4, JAK1/2	Replication of viral replicon	8.05
IRF3	Immune response of cells	4.899
IRF3	Infection of Mammalia	4.899

Abbreviations: APP, Amyloid precursor protein; CHUK, helix-loop-helix ubiquitous kinase; CSF2, colony stimulating factor 2; EF2AK2, eukaryotic translation initiation factor 2-alpha kinase 2; IFNAR, type I interferon (IFN) receptor; IL-10, Interleukin-10; IL-1β, Interleukin-β, MYD88, Myeloid differentiation primary response 88; REL, proto-oncogene c-Rel; IFN, Interferon; JAK, Janus kinases; IRF, Interferon regulatory factor 1.

2.20 Cytokine estimation by multiplex ELISA:

A panel of 13 cytokines namely IDO-1, TNF-α, eight Interleukins (IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-12p40, IL-12p70, and IL-123), type-I Interferon (IFN-α and IFN-β) and type-II Interferon (IFN-γ) was measured using the Luminex assay. The comparison of these 13 cytokines was performed at four different time points (post-infection), as early (12 and 24 hours) and late response (72 and 96 hours) **(Table 2.10)**.

At 12 hours post-infection, as compared to uninfected hMDMs, nine cytokines (i.e. IL-1α, IL-1β, IL-6, IL-12p40, IL-12p70, IL-23, IFN-α, IFN-γ, and TNF-α) had significantly higher levels after infection with *M. smegmatis* as compared to the uninfected samples. Also, we found significantly higher secretion of five cytokines (IFN-α, IFN-γ, IL-12p40, IL-12p70, and IL-6) post-infection to BCG. Similarly, after infection with R179, we found significantly higher levels of five cytokines (IFN-α. IFN-γ, IL-1α, IL-6 and TNF-α) as compared to uninfected hMDMs **(Table 2.10)**.

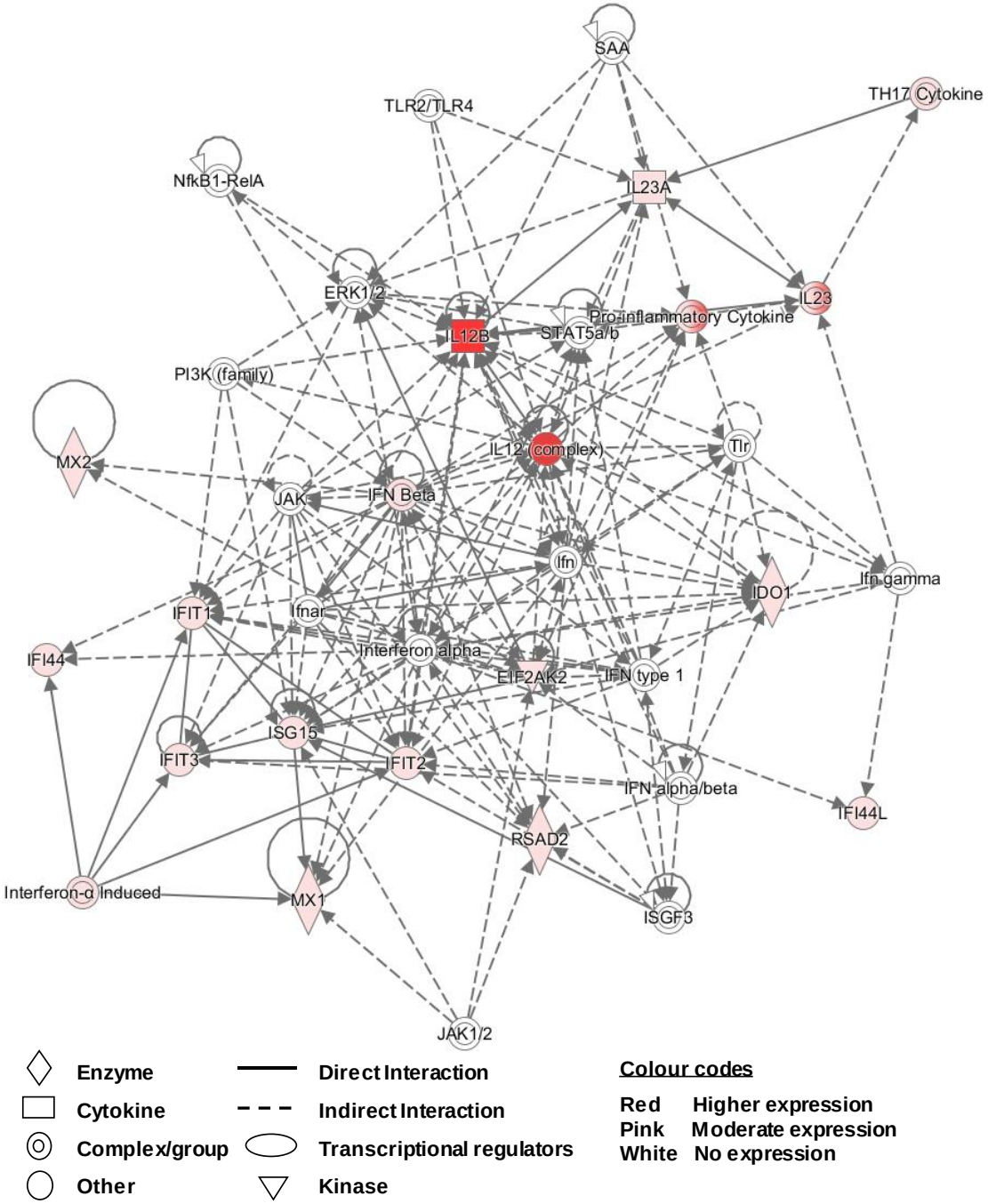


Figure 2.12: Canonical pathways generated through Ingenuity Pathway analysis (IPA) of DEGs depicting relationship between different proteins/factors.
Abbreviations: SAA, Serum amyloid A (SAA); EF2AK2, eukaryotic translation initiation factor 2-alpha kinase 2; IFNAR, type I interferon (IFN) receptor; IL, Interleukin; ISG, interferon stimulating gene; TLR, toll like receptor; NfKB1, nuclear factor kappa-light-chain-enhancer of activated B cells REL, proto-oncogene c-Rel; IFN, Interferon; JAK, Janus kinases; STAT, Signal Transducer and Activator of Transcription proteins; IRF, Interferon regulatory factor 1; MX2, human myxovirus resistance 2.

Table 2.10: Expression of early response cytokines from hMDMs before and after infection with pathogenic (BCG, R179) and non-pathogenic (*M. smegmatis*) mycobacteria measured at 12 and 96 hours

Cytokine (pg/ml)	Uninfected	*M. Smegmatis*	BCG	R179
at 12 hours				
IDO-1	1763.3 ± 753.2	1916.1 ± 741.5 0.954*	1843.6 ± 725.3 0.999* 0.994†	1870.6 ± 666.7 0.983* 0.998† 0.999‡
IFN-γ	639.7 ± 228.7	5710 ± 1533.5 <**0.0001***	2238.2 ± 781.8 **0.0008*** <**0.0001**†	1856.3 ± 733.9 **0.014*** <**0.0001**† 0.754‡
IL1-β	20.2 ± 19.1	119.4 ± 112.1 **0.021***	93.8 ± 111.5 0.129* 0.862†	84.9 ± 76.1 0.776* 0.180† 0.577‡
IL-12p70	17.8 ± 26.4	313.4 ± 143.8 <**0.0001***	198.4 ± 112.3 **0.0001*** **0.020**†	41.4 ± 38.9 0.927* <**0.0001**† **0.001**‡
IL-12p40	155.9 ± 142.5	3520.1 ± 2536 **0.0002***	2402.6 ± 2271.9 **0.020*** 0.439†	1302.7 ± 1228.4 0.416* **0.022**† 0.453‡
IL-4	571.2 ± 561.7	949.2 ± 362.4 0.170*	873 ± 339.8 0.349* 0.974†	860.3 ± 134.8 0.387* 0.960† 0.999‡
IL-6	1845.6 ± 663.1	7269.3 ± 2559.8 <**0.0001***	6957.6 ± 2611.1 <**0.0001*** 0.984†	5885.1 ± 2116.7 **0.000*** 0.397† 0.612‡
TNF-α	361.1 ± 211.6	28333.7 ± 24276 **0.0003***	8318.7 ± 4913.6 0.577* 0.925†	4196.4 ± 6515.1 **0.012*** **0.001**† 0.909‡
IL-23	1267.7 ± 190.7	2068.3 ± 475.6 <**0.0001***	1649.7 ± 436.3 0.090* 0.053†	1492.5 ± 387.7 0.495* **0.003**† 0.755‡
IFN-α	34.8 ± 4.4	83 ± 7.4 <**0.0001***	80.6 ± 10.8 <**0.0001*** 0.948†	78.6 ± 17 <**0.0001*** 0.753† 0.968‡
IFN-β	0 ± 0	0 ± 0 0.999*	0 ± 0 0.999* 0.999†	0 ± 0 0.999* 0.999† 0.999‡
IL-1α	241.6 ± 45.5	273.2 ± 15.8 **0.035***	266.4 ± 17.5 0.136* 0.930†	273.4 ± 19.9 **0.033*** 0.999† 0.923‡
IL-8	9953.3 ± 4555.2	12342.8 ± 2534.1 0.446*	12275.9 ± 4009.4 0.471* 0.999†	13677.8 ± 4203.4 0.104* 0.836† 0.815‡

Continue from last page

Cytokine (pg/ml)	Uninfected	BCG	R179
at 96 hours			
IDO-1	1634.5 ± 1176.1	1695.7 ± 583.2 0.980*	1552.9 ± 388.3 0.965* 0.898‡
IFN-γ	528.4 ± 331.6	1420.8 ± 444.1 <**0.0001***	87.4 ± 59 **0.005*** <**0.0001**‡
IL1-β	13.3 ± 7.9	136.1 ± 56 <**0.0001***	34.9 ± 17.9 0.282* <**0.0001**‡
IL-12p70	37.5 ± 59.6	115.6 ± 119.3 0.084*	51.3 ± 68.1 0.919* 0.178‡
IL-12p40	222.9 ± 486	463.4 ± 519.3 0.400*	324.4 ± 317.8 0.845* 0.731‡
IL-4	271.2 ± 404	554.6 ± 444.6 0.343*	481.5 ± 598.4 0.550* 0.929‡
IL-6	1759.2 ± 1792.3	3382 ± 1591.8 <**0.0001***	2532.8 ± 1003.4 **0.003*** 0.162‡
TNF-α	152.1 ± 181.6	173.7 ± 101.8 0.917*	167.7 ± 98.4 0.955* 0.993‡
IL-23	855.7 ± 612.8	1282.7 ± 630.5 0.192*	960 ± 515.1 0.901* 0.381‡
IFN-α	36.7 ± 26.3	64.7 ± 18.3 **0.006***	54.9 ± 16.3 0.095* 0.487‡
IFN-β	0 ± 0	0 ± 0 0.999*	0 ± 0 0.999* 0.999‡
IL-1α	185.8 ± 58	243.5 ± 53.4 **0.025***	237.4 ± 41.5 **0.049*** 0.955‡
IL-8	5820.3 ± 3736.6	9211.6 ± 4615 0.116*	8960.5 ± 3743.8 0.155* 0.987‡

p-values are shown for comparisons, *= Uninfected v/s Test (*M. smegmatis*, BCG, R179), † = *M. smegmatis* v/s BCG/R179, ‡ = BCG v/s R179.

Abbreviations: BCG, Bacillus Calmette–Guérin; IDO, Indoleamine 2,3-dioxygenase; IFN, Interferon gamma; IL, Interleukin

In *M. smegmatis* infected hMDMs, we found higher levels of IFN-γ and IL-12p70 as compared to post-infection with BCG and R179. Apart from these two cytokines, three other cytokines, IL-12p40, TNF-α and IL-23A had significantly lower levels after R179 infection when compared to *M. smegmatis*-infection.

At 24 hours, IL-1α and IL-6 had significantly higher levels of cytokine secretion in both BCG and R179-infected cells as compared to uninfected hMDMs. IL-12p40 showed higher secretion upon BCG infection as compared to the uninfected samples **(Appendix VII)**.

At 72 hours, IL-1α, IL-6, IFN-α, and IFN-γ showed higher expression in BCG and R179 infected cells as compared to uninfected hMDMs. IL-1β, IL-8, and TNF-α had higher levels upon BCG infection as compared to the uninfected hMDMs. It is noteworthy, that IL-1β shows a significant higher secretion upon R179 infection as compared to BCG infection **(Appendix VII)**.

At 96 hours, IL-1α and IL-6 showed higher expression in BCG and R179 infection as compared to uninfected hMDMs. IL-1β and IFN-α showed higher expression upon BCG infection as compared to uninfected hMDMs. Interestingly, IFN-γ had a significant difference in both BCG and R179 infected cells as compared to uninfected hMDMs. There was a significantly higher level of INF-γ found after infection with BCG as compared to R179 ($p<0.001$) **(Table 2.10)**.

In conclusion, we found a differed host response towards all three strains, which may be attributed to their pathogenicity. mRNA and protein level comparisons at different time points, depicted strong role of interferon and interleukin associated gene network. This network was able to successfully counter *M. smegmatis* but succumb to *M. bovis* BCG and R179.

CHAPTER 3

CHAPTER 3: *In vitro* knock-up (vector-based over-expression)/down of *IFITs* to study their role in mycobacterial survival

3.1 Study Design:

Nineteen DEGs were obtained after applying hexametric filter measures to AmpliSeq data of hMDMs infected with *M. smegmatis*, BCG and R179 mycobacterial strains. We selected a family of top up-regulated and closely related *IFITs* (*IFIT1*, *IFIT2* and *IFIT3*) for follow-up experiments using vector-based knock-up (vector-based over-expression) and knock-down via siRNA to study the role of *IFITs* in host immune response of hMDMs. **Figure 3.1** describe the flow of work for knock-up (vector-based over-expression)/knock-down intervention experiment. Following are the aim and objective of the intervention experiments:

Aim: To study the role of genes belonging to the interferon induced tetratricopeptide (*IFIT1*, *IFIT2* and *IFIT3*) family during mycobacterial infection.

Objective:

1. To knock-up (vector-based over-expression) selected *IFITs* (*IFIT1*, *IFIT2* and *IFIT3*) to study their role in intracellular survival of mycobacteria.
2. To knock-down (siRNA) selected *IFITs* (*IFIT1*, *IFIT2* and *IFIT3*) to study their role in intracellular survival of mycobacteria.
3. To validate the follow-up experiments at mRNA and protein expression level and to study the cytokines expression profile.

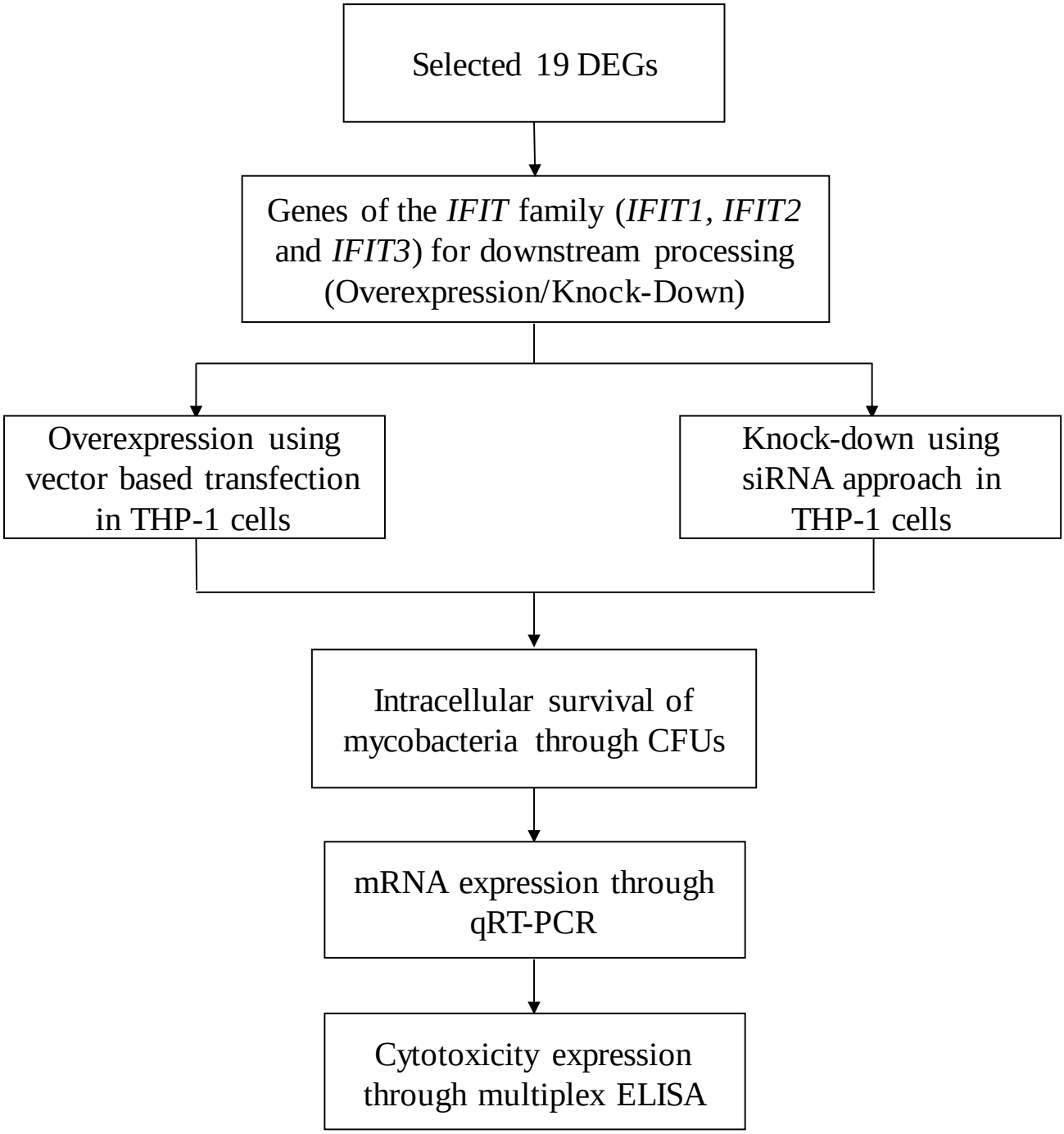


Figure 3.1: Flow of intervention experiments using vector-based knock-up (vector-based over-expression) and siRNA based knocking down of *IFIT1*, *IFIT2*, and *IFIT3*. Abbreviations: DEGs, differentially expressed genes; *IFIT*, interferon-induced protein with tetratricopeptide; KD, knock-down; KU, knock-up (vector-based over-expression); qRT-PCR, Reverse transcription polymerase chain reaction; ELISA, enzyme linked immunosorbent assay; siRNA, small interfering ribonucleic acid.

Materials and Methods

We selected an inter-related gene family of interferon induced protein with tetratricopeptides (*IFIT1*, *IFIT2* and *IFIT3*) from the list of 19 potential differentially expressed genes for knock-up (vector-based over-expression)/knock-down experiments. We performed knocking-up of *IFITs* via vector-based transfection and knocking-down via small inhibitory RNA (siRNA) approach to investigate their effect upon mycobacteria inside the host macrophages.

3.2 Culture of THP-1 cells for knock-up (vector-based over-expression)/knock-down experiments:

Commercially available human macrophage-like cells, THP-1 (ATCC-88081201), were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (Biochrome, Germany). The cells were incubated at 37^0C in a 5% CO_2 incubator. THP-1 cells were treated with a final concentration of 100nM phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich, USA) for 48 hours. PMA treatment helps monocytes to differentiate into macrophages. Cells were transferred to CO_2 incubator in a BSL3 laboratory and we proceeded with the infection. For infection experiments, human macrophage cells were seeded in 12-well plates with $0.7x10^6$ cells per well.

3.3 Preparation of detergent-free mycobacteria for infection

The three different strains of mycobacteria (*M. smegmatis*, BCG and R179) were cultured separately in T25 flasks with 10 ml volume in each flask (to have an appropriate air space). These were incubated in a 37^0C incubator for 2-3 weeks. The subcultures were grown up to an optical density of 0.4 as maximum. Agitation was required for *M. smegmatis* but not for BCG and R179 strains. The cultures were finally stocked at -80^0C for future use. Stock cultures of mycobacteria were brought out of -80^0C and thawed. Clumps in the thawed vials were disrupted by pipetting 10 times with 1 ml tip, passed through a syringe 20 times using

a G25 needle (Stokes et al., 2004). Major clumps were allowed to settle down (Cywes et al., 1997). The settling time was different for each strain (*M. smegmatis*: 30 seconds, BCG: 1 minute, R179: 1 minute). The top 750 µl was collected and added to 4.25 ml of RPMI 1640 media. This 5 ml bacterial suspension was then immediately filtered through 5.0 µm pore size filter (Merck Millipore, Germany) to which 10% human serum was added. The required volume (according to the titration and MOI calculation) was then added to THP-1 cells in complete medium (RPMI1640 + 10% human serum). *M. tuberculosis* stock titration was also done by this procedure (no human serum was added in this case), where an average CFU was obtained by processing 3 stock vials. **Table 3.1** provides a detailed description of bacterial strains and plasmids used in the study.

Table 3.1: Bacterial strains, plasmids used in the study;

Strains/Plasmids	**Description**	**Source/Reference**
M. smegmatis	*M. smegmatis* MC155	Laboratory collection (Harper et al., 2010)
BCG	*M. bovis* BCG strain Pasteur 1743P2	Laboratory collection (Viljoen et al., 2013)
R179	Beijing genotype strain R220	Clinical isolate (Johnson et al., 2006)
E. coli DH5α	ATCC53868	Laboratory collection
pcDNA3.1 3xFlag IFIT1	Mammalian expression vector with human *IFIT1* as an insert and without mutations	(Katibah et al., 2013)
pcDNA3.1 3xFlag IFIT2	Mammalian expression vector with human *IFIT2* as an insert without mutations	(Katibah et al., 2013)
pcDNA3.1 3xFlag IFIT3	Mammalian expression vector with human *IFIT3* as an insert without mutations	(Katibah et al., 2013)

Abbreviations: BCG, Bacillus Calmette–Guérin; *E. coli*, Escherichia coli; *IFIT*, interferon-induced protein with tetratricopeptide;

3.4 Infection with mycobacteria

Pathogenic (R179), facultative-pathogenic (BCG) and non-pathogenic (*M. smegmatis*) strains of mycobacteria were used for infection. Mycobacteria were cultured in 7H9 (added 10% OADC and 0.5% glycerol) without Tween 80. We avoid the use of Tween, as Tween is known to affect macrophage uptake and immune response to *M. tuberculosis* (Leisching et al., 2016). THP-1 cells were infected with each mycobacterial strain at MOI of 1 and permitted for four hours of uptake. The cells were washed three times with PBS to remove any extracellular mycobacteria.

We setup two separate *in vitro* infection experiments, in the first experiment, cells were infected with various mycobacterial strains and then washed after 4 hours of infection. Cells were then incubated for another 8 hours in 5% CO_2 incubator at 37^0C. Similarly, for another set of experiment, infected cells were washed after four hours of infection and then incubated for another 92 hours in 5% CO_2 incubator at 37^0C.

Uninfected THP-1 cells served as control/uninfected samples. Downstream processing of cells was carried out by knocking-up/down of *IFIT1*, *IFIT2* and *IFIT3* genes. Cells were processed for CFU analysis (at 12 and 96 hours), RNA extraction at 12- and 96- hours and processed for Western Blot by collecting cell lysate upon treating with RIPA buffer containing protease inhibitor at 12- and 96- hours post-infection. Cell supernatants were collected at 12- and 96- hours post-infection for multiplex ELISA Assay.

3.5 Knock-up (vector-based over-expression) of *IFITs (IFIT1, IFIT2* and *IFIT3*):

i **Cloning:** A high fidelity Taq Polymerase PCR was run to amplify the insert DNA (*IFIT1, IFIT2,* and *IFIT3* with a FLAG Tag at the N Terminus). *IFIT1* has three

isoforms (1, 2 and 3), *IFIT2* has no variants and *IFIT3* bears two isoforms (a and b). In our study, we used first isoform of *IFIT1* and the first isoform of *IFIT3*. A restriction digest was set up for the PCR product and recipient plasmid which is a mammalian expression vector pcDNA3.1 with neomycin as a selectable marker. The digested DNA was run on an agarose gel and gel purification was performed to isolate the DNA.

The insert was ligated into the recipient plasmid. An aliquot of 1-2 µl of the ligation reaction was transformed into DH5alpha competent cells. DNA purification was performed by picking up a single colony which was grown overnight for DNA purification. After purifying the DNA, a restriction digest was performed (5' cloning site *Nhel*, 3' cloning site *Xhol*, 5' sequence primer CMV-F, 3' sequence primer BGH Reverse) and run on an agarose gel to isolate the plasmid.

ii Overexpression in *E. coli*: The bacterial stock containing cloned plasmid was streaked on LB Agar plate with 100 µg/ml of ampicillin antibiotic as the plasmid designed was resistant to Ampicillin. The agar plate was incubated overnight at 37^0C in a 5% CO_2 incubator. A single colony was picked up and inoculated overnight in Luria Bertani (LB) broth at 37^0C in a 5% CO_2 shaking incubator. The final product which had ampicillin-resistance was sequenced in order to verify the correct plasmid. pcDNA3.1 3xFlag IFIT1, pcDNA3.1 3xFlag IFIT2, and pcDNA3.1 3xFlag IFIT3 was a gift from Kathleen Collins (Addgene plasmid # 53554; http://n2t.net/addgene :53554; RRID: Addgene_53554). **Figure 3.2** describes the detailed flow of vector-based knock-up (vector-based over-expression) of *IFITs*.

iii Plasmid purification: Plasmid purification was carried out for the high-copy plasmid using the QIAGEN plasmid mini kit. The starter LB broth culture was processed by spinning at 6000 x g for 15 minutes at 4^0C in order to harvest the

bacterial cells. The bacterial cells were re-suspended in 0.3 ml of buffer P1. A total of 0.3 ml of buffer P2 was mixed thoroughly by vigorously inverting the sealed tube 4-6 times and incubating at room temperature for 5 minutes. 0.3 ml of ice-cold buffer P3 was added immediately and mixed thoroughly in order to neutralize the solution completely. This mixture was incubated on ice for 5 minutes. The mixture was centrifuged at a maximum speed in a micro centrifuge for 10 minutes. This helps to

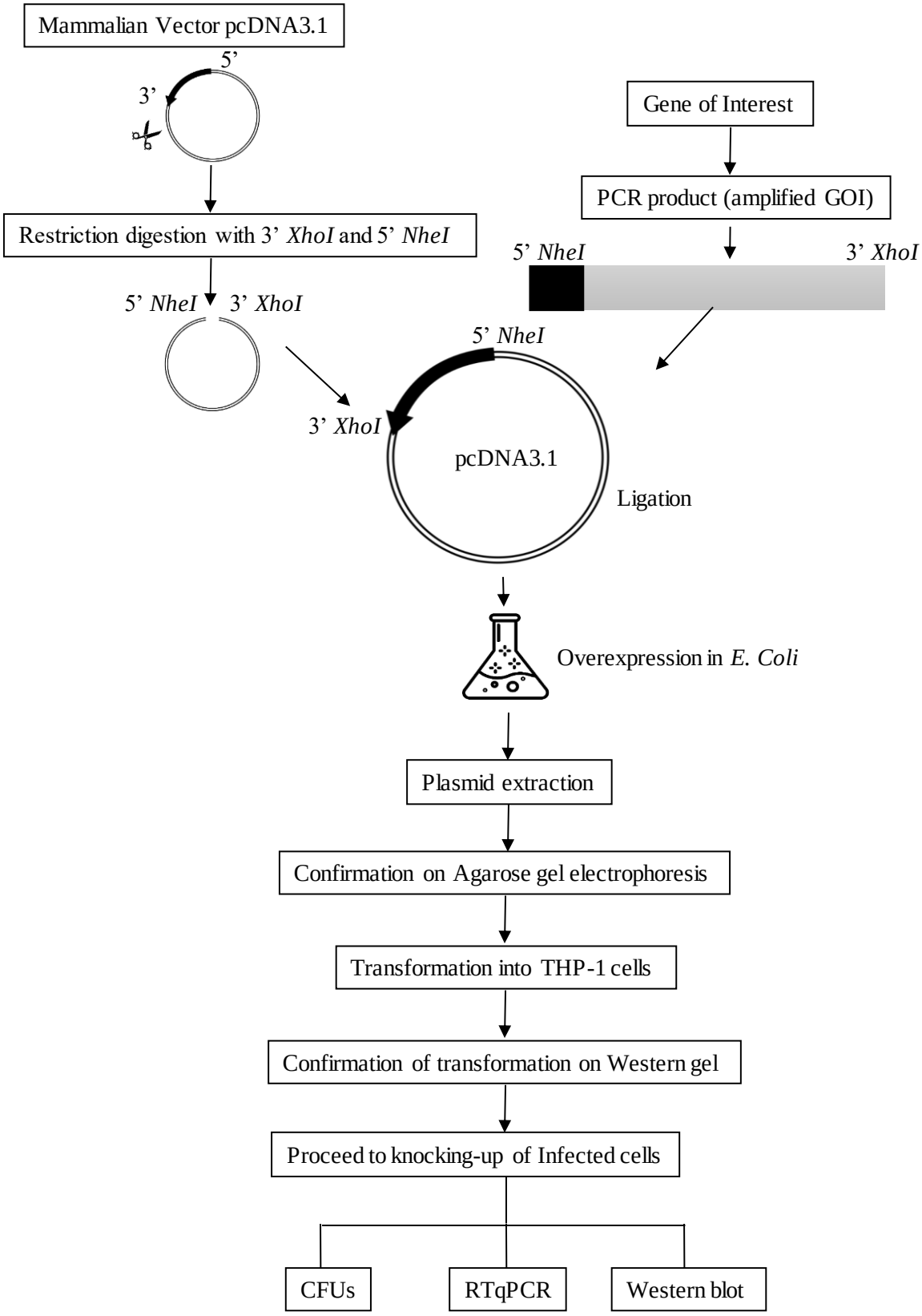


Figure 3.2 Depiction of methodology adopted for vector based knock-up (vector-based over-expression) of IFITs.

Abbreviations: pc, vector constrict; GOI, gene of interest; *Xhol, Xanthomonas holcicola; Nhel, Neisseria mucosa heidelbergensis; E. coli, Escherichia coli*; CFUs, colony forming units; qRT-

bind the DNA to the column. The column was washed with 2 ml of buffer QC. Plasmid DNA was finally eluted with 0.5 ml of double distilled water. The purity and DNA intactness was analysed using 0.8% agarose gel electrophoresis. **Figure 3.3** depicts the agarose gel with plasmids *IFIT1, IFIT2* and *IFIT3*.

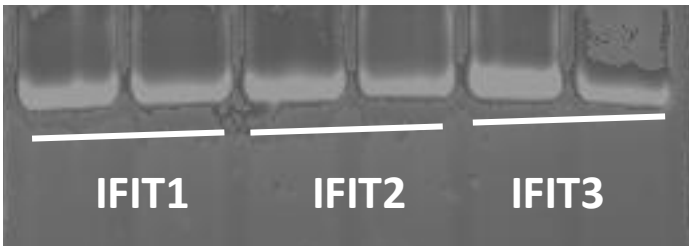


Figure 3.3: Agarose gel electrophoresis (0.8%) for confirmation of intactness of plasmids *IFIT1, IFIT2* and *IFIT3* run in duplicate
Abbreviations: *IFIT,* interferon-induced protein with tetratricopeptide.

iv Transfection: Optimization of transfection in THP-1 cells was performed using different transfection reagents including Lipofectamine 2000 (ThermoFisher Scientific, Cat. No. 11668019), Lipofectamine 3000 (ThermoFisher Scientific, Cat. No. L3000015) and Mission siRNA Transfection Reagent (Sigma-Aldrich, Cat No: S1452). A successful plasmid transfection into THP-1 cells was achieved by Mission siRNA transfection reagent which was confirmed by Western Blot. Transfection was performed in a 48-well plate (Greiner Bio One, cat. No. 677180) with $0.05x10^6$ cells/well in a 300 µl volume of complete media (RPMI + 10% FBS).

For 1 well of a 48-well plate, 2 µl of transfection reagent was added to 125 ng of plasmid DNA (titrated for transfection) and finally mixed with 100 µl of DMEM. This mixture was vortexed and incubated for 15 minutes at room temperature. A total of 100 µl of the final mixture was added to each well and the plate was swirled slowly for mixing. Vector only (pcDNA3.1) was used as a control in this experiment. The cells were then incubated at 37^0C in a 5% CO_2 incubator for respective time points.

Detailed representation of plasmids *IFIT1*, *IFIT2* and *IFIT3* are provided in **Figure 3.4, Figure 3.5 and Figure 3.6.**

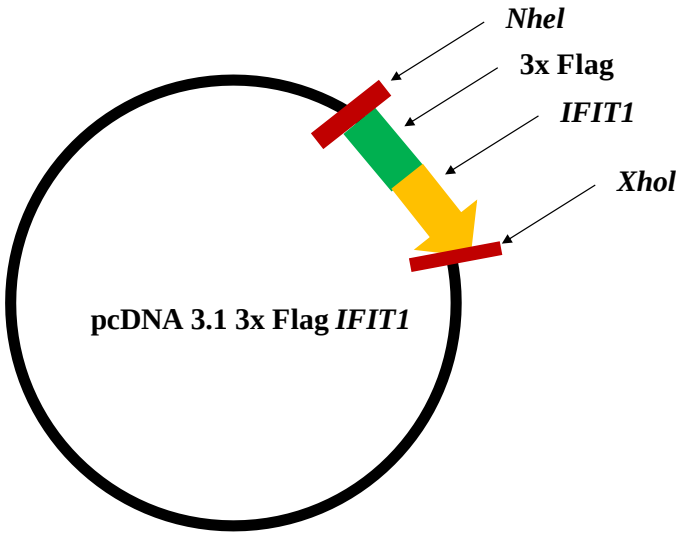


Figure 3.4: Mammalian expression vector (pcDNA3.1) cloned with *IFIT1* and 3x flag into *Nhel* and *Xhol* restriction sites
Abbreviations: pc, vector constrict; *Xhol, Xanthomonas holcicola; Nhel, Neisseria mucosa heidelbergensis; IFIT,* interferon-induced protein with tetratricopeptide.

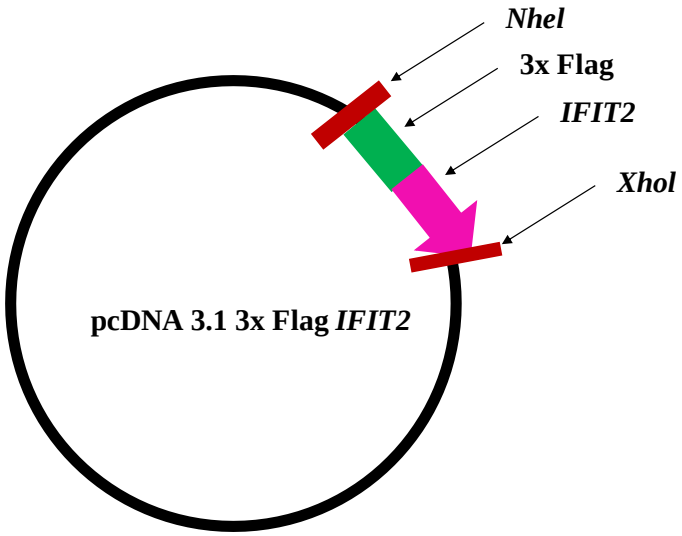


Figure 3.5: Mammalian expression vector (pcDNA3.1) cloned with *IFIT2* and 3x flag into *Nhel* and *Xhol* restriction sites
Abbreviations: pc, vector constrict; *Xhol, Xanthomonas holcicola; Nhel, Neisseria mucosa heidelbergensis; IFIT,* interferon-induced protein with tetratricopeptide.

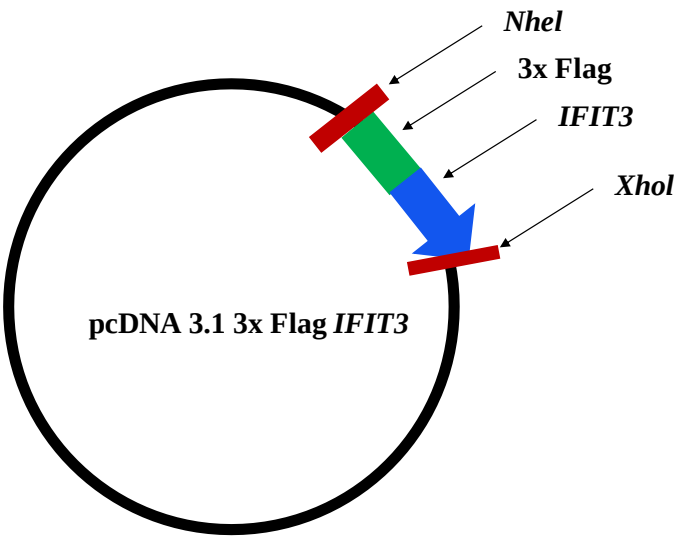


Figure 3.6: Mammalian expression vector (pcDNA3.1) cloned with *IFIT3* and 3x flag into *Nhel* and *Xhol* restriction sites
Abbreviations: pc, vector constrict; *Xhol, Xanthomonas holcicola; Nhel, Neisseria mucosa heidelbergensis; IFIT*, interferon-induced protein with tetratricopeptide.

3.6 Knock-down of *IFITs (IFIT1, IFIT2* and *IFIT3)*

THP-1 cells were seeded with 5×10^4 cells per well of a 48 well plate in 0.3 ml of RPMI supplemented with 10% FBS. A final concentration of 100 nM PMA was added and mixed well. Cells were incubated for 18-20 hours at 37^0C in a 5% CO_2 incubator. After incubation, cells were infected with different mycobacterial strains at pre-specified MOIs (MOI=1). After 4 hours of bacterial uptake, cells were washed thoroughly with PBS and fresh complete media was added to each well (300 µl per well of a 48-well plate). A total of 6.25 µl Flexi Tube siRNA Premix was added drop-wise to the cells which made a final siRNA concentration of 25 nM in each well (**Table 3.2** provides the detailed list of siRNA premix used). For negative control, a scrambled sequence was used as a negative siRNA Premix (Cat. No. S103650325). Culture plates were swirled slowly to mix the premix in complete media. Cells were incubated with the transfection complexes under their optimal growth conditions (37^0C, 5% CO_2 incubator). Gene silencing was measured at 24 and 96 hours post transfection **(Figure 3.7)**. Silencing was performed using Qiagen FlexiTube siRNA

Premix. *In vitro* gene silencing for each gene was performed targeting two different silencing sites. The latter results including CFUs and mRNA expression levels were taken as an average of the values generated by silencing two different target sequences.

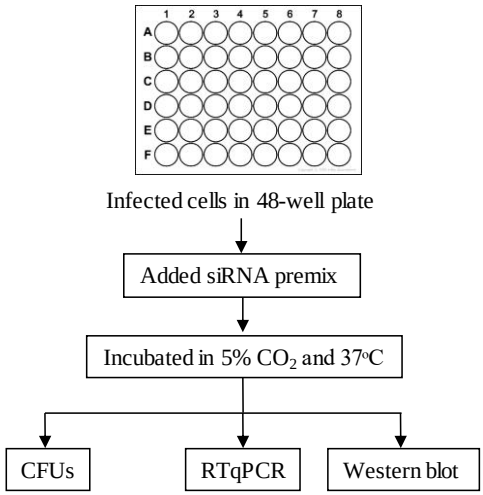


Figure 3.7: Depiction of knock-down of infected THP-1 cells using siRNA pre-mix and confirmation using colony forming units, qRT-PCR and Western blot.
Abbreviations: siRNA, small interfering ribonucleic acid; CO_2, carbon dioxide; °C, degree Celsius; CFUs, colony forming units; QRT-PCR, reverse transcription polymerase chain

Table 3.2: Detailed information of siRNA pre-mix of *IFITs* used for knocking-down experiments

FlexiTube siRNA Premix	Entrez Gene ID	Target Sequence (5' to 3')	Catalogue Number
Hs-IFIT1	3434	CAGGCTGTCCGCTTAAATCCA	S100445879
Hs-IFIT1	3434	TACATGGGAGTTATCCATTGA	S103224284
Hs-IFIT2	3433	AAAGAAAGTTACTGGAACTAA	S104145372
Hs-IFIT2	3433	CCCATAGAGGTTAGTCCTGCA	S104259010
Hs-IFIT3	3437	ATGCTATGGACTATTCGAATA	S103152737
Hs-IFIT3	3437	AGAGATGATTGAAGCACTAAA	S104197788
Negative	3434	Scrambled Sequence	S103650325

Abbreviations: Hs, Homosapiens; *IFIT*, Interferon induced tetratricopeptide; bp, base pair.

3.7 Determination of bacterial counts after knock-up (vector-based over-expression)/knock-down of *IFITs*

CFUs were determined in mycobacteria infected THP-1 cells knocked-up/down with *IFITs* family. These cells were lysed using 0.1% Triton X-100. Bacterial counts was determined by serial dilution (10^{-1} – 10^{-4}) and plating out of mycobacteria onto 7H11 agar plates. The agar plates were incubated at 37 °C for 5 weeks and CFUs/ml was determined. Mycobacteria survival within the treated cells was monitored at 12 and 96 hours post-infection (with *M. smegmatis* not measured at 96 hours).

3.8 Cytotoxicity assay after knock-up (vector-based over-expression)/knock-down of *IFITs*

Cell cytotoxicity was tested with the Roche water soluble tetrazolium (WST-1) cell cytotoxicity reagent (Roche, USA) in 1:10 dilution of WST-1 reagent to RPMI complete media (RPMI + 10% Human Serum). Cells post-infection and at two time points (12 and 96 hours) were processed for cell cytotoxicity. A total of 300 µl of 1:10 dilution of WST-1 reagent to complete media was added to the wells of a 12-well plate. The culture plates were covered properly with aluminium foil as the cytotoxicity reagent is light sensitive. Cells were incubated for 1 hour at 37 °C and 5% CO_2. Cells were then transferred to a multi-mode reader placed in the dark room. Absorbance was measured at 450 and 630 nm (wavelength correction). The difference between the two absorbance readings was determined and plotted in Microsoft Excel as percentage values.

3.9 RNA extraction after knock-up (vector-based over-expression)/knock-down of *IFITs*

Total RNA from human macrophages was extracted using a kit RNeasy Plus Mini Kit (Cat. No. 74134, Qiagen, Limburg, Netherlands). The cell culture medium was completely aspirated from the culture plates. Cells were washed three times with ice-cold 1X PBS. 350µl of RLT Plus Buffer (with 10 µl/1ml β-mercaptoetanol) was added to the wells and scrapped with a pipette tip to disrupt the cells. The lysate was then pipetted into a micro

centrifuge tube and vortexed to ensure that no cell clumps were visible. The lysate was then loaded directly to a QIAshredder spin column and centrifuged for 2 minutes at maximum speed.

The homogenised lysate was transferred to a gDNA eliminator spin column and centrifuged for 1 minute at 8000 x g. The gDNA eliminator column ensures removal of any genomic DNA from all the samples. A total of 350 µl of 70 % ethanol was added to the flow through and mixed well by pipetting. Up to 700 µl of the sample (including any precipitate) was transferred to RNeasy spin column placed in a 2 ml collection tube and the lid was closed gently. This was then centrifuged for 15 seconds at 8000 x g and the flow through was discarded.

A total of 700 µl of RW1 Buffer was then added to RNeasy spin column and the lid was gently closed. This was then centrifuged for 15 seconds at 8000 x g to wash the spin column membrane and the flow through was discarded. Column washing was performed as explained in the RNA extraction section of chapter 2 (mentioned previously). The RNeasy spin column was then placed in a 2ml collection tube and centrifuged at full speed for 1 minute. The RNeasy spin column was then placed in a 1.5 ml collection tube. A total of 30 µl of RNase-free water was then added directly to the spin column membrane and then centrifuged for 1 minute at 8000 x g to elute RNA.

For each experiment, RNA quantity and quality were determined using Agilent 2100 Bioanalyzer. The RNA with RIN ≥ 9 was used for AmpliSeq and qRT-PCR experiments.

3.10 Quantitative real time PCR after knock-up (vector-based over-expression)/knock-down of *IFITs*

Good quality RNA (RIN>9, 0.8 µg) was converted to cDNA using a kit (Quantitect ^{R} Reverse Transcription Kit). To ensure the removal of genomic DNA, ‘gDNA wipe-out buffer’ was added to RNA (included in the kit) prior to the RNA conversion step. qRT-

PCR amplification was run on a LightCycler^{R} 96 system (Roche, Germany). LightCycler^{R} 480 SYBR Green I Master was used for various differentially expressed genes using QuantiTect^{R} primer assays with 20 µl reaction volume.

Hs-GAPDH and Hs-UBC were selected as reference genes with stable expression levels. The amplification process involved 45 cycles of 95 °C for 10 s (denaturation) followed by 60 °C for 10s (annealing) and finally 72 °C for 10s (extension). Gene expression fold-changes were computed (using ΔΔ Ct method) for pathogenic infected and non-pathogenic infected macrophages using calibrated normalized relative quantities using the equation $N = N_0 \times 2^{Cp}$. All qRT-PCRs were performed on RNA extracted from three different experiments. All biological replicates having a positive control and a non-reverse transcription control were run in triplicate (along with calibrator) as per the MIQE Guidelines.

3.11 Ingenuity pathway analysis (IPA) after knock-up (vector-based over-expression)/knock-down of *IFITs*

Ingenuity pathway analysis (IPA) was performed using IPA Gene View Software version 01-13. To identify potential targets from the AmpliSeq data, it was essential to filter the list of DEGs obtained after analysing the AmpliSeq data. IPA was one of the filtering measures applied on the DEG list. Thoroughly analysed AmpliSeq data with strong FDR, p-values and fold changes was used for IPA analysis. The baseline data were uploaded on IPA. Statistical measures reflecting dataset genes interacting with each other (overlap p-value, <0.001) and activation (positive z-score, fold change of >1.5) were based on the known direction of up and down regulated genes. For detailed pathway evaluation DEGs with fold changes > 1.5 and adjusted p-value ≤ 0.001 was used in the analysis.

3.12 Multiplex ELISA Assay after knock-up (vector-based over-expression)/knock-down of *IFITs*

A panel of 13 cytokines including IDO-1, TNF-α, eight interleukins (IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-12p40, IL-12p70 and IL-23), type-I interferon (IFN-α and IFN-β) and type-II interferon (IFN-γ) was designed for studying the host response upon knocking-up/down of IFITs in THP-1 macrophages infected with mycobacteria at different time points using the Luminex platform. This panel was designed based on the 19 DEGs obtained after AmpliSeq analysis. Cytokine concentrations were measured using Procarta Luminex kits - ThermoFisher on the Bioplex platform (BioRad Laboratories, Hercules, CA, USA) according to the instructions of the kit manufacturer (ThermoFisher).

Samples were evaluated in duplicate by a single technician. The coefficient of variation for duplicate runs was <20% for all samples (range, 5.2-19.6%) and the levels of all analytes in the quality control reagents were within their expected ranges. The standard curve for all samples ranged from 3.6-10000 pg/ml. Bioplex Manager Software version 1 was used for analysis of median fluorescent intensities.

The Procarta ThermoFisher Luminex kit is recommended to use with serum, plasma and culture supernatants. Here, we used the culture supernatants collected at different time points post-infection and stored at -80^{0}C. Samples were brought out of -80^{0}C and thawed 30 minutes before setting up the experiment.

i Preparation of standards

Each antigen standard set vials was centrifuged at 2000xg for 10 seconds. A total of 50µl of sample type specific buffer was added into each standard vial. Vials were vortexed for 10 seconds and centrifuged at 2000xg for 10 seconds to collect contents at the bottom of the vial. Vials were then incubated on ice for 10 minutes to ensure complete reconstitution. The entire content of each vial was pooled into one of the vials and topped up with sample type specific buffer to a total volume of 250 µl. The vial was gently vortexed for 10 seconds

and centrifuged at 2000xg for 10 seconds to collect the contents at the bottom of the vial, followed by a 4-fold serial dilution.

ii Luminex assay protocol

Magnetic capture beads were vortexed for 30 seconds. A total of 50 µl of the magnetic beads was added to each well of the 96-well plate. The magnetic beads were washed by securely placing the 96-well flat bottom plate into the magnetic plate washer. The liquid from the wells was removed by quickly inverting the 96-well plate assembly over a sink. For cell culture supernatants, 50 µl of prepared standards and the samples were added into each respective wells. For the wells designated as blanks, 50 µl of cell culture medium was added. The plate was sealed and incubated on a shaker at room temperature for 2 hours. Plates were washed twice and 25 µl of detection antibody was added to each well. The plates were sealed and incubated on a shaker at room temperature at 500 rpm for 30 minutes. The plate was washed twice. A total of 50 µl of SAPE solution was added to each well. The plates were sealed and incubated on a shaker at room temperature at 500 rpm for 30 minutes. The plate was washed twice. A total of 100 µl of reading buffer was added to each well. The plates were sealed and incubated on a shaker at room temperature at 500 rpm for 5 minutes. The plate seal was removed and projected to Luminex instrument for plate reading.

3.13 Western blot

After the infection and subsequent transfection period, protein was extracted using RIPA buffer (0.5% sodium deoxycholate, 150 mM NaCl, 0.1% SDS and 50 mM Tris and 1.0% Triton X-100). A total of 50 µl of protease inhibitor (Roche, Switzerland) was added to 1 ml of lysis buffer. Complete cell lysis was achieved by scraping the cells using a cell scraper and pipetting 5 – 10 times with a 1 ml pipet tip. Cells were left on ice for 10 minutes

followed by centrifugation at 200× g for 10 minutes at 4°C., to achieve at least 80% cell lysis. The supernatant was collected and centrifuged at 8000× g for 30 minutes, the post-nuclear supernatant (PNS) was collected, filtered through a 0.2 µm acrodisc syringe filter, (Sigma-Aldrich, USA) into 1.5 ml Eppendorf tubes and stored at -80˚C. The Protein quantity and quality was assessed using the Bradford assay and SDS-PAGE.

For Bradford assay, a standard curve was generated by using 10% bovine serum albumin (BSA), Cat No. HD14-4 (QIAGEN, USA). A working stock of 1 mg/ml was made, and 6 BSA concentrations were used to generate the standard curve 2 µg, 4 µg, 8 µg, 12 µg, 16 µg and 20 µg, with 900 µl of 1× Bradford dye reagent Cat No. 500-0205 (Bio-Rad, USA). Protein samples were kept on ice. A total of 5 µl of the sample, 95 µl distilled water and 900 µl of 1× Bradford dye reagent were mixed to make 1 ml and the absorbance read at OD 595 on a spectrophotometer (MRCLAB Spectro UV-16, Israel). A control sample contained water and Bradford reagent.

A total of 10 µg of each protein sample was used. The sample was mixed with 1× XT sample buffer (Bio-Rad, USA) in an Eppendorf tube, heated at 90˚C on a heating block for 2 minutes. Samples were loaded in a 10% Precast SDS gel (Bio Rad Mini TGX Gels, Bio-Rad, USA, Cat No. 456-1044), in 1× running buffer XT MOPS, Bio-Rad, USA). The gel was run at 90 Volts for 30 minutes to quantify the protein levels. Gel was then transferred onto a Polyvinylidene difluoride (PVDF) membrane (BioRad, Cat No. 1620174) for 7 minutes using the TURBO Blot Transfer machine (BioRad Trans Blot Turbo transfer system, serial no. 690BR016386). The blotted membranes were blocked with blocking solution (5% BSA in TBST buffer) for 2 hours at room temperature on a 15 rpm shaker. The membrane was then washed three times using TBST buffer (TBS – 10x – 24mM Tris base, 88mM NaCl, pH 7.6, 0.1% Tween) (each wash for 5 minutes on a shaker at 20 rpm

room temperature). The membrane was then incubated with different primary antibodies **(Table 3.3)** overnight at 4^0C.

Table 3.3: List of primary antibodies used for Western blot.

Primary Antibody	**Company**	**Catalog Number**	**Dilution in TBST**
anti-Flag	Sigma Aldrich	F3165	1:5000
anti-*IFIT1*	Sigma Aldrich	SAB4501508	1:1000
anti-*IFIT2*	Sigma Aldrich	SAB2101128	1:1000
anti-*IFIT3*	Sigma Aldrich	AV46034	1:1000

Abbreviations: *IFIT*, interferon induced proteins with tetratricopeptide; TBST, tris buffered saline with tween 20.

Membrane was washed three times using TBST buffer (each wash for 5 minutes on a 20 rpm shaker at room temperature. Membrane was then incubated with respective secondary antibody for 1 hour at room temperature (anti mouse monoclonal antibody (Santa Cruz Biotechnology, Sc516102) – 1:5000 dilution in TBST used against anti FLAG primary antibody and anti-rabbit monoclonal antibody (Santa Cruz Biotechnology, Sc2030) – 1:5000 dilution in TBST used against anti *IFIT1*, anti *IFIT2* and anti *IFIT3* Primary antibody). Membrane was washed three times using TBST buffer (each wash for 5 minutes) on a 20 rpm shaker at room temperature. Membrane was conjugated with horseradish peroxidase (BioRad). The bound secondary antibody was spotted using an improved chemiluminescence detection kit Clarity Max Western ECL substrate (Cat. No. 1705062, BioRad). Furthermore, to ensure equal loading of proteins, the membranes were stripped for 30 minutes at room temperature with stripping buffer (100mM 2-mercaptoethanol, 62.5mM Tris, and pH 6.8, 2% SDS) and were re-probed with beta-actin/GAPDH antibody (Santa Cruz Biotechnology, Sc32233) for 2 hrs at room temperature.

3.14 Statistical analysis

Real time QRT-PCR data were analysed using Light Cycler 96 SW 1.1 Software and Graph-pad Prism V7. Relative expression which measures target transcript in a treatment group to that of the untreated group was analyzed through the software in response to the calibrator and non-transcription control. The relative expression data of the cytokines was further analysed through Graph-pad Prism to generate the p-values through one-way ANOVA. The final p-values were generated through Multiple Testing correction using Tukey corrections. The data (in technical triplicate) was plotted in histograms with respective mean and standard deviations.

Cytotoxicity graphs and CFUs were plotted with an average of the technical triplicates leading to the mean of all the biological replicates. Statistical analysis was performed through Graph-pad Prism V7 software where the percentage of every expressing cell was generated and p value was calculated using two-way ANOVA with Tukey's correction. Luminex data were analysed by two-way ANOVA with Tukey's correction using Graph-pad Prism V7 for Windows (Graph-pad Software, San Diego California, USA).

Results

3.15 CFUs and cell cytotoxicity after knocking-up and knocking-down of *IFITs*:

We have determined CFUs of *M. smegmatis* after 12 and 24 hours, while CFUs of BCG and R179 were determined after 12 and 96 hours of infection to hMDMs with knock-down and knock-up (vector-based over-expression) of *IFIT1, IFIT2* and *IFIT3*. At 12 hours, we found significantly higher ($p<0.0001$) CFUs after knock-down of all three *IFITs,* whereas, knock-up (vector-based over-expression) resulted in significantly reduced ($p<0.0001$) number of CFUs for all three strains of mycobacteria.

Subsequently, CFUs of *M. smegmatis* at 24 hours, while BCG and R179 at 96 hours post-infection upon knocking down with *IFITs* showed significantly higher CFUs ($p<0.001$), on the other hand, knocking-up, showed significantly reduced CFUs ($p<0.001$). Comparison of CFUs across the strains was found to be similar for the scrambled sequence for knock-down and vector control for knock-up (vector-based over-expression) of *IFITs* **(Figure 3.8, Figure 3.9 & Figure 3.10)**.

At 12 hours, comparison of *M. smegmatis* CFUs across knock down of *IFIT* family showed significantly higher counts after knock-down with *IFIT2* ($p=0.007$) and *IFIT3* ($p=0.024$) as compared to *IFIT1*.

Also, comparison of CFUs across knock-down of *IFIT2* and IFIT3 were found to be similar ($p=0.996$). At 12 hours, comparison of CFUs across knock up of *IFIT* family showed significantly higher counts after knock-up (vector-based over-expression) with *IFIT2* ($p=0.014$) and *IFIT3* ($p=0.001$) as compared to *IFIT1*. **Figure 3.11** shows a representative image of CFUs after knocking-up of *IFITs.*

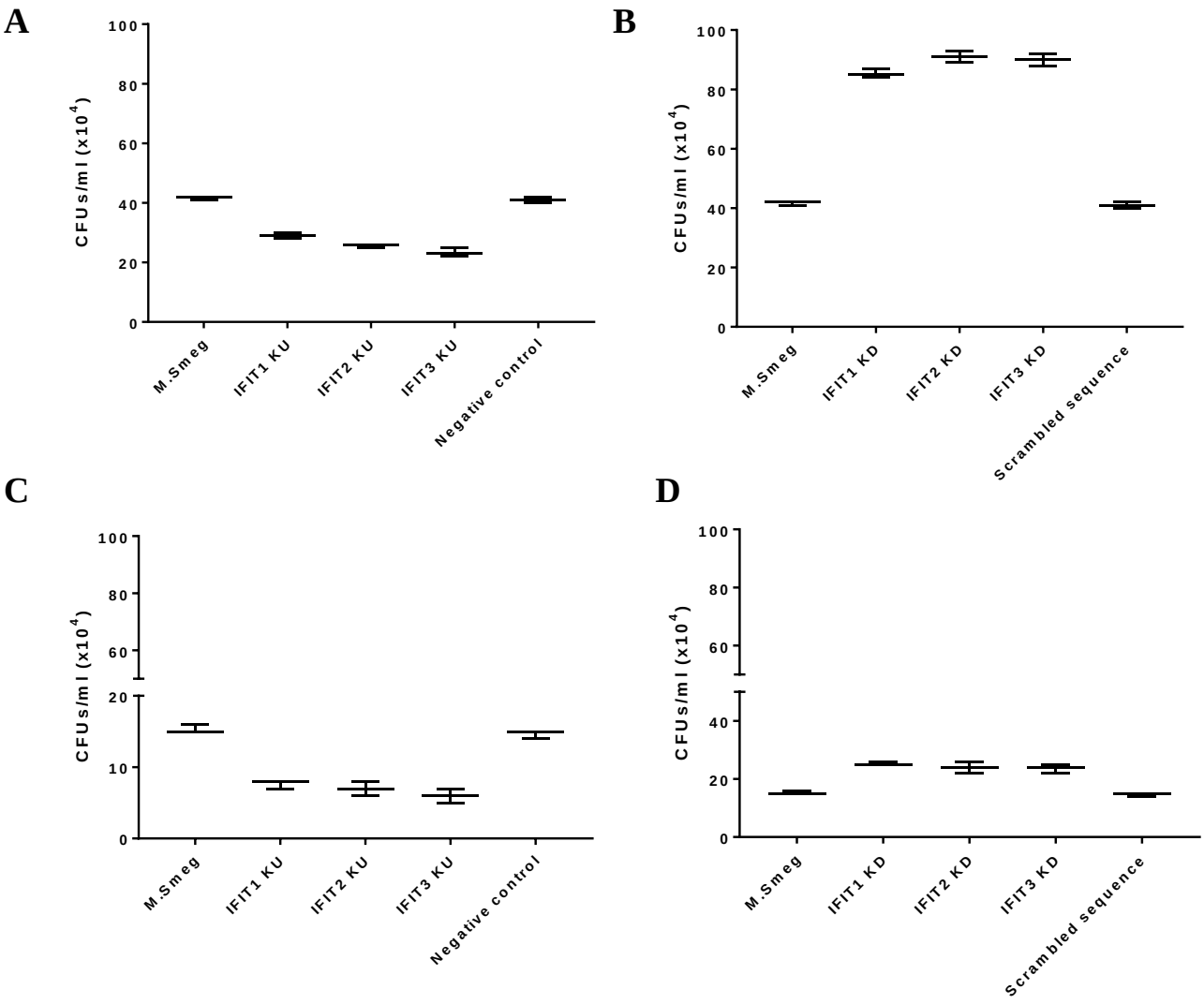


Figure 3.8: CFUs obtained with and without knock-up (vector-based over-expression) and knock-down of IFITs in hMDMs infected with *M. smegmatis*; A, 12 hours IFIT Knock-up (vector-based over-expression); B, 12 hours IFIT Knock-down; C, 24 hours IFIT Knock-up (vector-based over-expression); D, 24 hours IFIT Knock-down; KU, knock-up (vector-based over-expression); KD, knock-down.

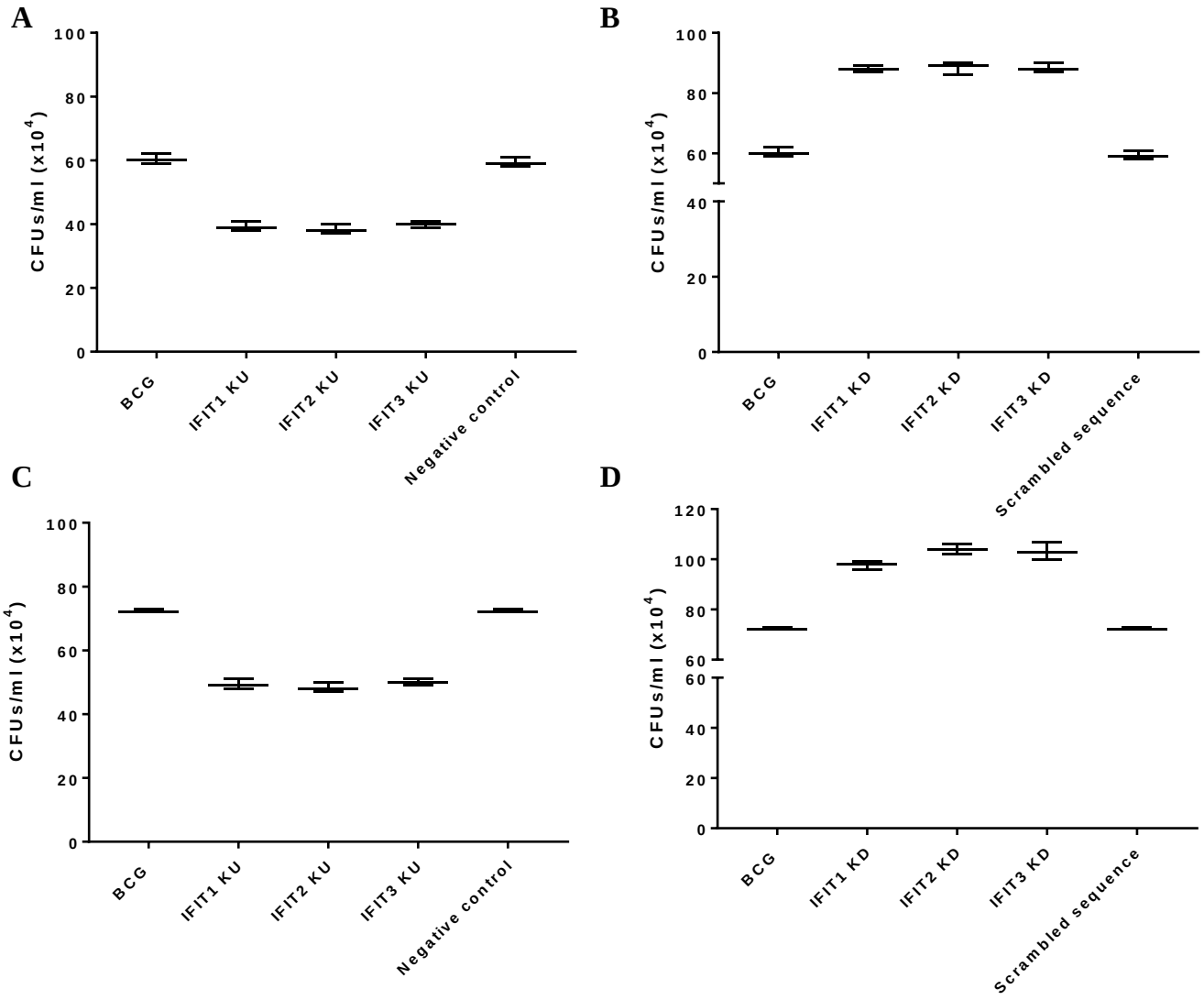


Figure 3.9: CFUs obtained with and without knock-up (vector-based over-expression) and knock-down of IFITs in hMDMs infected with BCG*;* A, 12 hours IFIT Knock-up (vector-based over-expression); B, 12 hours IFIT Knock-down; C, 96 hours IFIT Knock-up (vector-based over-expression); D, 96 hours IFIT Knock-down; KU, knock-up (vector-based over-expression); KD, knock-down.

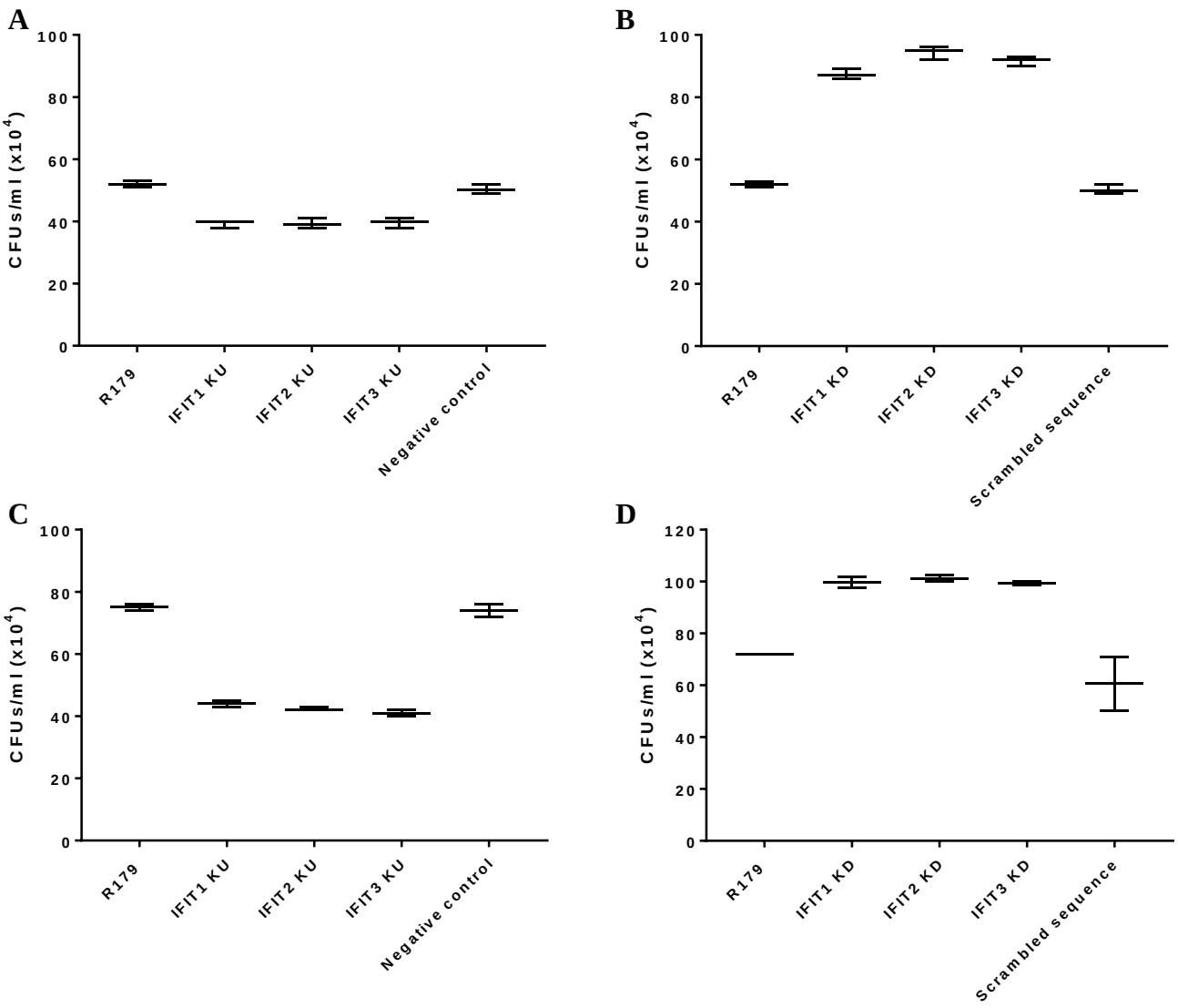


Figure 3.10: CFUs obtained with and without knock-up (vector-based over-expression) and knock-down of IFITs in hMDMs infected with R179*;* A, 12 hours IFIT Knock-up (vector-based over-expression); B, 12 hours IFIT Knock-down; C, 96 hours IFIT Knock-up (vector-based over-expression); D, 96 hours IFIT Knock-down; KU, knock-up (vector-based over-expression); KD, knock-down.

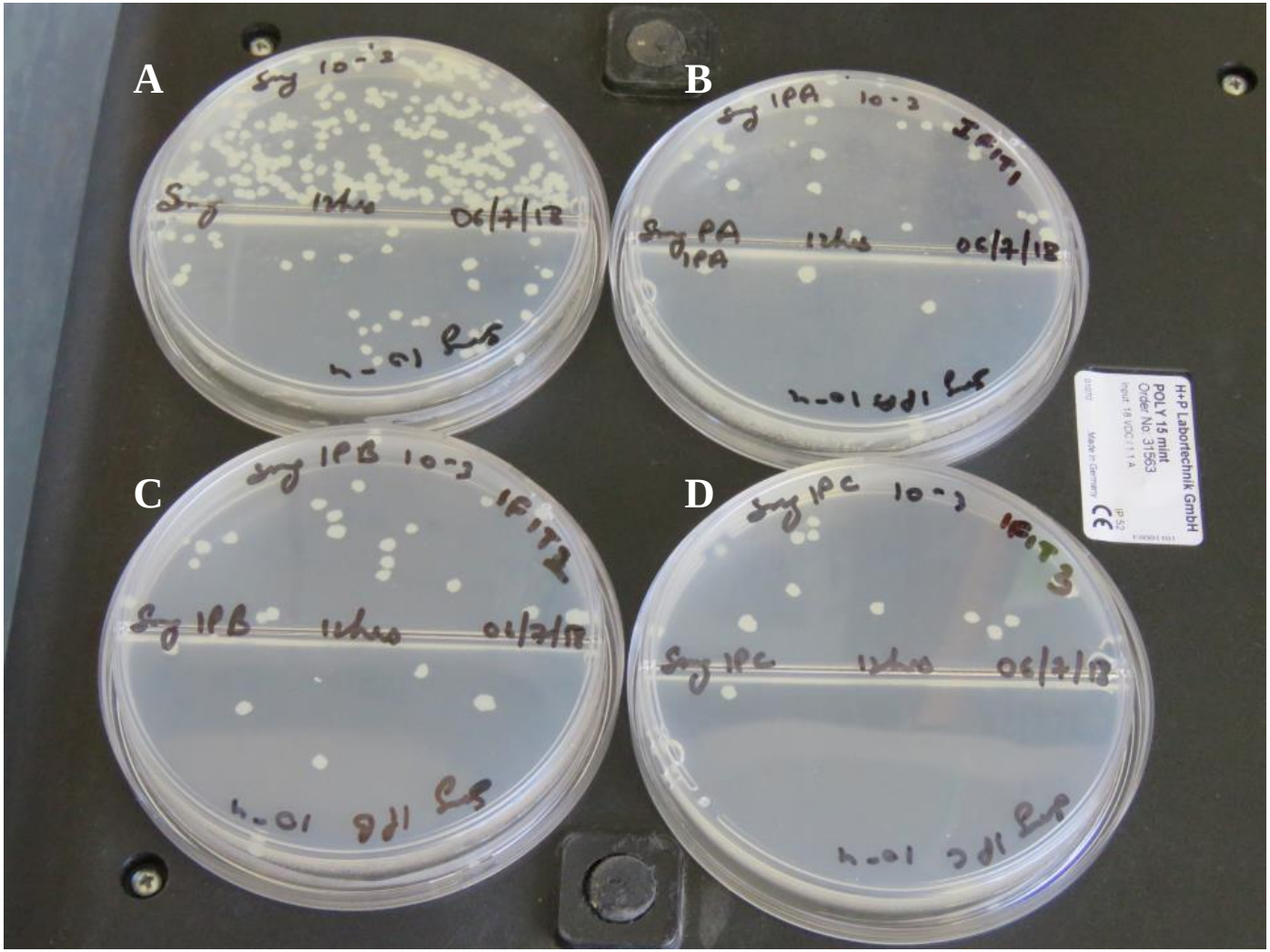


Figure 3.11: A representative image of CFUs obtained after a) *M. smegmatis* infection in hMDMs after 12 hours, b) knock-up (vector-based over-expression) of *IFIT-1* in hMDMs infected with *M. smegmatis* at 12 hours post-infection, c) knock-up (vector-based over-expression) of *IFIT-2*, and d) knock-up (vector-based over-expression) of *IFIT-3*.

Subsequently, at 96 hours (4 days), BCG CFUs across knock down of *IFIT* family showed significantly higher colony counts after knock-down with *IFIT2* (p=0.018) and *IFIT3* (p=0.034) as compared to *IFIT1*.

At 12 hours, comparison of R179 CFUs across knock down of *IFIT* family showed higher colony counts after knock-down with *IFIT2* (p=0.002) and *IFIT3* (p=0.043) as compared to *IFIT1*.

3.16 Viability of THP-1 cells

Viability of THP-1 cells determined by cell cytotoxicity assay after infection with *M. smegmatis*, BCG and R179 were found to be statistically similar after knocking-up and knocking-down with *IFITs*. Further, comparison of viability at 12 and 24 hours for *M. smegmatis*, and 12 and 96 hours for BCG, R179 revealed consistent viability independent of knocking-up/down with *IFITs*. **Figure 3.12, Figure 3.13 and Figure 3.14** show the comparison of viability of hMDMs upon knocking-up and knocking-down of *IFITs* after infection with *M. smegmatis*, BCG and R179, respectively. The viability of THP-1 cells at all-time points ranged from 85-95%. This indicated that knock-up (vector-based over-expression)/knock-down was not cytotoxic to THP-1 cells under all conditions.

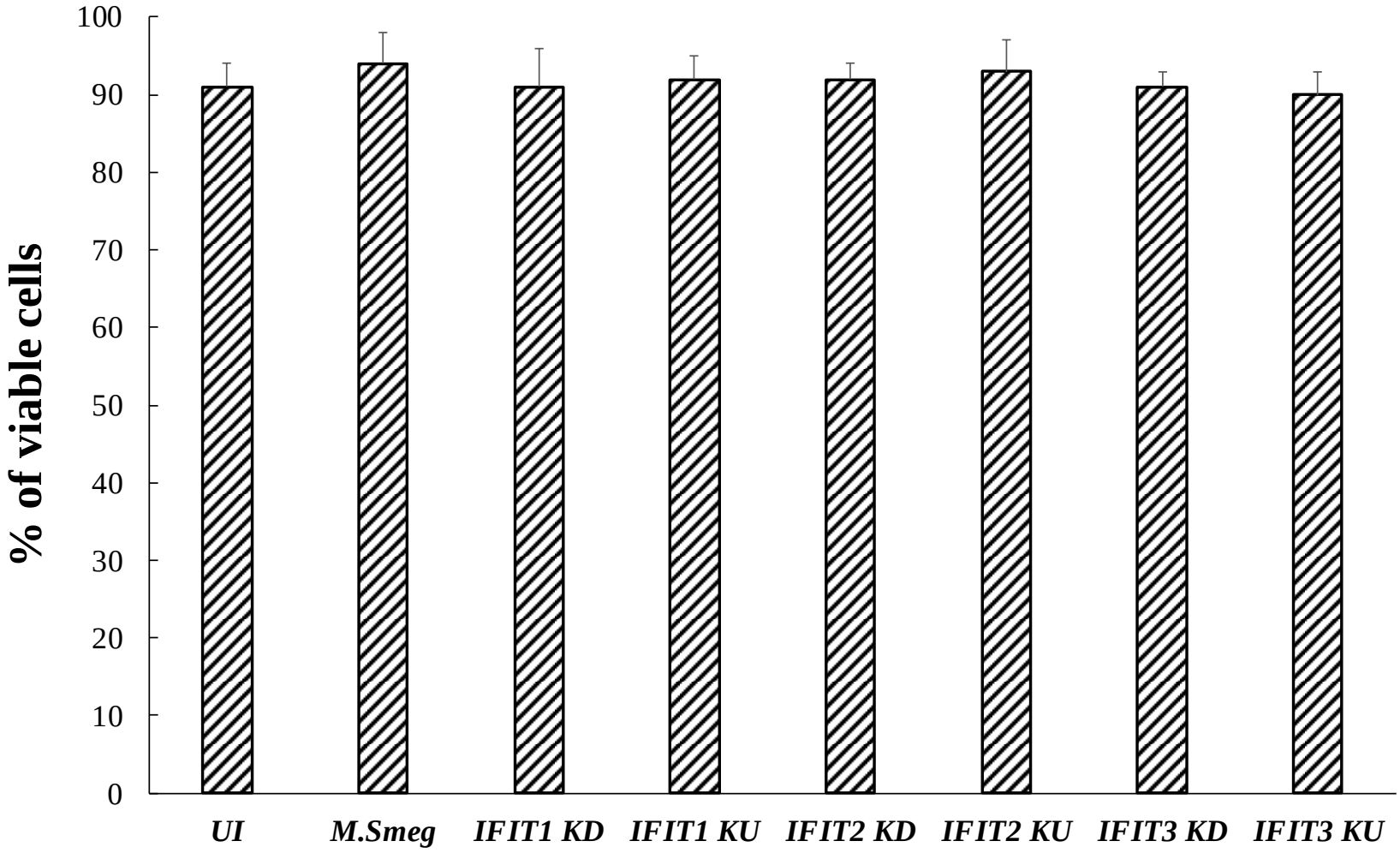


Figure 3.12: Results of cytotoxicity analysis of hMDMs at 12 hours post *M. smegmatis* infection. KU-Knock-up (vector-based over-expression), KD-Knock-down. There was no significant difference observed here.

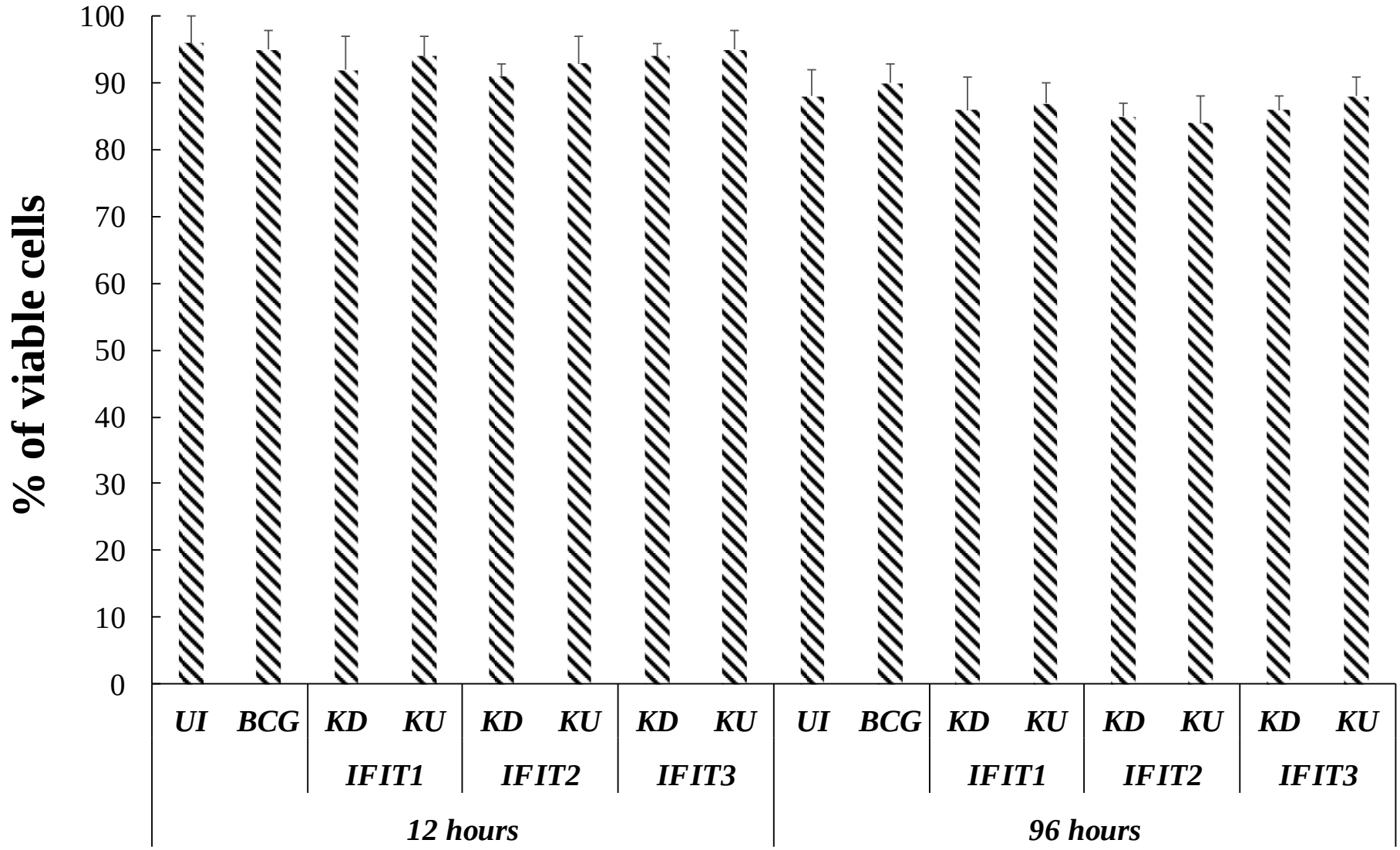


Figure 3.13: Results of cytotoxicity analysis of hMDMs at 12 and 96 hours post BCG infection. KU-Knock-up (vector-based over-expression), KD-Knock-down. There was no significant difference observed here.

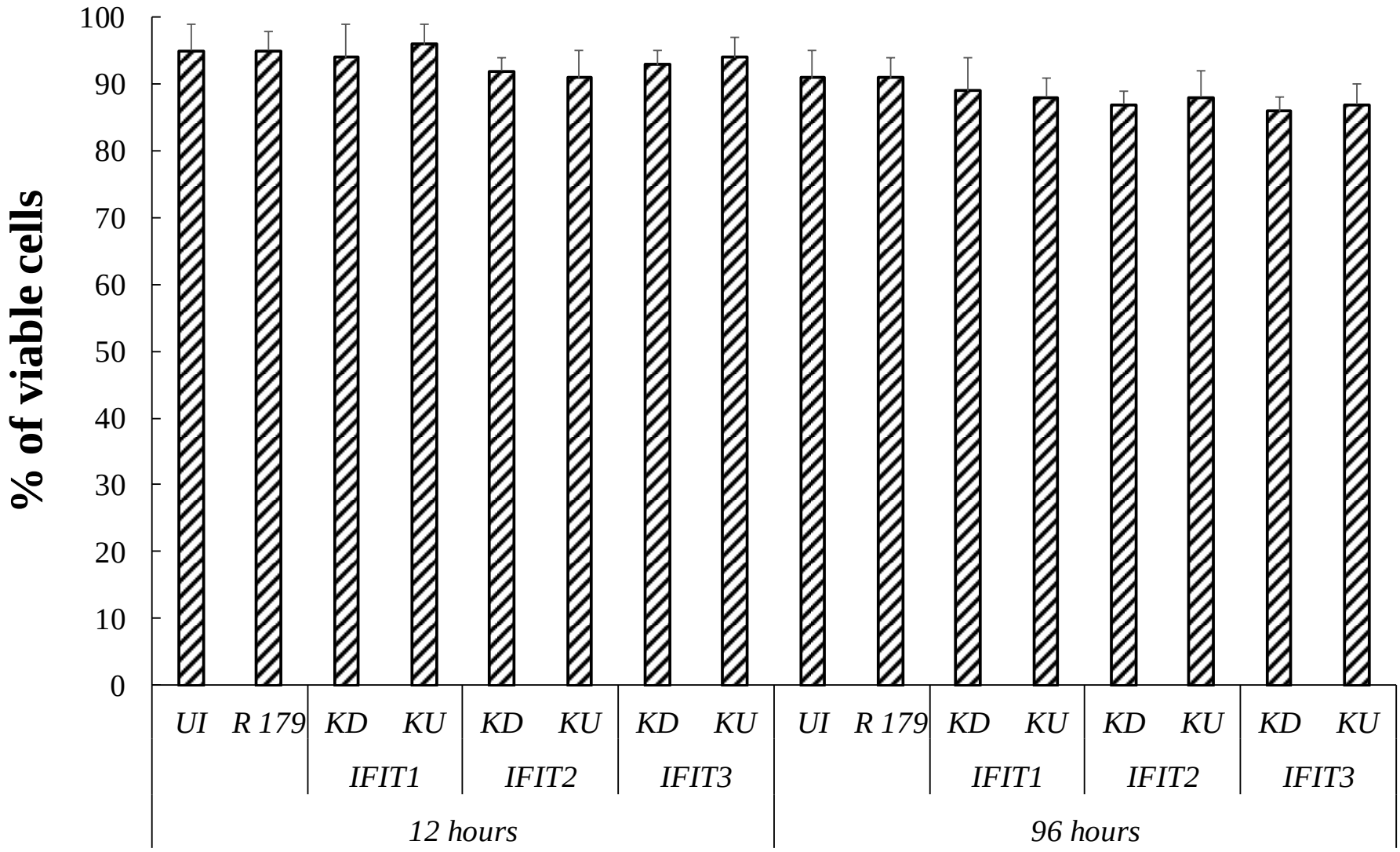


Figure 3.14: Results of cytotoxicity analysis of hMDMs at 12 and 96 hours post R179 infection. KU-Knock-up (vector-based over-expression), KD-Knock-down. There was no significant difference observed here.

3.17 Western Blot after knock-up (vector-based over-expression)/knock-down of *IFITs*

We also confirmed knocking-up and knocking-down of *IFITs* by investigating protein expression through Western blotting **(Figure 3.15)**. GAPDH protein expression was used as internal quality control. The blots clearly depict that after knocking-up there was an increase in band intensity across all three strains, whereas, we observed a decrease in intensity after knocking down across the strains.

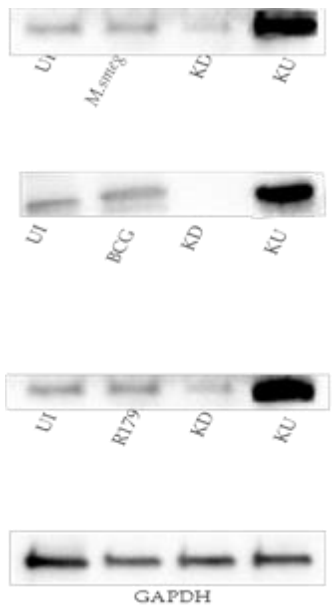


Figure 3.15: Representative Western blot confirming knock-down and knock-up (vector-based over-expression) of *IFIT1* in hMDMs infected with *M. smegmatis*, BCG and R179 at 12 hours post-infection (*IFIT1* – 55 KDa, GAPDH – 37 KDa); KU-Knock-up (vector-based over-expression), KD-Knock-down.

3.18 Results of qRT-PCR after knock-up (vector-based over-expression)/knock-down of *IFITs*

We determined the relative expression of *IFITs* (1, 2 & 3) upon infection with *M. smegmatis* (at 12 hours), BCG and R179 (at 12 and 96 hours) through qRT-PCR after knocking-down and knocking-up of these *IFITs*. As expected, knocking-down *IFITs* across all three strains showed significantly lower relative expression ($p<0.001$) and upon knocking-up showed statistically higher relative expression ($p<0.001$) of respective *IFITs.*

For *M. smegmatis*/*M. bovis* BCG/*M. tb* R179, at 12 hours post-infection, the mRNA expression level for *IFIT1* was very low upon knocking-down ($p<0.001$), while it was higher upon knocking-up ($p<0.001$) when compared to the mRNA expression levels of cells only infected with *M. smegmatis*/BCG/R179. The mRNA expression level for *IFIT2* was very low upon knocking-down ($p<0.001$) while, was higher upon knocking-up

($p<0.001$) when compared to the mRNA expression levels of cells only infected with *M. smegmatis*/BCG/R179. This was also true for *IFIT3* where the mRNA expression level was very low upon knocking-down ($p<0.001$) and higher upon knocking-up ($p<0.001$). The expression levels among the *IFIT* family was similar upon knocking-up/down of *IFIT1*, *IFIT2* and *IFIT3*.

In BCG/R179, at 96 hours post-infection, the mRNA expression level for *IFIT1* was very low upon knocking-down ($p<0.001$) while, expression was higher upon knocking-up ($p<0.001$) when compared to the mRNA expression levels of cells only infected with BCG/R179. This was also observed in *IFIT2/IFIT3* where the mRNA expression level was very low upon knocking-down ($p<0.001$) while, expression was higher upon knocking-up ($p<0.001$) when compared to the mRNA expression levels of cells only infected with BCG/R179. The expression levels among the *IFIT* family was similar upon knocking-up/down of *IFIT1*, *IFIT2* and *IFIT3*.

Figure 3.16, Figure 3.17 and Figure 3.18 show the comparison of mRNA expression upon knocking-up and knocking-down of *IFITs* in hMDMs after infection with *M. smegmatis*, BCG and R179, respectively.

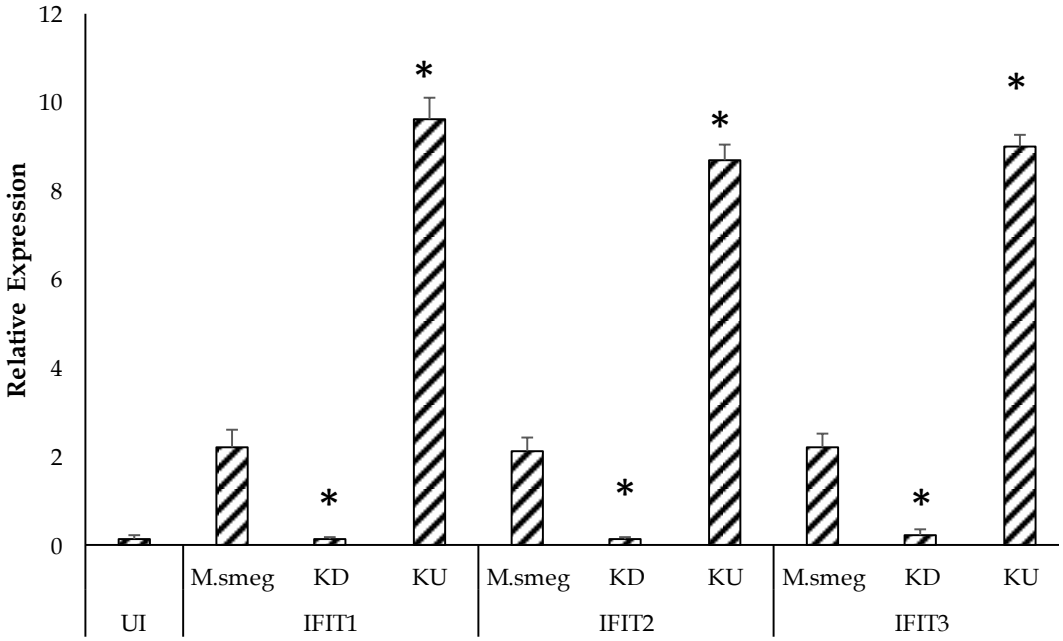


Figure 3.16: Comparison of relative expression after knock-down and knock-up (vector-based over-expression) of IFIT1, IFIT2 and IFIT3 in hMDMs infected with *M. smegmatis;* KU-Knock-up (vector-based over-expression), KD-Knock-down. * indicates p<0.001 as compared to uninfected.

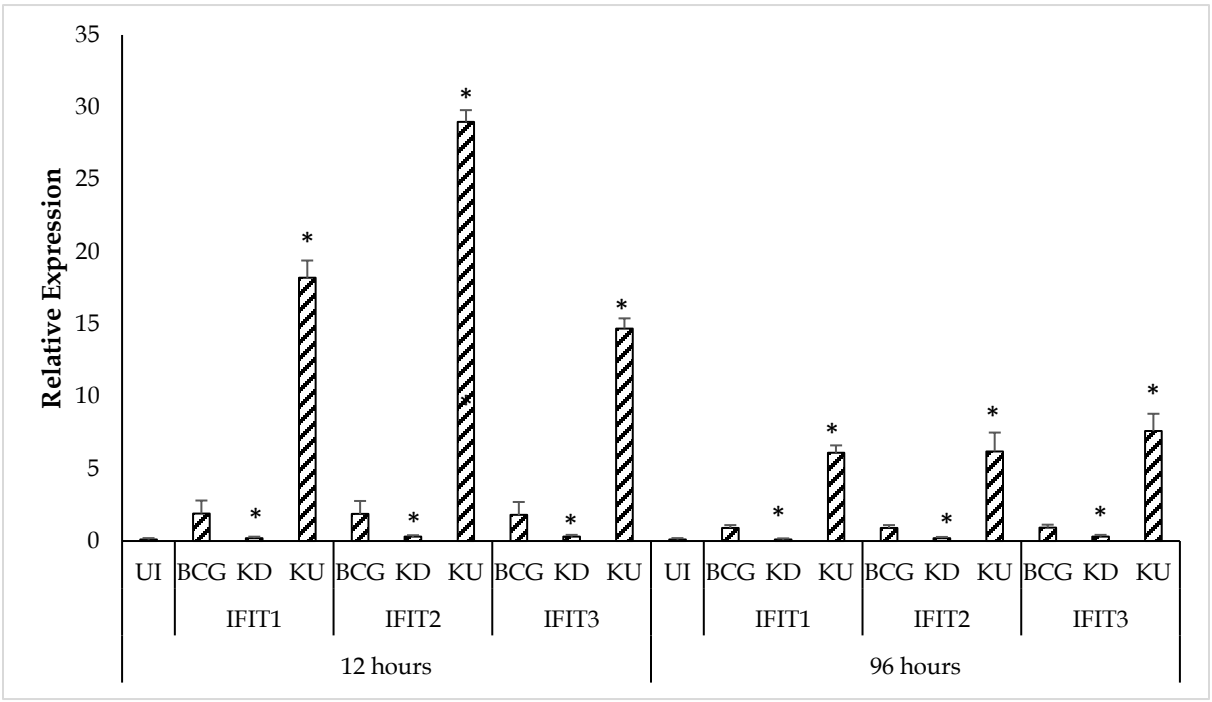


Figure 3.17: Comparison of relative expression after knock-down and knock-up (vector-based over-expression) of IFIT1, IFIT2 and IFIT3 in hMDMs infected with BCG; KU-Knock-up (vector-based over-expression), KD-Knock-down. * indicates p<0.001 as compared to uninfected.

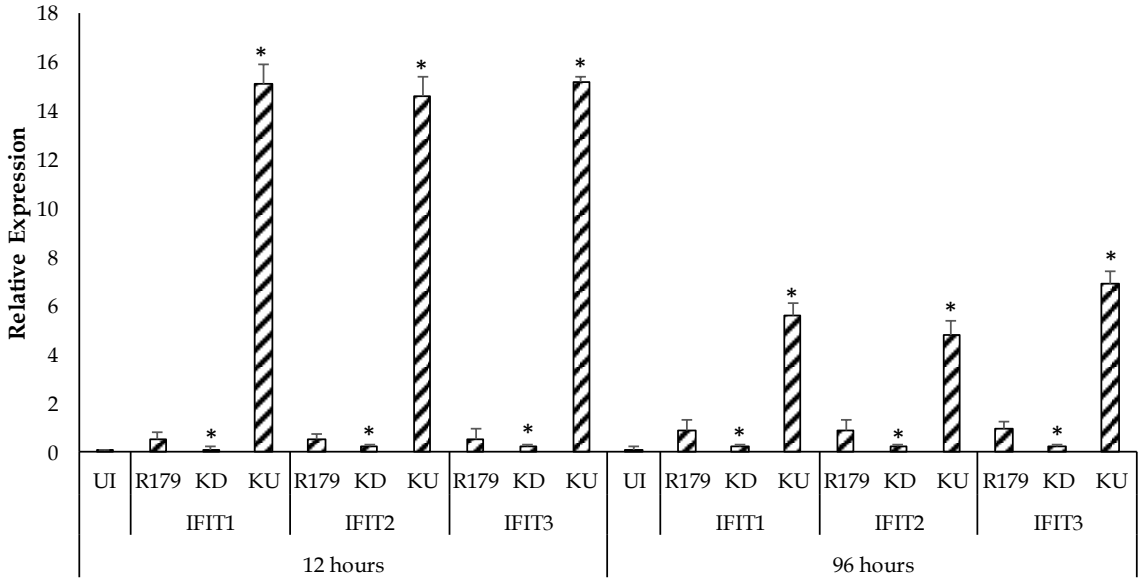


Figure 3.18: Comparison of relative expression after knock-down and knock-up (vector-based over-expression) of IFIT1, IFIT2 and IFIT3 in hMDMs infected with R179; KU-Knock-up (vector-based over-expression), KD-Knock-down. * indicates $p<0.001$ as compared to uninfected.

3.19 Measurement of cytokine levels by multiplex ELISA after knock-up (vector-based over-expression)/down of *IFITs*

A panel of 13 cytokines namely IDO-1, TNF-α, eight Interleukins (IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-12p40, IL-12p70, and IL-123), type-1 interferon (IFN-α and IFN-β) and type-II interferon (IFN-γ) were measured using the Luminex assay which is a multiplex ELISA platform. The comparison of these 13 cytokines was performed at 12 and 24 hours post-infection. **Table 3.4, Table 3.5 and Table 3.6** provide cytokine levels after knocking-up and knocking down of *IFITs* post-infection with *M. smegmatis*, BCG and R179 respectively.

Table 3.4: Cytokines expression (pg/ml) 12 hours post *M. smegmatis* infection[†]

Cytokines (pg/mL)	UI	*M.Smeg*	Knock-down			Knock-up (vector-based over-expression)		
			IFIT1	***IFIT2***	***IFIT3***	***IFIT1***	***IFIT2***	***IFIT3***
IDO-1	971 ± 13.4	1550.3 ± 16.4 p=0.047 [*]	0.3 ± 0.5 **p<0.001**[*] **p<0.001**[†]	8.6 ± 1.5 **p<0.001**[*] **p<0.001**[†]	9 ± 1 **p<0.001**[*] **p<0.001**[†]	1864.3 ± 5.8 **p<0.001**[*] **p<0.001**[†]	2343 ± 7.5 **p<0.001**[*] **p<0.001**[†]	2865 ± 9.8 **p<0.001**[*] **p<0.001**[†]
IFN-α	29 ± 1	82 ± 6.2[*] p>0.999[*]	0.3 ± 0.5[*] p>0.999[*] p>0.999[†]	6 ± 1[*] p>0.999[*] p>0.999[†]	12.5 ± 2[*] p>0.999[*] p>0.999[†]	1085 ± 9.8 **p<0.001**[*] **p<0.001**[†]	1255.6 ± 9.4 **p<0.001**[*] **p<0.001**[†]	984.6 ± 15.6 **p<0.001**[*] **p<0.001**[†]
IFN-β	0	0[*] p>0.999 [*]	0[*] p>0.999[*] p>0.999[†]	0[*] p>0.999[*] p>0.999[†]	0[*] p>0.999[*] p>0.999[†]	635 ± 12.5 p= 0.019[*] p=0.019 [†]	414.6 ± 8.5 p=0.354 [*] p= 0.354[†]	488.3 ± 8 p=0.163 [*] p= 0.163[†]
IFN-γ	527.3 ± 12	1361 ± 7.5 **p<0.001**[*]	0 p>0.999[*] **p<0.001** [†]	0 p>0.999[*] **p<0.001** [†]	0 p>0.999[*] **p<0.001** [†]	7599 ± 946.8 **p<0.001** [*] **p<0.001** [†]	7405.3 ± 1029.9 **p<0.001** [*] **p<0.001** [†]	7670 ± 908.8 **p<0.001** [*] **p<0.001** [†]
IL-4	7.6 ± 4.7	487.3 ± 9.6 p= 0.181[*]	0 p>0.999[*] p=0.165 [†]	0.3 ± 0.5 p>0.999[*] p= 0.166[†]	2.3 ± 2.5 p>0.999 [*] p=0.17 [†]	5164.3 ± 179.8 **p<0.001**[*] **p<0.001**[†]	5134.3 ± 305.1 **p<0.001**[*] **p<0.001**[†]	4954.6 ± 492.2 **p<0.001**[*] **p<0.001**[†]

[*]vs. Uninfected, [†]vs. *M. smegmatis*

IL-6	239.3 ± 19.8	1983.6 ± 4 **p<0.001***	0.6 ± 0.5 p=909 * **p<0.001** †	9 ± 8.1 p=0.924 * **p<0.001** †	0.6 ± 1.1 p=0.909 * **p<0.001** †	3530.6 ± 467.3 **p<0.001*** **p<0.001** †	3567.3 ± 216.7 **p<0.001*** **p<0.001** †	2921 ± 84.1 **p<0.001*** **p<0.001** †
IL-8	657 ± 19.6	9442.6 ± 489.8 **p<0.001** *	1300.6 ± 3 p= 0.016* **p<0.001** †	676.3 ± 19.8 p>0.999 * **p<0.001** †	991.6 ± 38.2 p=0.635 * **p<0.001** †	8680.6 ± 84.5 **p<0.001*** **p= 0.001**†	8932.6 ± 52.5 **p<0.001*** p=0.125 †	7451.6 ± 797.8 **p<0.001*** **p<0.001** †
IL-12p40	94.6 ± 6.6	753.6 ± 9.7 p=0.013 *	3.6 ± 3.2 p=0.999 * **p= 0.002**†	9.3 ± 4.5 p=0.924 * **p<0.001** †	0 p=0.909 * **p<0.001**†	3421.6 ± 477.3 **p<0.001*** **p<0.001** †	2591 ± 360.2 **p<0.001*** **p<0.001** †	3828 ± 164.8 **p<0.001*** **p<0.001** †
IL-12p70	16.6 ± 4.1	406.3 ± 14.7 p=0.437 *	0.3 ± 0.5 p>0.999 * p=0.382 †	0 p>0.999 * p=0.381 †	0 p>0.999 * p=0.381 †	561.6 ± 8.7 p=0.078 * p= 0.991†	844.6 ± 32 p<0.001 * p= 0.283†	767.6 ± 32.6 p=0.002 * p=0.539 †
IL-23	1245 ± 52.7	2069.6 ± 122.9 **p<0.001** *	0.6 ± 1.1 **p<0.001** * **p<0.001** †	0 **p<0.001** * **p<0.001** †	2 ± 3.4 **p<0.001** * **p<0.001** †	4599.3 ± 510.1 **p<0.001** * **p<0.001** †	4547.3 ± 253.6 **p<0.001** * **p<0.001** †	3207.3 ± 179.4 **p<0.001** * **p<0.001** †

*vs. Uninfected, †vs. *M. smegmatis*

IL-1α	243.3 ± 9.4	254.3 ± 40.5 p<0.999 *	67.3 ± 3 p= 0.982* p= 0.975†	74.6 ± 10.9 p=0.986 * p= 0.980†	75 ± 8.7 p=0.986 * p= 0.980†	6932.3 ± 79 **p<0.001** * **p<0.001** †	5453 ± 380.7 **p<0.001** * **p<0.001** †	4562.6 ± 312.5 **p<0.001** * **p<0.001** †
IL-1β	22 ± 4.3	131 ± 13.1 p<0.999 *	8.6 ± 3.2 p<0.999 * p=0.998 †	7 ± 2.6 p<0.999 * p=0.997 †	1 ± 1 p<0.999 * p=0.997 †	1278 ± 151.6 **p<0.001** * **p<0.001** †	1484.6 ± 41.7 **p<0.001** * **p<0.001** †	1160.6 ± 165.7 **p<0.001** * **p<0.001** †
TNF-α	30 ± 5.5	634.3 ± 33.8 p=0.032 *	139.3 ± 23.1 p= 0.999* p= 0.151†	264.6 ± 20.6 p= 0.916* p=0.508 †	113.6 ± 9.5 p<0.999 * p= 0.109†	4456.6 ± 318.1 **p<0.001** * **p<0.001** †	6202 ± 168.3 **p<0.001** * **p<0.001** †	4154.6 ± 123.1 **p<0.001** * **p<0.001** †

*vs. Uninfected, †vs. *M. smegmatis*

Table 3.5: Cytokine expression (pg/ml) after 12 and 96 hours of BCG infection

Cytokines	UI	BCG	Knock-down			Knock-up (vector-based over-expression)		
			IFIT1	*IFIT2*	*IFIT3*	*IFIT1*	*IFIT2*	*IFIT3*
at 12 hours								
IDO-1	971 ± 13.4	1489.3 ± 112.2 p =0.073*	0.3 ± 0.5 **p<0.001*** **p<0.001**†	8.6 ± 1.5 **p<0.001*** **p<0.001**†	9 ± 1 **p<0.001*** **p<0.001**†	3201.3 ± 86.6 **p<0.001*** **p<0.001**†	3198 ± 171.4 **p<0.001*** **p<0.001**†	4069.3 ± 141.9 **p<0.001*** **p<0.001**†
IFN-α	29 ± 1	83.6 ± 9 p>0.999 *	0.3 ± 0.5 p>0.999 * p>0.999†	6 ± 1 p>0.999 * p>0.999†	12.6 ± 2 p>0.999 * p>0.999†	769.6 ± 28.5 **p=0.001*** **p=0.003**†	579.6 ± 36.1 **p=0.044*** p=0.101†	872 ± 103.7 **p<0.001*** **p<0.001**†
IFN-β	0	0 p>0.999*	0 p>0.999* p>0.999†	0 p>0.999* p>0.999†	0 p>0.999* p>0.999†	8225.6 ± 413.2 **p<0.001*** **p<0.001**†	5366.3 ± 304 **p<0.001*** **p<0.001**†	6498.6 ± 160.9 **p<0.001*** **p<0.001**†
IFN-γ	527.3 ± 12	1691 ± 84.7 **p<0.001***	0 p=0.064* **p<0.001**†	0 p=0.064* **p<0.001**†	0 p=0.064* **p<0.001**†	6937.3 ± 369.8 **p<0.001*** **p<0.001**†	8228.3 ± 404.2 **p<0.001*** **p<0.001**†	6273.3 ± 370.5 **p<0.001*** **p<0.001**†
IL-4	7.6 ± 4.7	829 ± 31.9 **p<0.001***	0 p>0.999* **p<0.001**†	0.3 ± 0.5 p>0.999* **p<0.001**†	2.3 ± 2.5 p>0.999* **p<0.001**†	5797.6 ± 810.4 **p<0.001*** **p<0.001**†	5019.6 ± 290.5 **p<0.001*** **p<0.001**†	4559.6 ± 252.1 **p<0.001*** **p<0.001**†
IL-6	239.3 ± 19.8	2376 ± 319 **p<0.001***	0.6 ± 0.5 p=0.880* **p<0.001**†	9 ± 8.1 p=0.898* **p<0.001**†	0.6 ± 1.1 p=0.880* **p<0.001**†	9253.3 ± 378.3 **p<0.001*** **p<0.001**†	6491 ± 157.4 **p<0.001*** **p<0.001**†	8353.6 ± 225.3 **p<0.001*** **p<0.001**†
IL-8	657 ± 19.6	9113 ± 572 **p<0.001***	1854.6 ± 127.1 **p<0.001** * **p<0.001**†	1106.6 ± 72.5 p=0.186 * **p<0.001**†	1799.3 ± 56.5 **p<0.001** * **p<0.001**†	8328 ± 616.3 **p<0.001** * **p<0.001**†	8526 ± 677.8 **p<0.001** * **p=0.024**†	7928 ± 54.5 **p<0.001** * **p<0.001**†
IL-12p40	94.6 ± 6.6	493.6 ± 60 p=0.327*	3.6 ± 3.2 p<0.999* p=0.110†	9.3 ± 4.5 p<0.999* p=0.110†	0 p<0.999* p=0.110†	3241.3 ± 652.6 **p<0.001*** **p<0.001**†	2480 ± 419.2 **p<0.001*** **p<0.001**†	1080.6 ± 61.2 **p<0.001*** p=0.024†
IL-12p70	16.6 ± 4.1	375.3 ± 59.9 p=0.469*	0.3 ± 0.5 p<0.999* p=0.409†	0 p<0.999* p=0.408†	0 p<0.999* p=0.408†	1538.6 ± 26.6 **p<0.001*** **p<0.001**†	1087 ± 17.6 **p<0.001*** **p<0.001**†	1968 ± 195 **p<0.001*** **p<0.001**†

*vs. Uninfected, †vs. BCG

IL-23	1245 ± 52.7	2205 ± 213.6 **p<0.001***	528 ± 102.8 **p<0.001*** **p<0.001†**	147.3 ± 25.5 **p<0.001*** **p<0.001†**	376.3 ± 39.4 **p<0.001*** **p<0.001†**	6530 ± 453.2 **p<0.001*** **p<0.001†**	6558 ± 258.9 **p<0.001*** **p<0.001†**	4408.6 ± 495.2 **p<0.001*** **p<0.001†**
IL-1α	243.3 ± 9.4	254.6 ± 40.5 p>0.999*	72.6 ± 11.7 p=0.979* p=0.970†	67.6 ± 21.1 p=0.974* p=0.965†	57.3 ± 26.3 p=0.966* p=0.953†	786.3 ± 71.2 p=0.050* p=0.060†	621.6 ± 95 p=0.390* p=0.438†	1139.6 ± 122.5 **p<0.001*** **p<0.001†**
IL-1β	22 ± 4.3	127 ± 15.6 p=0.998*	12.3 ± 8.7 p>0.999* p=0.998†	12 ± 7.2 p>0.999* p=0.998†	2.3 ± 1.5 p>0.999* p=0.996†	752.3 ± 33.2 **p=0.001*** **p=0.012†**	743.3 ± 26.8 **p=0.001*** **p=0.014†**	597.3 ± 65.2 **p=0.029*** **p=0.144†**
TNF-α	30 ± 5.5	679.3 ± 103.3 **p=0.007***	104 ± 11 p>0.999* **p=0.029†**	56.3 ± 16.6 p>0.999* **p=0.012†**	14 ± 2 p>0.999* **p=0.005†**	8295 ± 303.9 **p<0.001*** **p<0.001†**	8200.6 ± 608.7 **p<0.001*** **p<0.001†**	6406 ± 168 **p<0.001*** **p<0.001†**

Cytokines	UI	BCG	Knock-down			Knock-up (vector-based over-expression)		
			IFIT1	***IFIT2***	***IFIT3***	***IFIT1***	***IFIT2***	***IFIT3***
at 96 hours								
IDO-1	971 ± 13.4	1504.3 ± 86.5 **p=0.112***	0.3 ± 0.5 **p<0.001*** **p<0.001†**	8.6 ± 1.5 **p<0.001*** **p<0.001†**	9 ± 1 **p<0.001*** **p<0.001†**	2525.3 ± 346.7 **p<0.001*** **p<0.001†**	1236.6 ± 185.1 **p<0.001*** **p<0.001†**	2724 ± 249 **p<0.001*** **p<0.001†**
IFN-α	29 ± 1	74 ± 7.9 p>0.999 *	0.3 ± 0.5 p>0.999 * p>0.999 †	6 ± 1 p>0.999* p>0.999†	12.6 ± 2 p>0.999* p>0.999†	534 ± 74 p=0.158* p=0.258†	131.6 ± 9 p=0.995* p>0.999 †	664.6 ± 34 **p=0.026*** p=0.051†
IFN-β	0	0 p>0.999*	0 p>0.999 * p>0.999 †	0 p>0.999* p>0.999†	0 p>0.999* p>0.999†	6310.3 ± 248.4 **p<0.001*** **p<0.001†**	5512.6 ± 101 **p<0.001*** **p<0.001†**	4534 ± 365.8 **p<0.001*** **p<0.001†**
IFN-γ	527.3 ± 12	964.3 ± 30 p=0.322*	0 p=0.121* **p<0.001†**	0 p=0.121* **p<0.001†**	0 p=0.121* **p<0.001†**	5936.3 ± 635.3 **p<0.001*** **p<0.001†**	8237.3 ± 459.7 **p<0.001*** **p<0.001†**	7457.6 ± 298.2 **p<0.001*** **p<0.001†**
IL-4	7.6 ± 4.7	492 ± 13.4 p=0.200*	0 p>0.999 * p=0.184†	0.3 ± 0.5 p>0.999* p=0.184†	2.3 ± 2.5 p>0.999* p=0.188†	4410.3 ± 177.9 **p<0.001*** **p<0.001†**	4216 ± 102.8 **p<0.001*** **p<0.001†**	3453.6 ± 366.5 **p<0.001*** **p<0.001†**

*vs. Uninfected, †vs. BCG

IL-6	239.3 ± 19.8	2347.6 ± 315.4 **p<0.001***	0.6 ± 0.5 p=0.921* **p<0.001†**	9 ± 8.1 p=0.934* **p<0.001†**	0.6 ± 1.1 p=0.921* **p<0.001†**	6586 ± 492.9 **p<0.001* p<0.001†**	7373.3 ± 163.5 **p<0.001* p<0.001†**	4651 ± 385.9 **p<0.001* p<0.001†**
IL-8	657 ± 19.6	9296.6 ± 445.6 **p<0.001***	1208.3 ± 160.5 p=0.089* **p<0.001†**	1479.3 ± 153.8 **p<0.001* p<0.001†**	979.6 ± 20.9 p=0.708* **p<0.001†**	3270.6 ± 1025.6 **p<0.001* p<0.001†**	2677 ± 417.2 **p<0.001* p<0.001†**	2662.3 ± 263.8 **p<0.001* p<0.001†**
IL-12p40	94.6 ± 6.6	589.6 ± 59 p=0.177*	3.6 ± 3.2 p>0.999 * p=0.055†	9.3 ± 4.5 p>0.999* p=0.055†	0 p>0.999* p=0.055†	1355 ± 527.6 **p<0.001* p<0.001†**	2553.3 ± 370.2 **p<0.001* p<0.001†**	2358.3 ± 433.3 **p<0.001* p<0.001†**
IL-12p70	16.6 ± 4.1	23.6 ± 1.1 p>0.999*	0 p>0.999 * p>0.999†	0.3 ± 0.5 p>0.999* p>0.999†	0 p>0.999* p>0.999†	1448 ± 264.7 **p<0.001* p<0.001†**	1873.6 ± 126.1 **p<0.001* p<0.001†**	2923 ± 346.1 **p<0.001* p<0.001†**
IL-23	1245 ± 52.7	1077.6 ± 49.6 p=0.988*	407.3 ± 12 **p<0.001* p=0.014†**	410.3 ± 13.5 **p<0.001* p=0.015†**	231.6 ± 46.1 **p<0.001* p<0.001†**	5143 ± 409.8 **p<0.001* p<0.001†**	4478 ± 251.8 **p<0.001* p<0.001†**	4507 ± 319.5 **p<0.001* p<0.001†**
IL-1α	243.3 ± 9.4	262.6 ± 42.8 p>0.999*	14.6 ± 3 p=0.936* p=0.904†	14.3 ± 3 p=0.936* p=0.904†	38.3 ± 3 p=0.964* p=0.942†	732.6 ± 112.4 p=0.180* p=0.230†	551.6 ± 112.3 p=0.750* p=0.810†	469.3 ± 45.4 p=0.940* p=0.960†

*vs. Uninfected, †vs. BCG

IL-1β	22 ± 4.3	125.3 ± 17.3 p>0.999*	8.6 ± 3.2 p>0.999 * p=0.998†	7 ± 2.6 p>0.999* p=0.998†	1 ± 1 p>0.999* p=0.998†	456 ± 16 p=0.331* p=0.680†	504.3 ± 32.1 p=0.204* p=0.510†	833 ± 96.3 **p=0.001*** **p=0.001†**
TNF-α	30 ± 5.5	352.6 ± 40.7 p=0.708*	54.3 ± 11.5 p>0.999 * p=0.784†	52.3 ± 18.7 p>0.999* p=0.778†	21 ± 6.2 p>0.999* p=0.678†	4875.6 ± 969.5 **p<0.001*** **p<0.001†**	5929 ± 477.6 **p<0.001*** **p<0.001†**	3955.6 ± 312.9 **p<0.001*** **p<0.001†**

*vs. Uninfected, †vs. BCG

Table 3.6: Cytokines expression (pg/ml) after 12 and 96 hours of R179 infection

Cytokines	UI	R179	Knock-down			Knock-up (vector-based over-expression)		
			IFIT1	*IFIT2*	*IFIT3*	*IFIT1*	*IFIT2*	*IFIT3*
at 12 hours								
IDO-1	971 ± 13.4	1338.8 ± 30 p=0.902*	0.3 ± 0.5 p=0.017* **p<0.001**†	8.6 ± 1.5 p=0.019* **p<0.001**†	9 ± 1 p=0.019* **p<0.001**†	2849.3 ± 562.2 **p<0.001*** **p<0.001**†	3148.6 ± 87.6 **p<0.001*** **p<0.001**†	3693.6 ± 511.2 **p<0.001*** **p<0.001**†
IFN-α	29 ± 1	77.3 ± 2.5 p>0.999*	0.3 ± 0.5 p>0.999* p>0.999†	6 ± 1 p>0.999* p>0.999†	12.6 ± 2 p>0.999* p>0.999†	739.3 ± 64.6 p=0.205* p=0.287†	574.3 ± 40.5 p=0.544* p=0.659†	836.3 ± 114.3 p=0.093* p=0.140†
IFN-β	0	0 p>0.999*	0 p>0.999* p>0.999†	0 p>0.999* p>0.999†	0 p>0.999* p>0.999†	8143.6 ± 271.5 **p<0.001*** **p<0.001**†	5759.6 ± 781.3 **p<0.001*** **p<0.001**†	6483.6 ± 158.5 **p<0.001*** **p<0.001**†
IFN-γ	527.3 ± 12	927.3 ± 84 p=0.855*	0 p=0.587* **p=0.028**†	0 p=0.587* **p=0.028**†	0 p=0.587* **p=0.028**†	7077.3 ± 583 **p<0.001*** **p<0.001**†	7868.3 ± 221.9 **p<0.001*** **p<0.001**†	7563.3 ± 1206.4 **p<0.001*** **p<0.001**†
IL-4	7.6 ± 4.7	800 ± 50.8 p=0.106*	0 p>0.999* p=0.099†	0.3 ± 0.5 p>0.999* p=0.099†	2.3 ± 2.5 p>0.999* p=0.100†	5379.6 ± 143.6 **p<0.001*** **p<0.001**†	8436.3 ± 453.5 **p<0.001*** **p<0.001**†	4521.3 ± 285.2 **p<0.001*** **p<0.001**†
IL-6	239.3 ± 19.8	918 ± 19.6 p=0.257*	0.6 ± 0.5 p= 0.990* **p=0.032**†	9 ± 8.1 p=0.992* **p=0.032**†	0.6 ± 1.1 p=0.992* **p=0.032**†	6532.6 ± 470.2 **p<0.001*** **p<0.001**†	5303.3 ± 329.7 **p<0.001*** **p<0.001**†	7818.3 ± 291.9 **p<0.001*** **p<0.001**†
IL-8	657 ± 19.6	8822.3 ± 972.2 **p<0.001***	794 ± 32 p>0.999* **p<0.001**†	1066.3 ± 74.1 p=0.084* **p<0.001**†	1664.6 ± 187.3 p=0.017* **p<0.001**†	6573.6 ± 204.5 **p<0.001*** **p<0.001**†	6886.6 ± 50.6 **p<0.001*** **p<0.001**†	2778.6 ± 2096.4 **p<0.001*** **p<0.001**†

*vs. Uninfected, †vs. R179

IL-12p40	94.6 ± 6.6	245 ± 13.5 p>0.999*	3.6 ± 3.2 p>0.999* p=0.990†	9.3 ± 4.5 p>0.999* p=0.991†	0 p>0.999* p=0.989†	3463.6 ± 458 **p<0.001*** **p<0.001†**	3054.3 ± 858 **p<0.001*** **p<0.001†**	4108 ± 35.5 **p<0.001*** **p<0.001†**
IL-12p70	16.6 ± 4.1	302.6 ± 17.7 p=0.973*	0 p>0.999* **p=0.001†**	3.3 ± 5.7 p>0.999* **p=0.001†**	0 p>0.999* **p=0.001†**	4589.6 ± 54 **p<0.001*** **p<0.001†**	4112 ± 61.7 **p<0.001*** **p<0.001†**	2766.6 ± 201.6 **p<0.001*** **p<0.001†**
IL-23	1245 ± 52.7	2009.3 ± 94.4 p=0.134*	332.3 ± 27.7 **p=0.036*** **p<0.001†**	350.6 ± 12.6 **p=0.040*** **p<0.001†**	346.3 ± 43 **p=0.030*** **p<0.001†**	6610.3 ± 521.1 **p<0.001*** **p<0.001†**	7477.6 ± 848.2 **p<0.001*** **p<0.001†**	7405 ± 489 **p<0.001*** **p<0.001†**
IL-1α	243.3 ± 9.4	249.3 ± 41.4 p>0.999*	71.3 ± 9.4 p=0.998* p=0.998†	68 ± 20.6 p=0.998* p=0.998†	77 ± 11.1 p>0.999 * p=0.945†	702.3 ± 156.7 p=0.169* p=0.821†	601.3 ± 90.1 p=0.267* p=0.912†	438.6 ± 24.1 p=0.361* p=0.959†
IL-1β	22 ± 4.3	337.6 ± 23.4 p=0.954*	39 ± 7.9 p>0.999* p=0.966†	66 ± 12 p>0.999* p=0.980†	10.3 ± 7.3 p>0.999* p=0.945†	757.6 ± 38.2 p=0.169* p=0.821†	697 ± 53.8 p=0.267* p=0.912†	647 ± 24 p=0.361* p=0.959†
TNF-α	30 ± 5.5	1222.3 ± 30.7 **p=0.001***	30.3 ± 4.5 p>0.999* **p=0.001†**	61 ± 17.5 p>0.999* **p=0.001†**	17 ± 7 p>0.999* **p=0.001†**	5956 ± 281.6 **p<0.001*** **p<0.001†**	8084 ± 411.6 **p<0.001*** **p<0.001†**	9036.6 ± 839.7 **p<0.001*** **p<0.001†**

*vs. Uninfected, †vs. R179

Cytokines	UI	R179	Knock-down			Knock-up (vector-based over-expression)		
			IFIT1	*IFIT1*	*IFIT2*	*IFIT1*	*IFIT1*	*IFIT2*
at 96 hours								
IDO-1	971 ± 13.4	1798 ± 63.6 **p=0.008***	0.3 ± 0.5 **p<0.001*** **p<0.001†**	8.6 ± 1.5 **p<0.001*** **p<0.001†**	9 ± 1 **p<0.001*** **p<0.001†**	2445.3 ± 368.3 **p<0.001*** **p<0.001†**	2313.6 ± 52.2 **p<0.001*** **p<0.001†**	2680.6 ± 324.5 **p<0.001*** **p<0.001†**
IFN-α	29 ± 1	71.3 ± 12.5 p>0.999*	0.3 ± 0.5 p>0.999* p>0.999†	6 ± 1 p>0.999* p>0.999†	12.6 ± 2 p>0.999* p>0.999†	127.3 ± 26 p>0.999* p>0.999†	275.6 ± 44.2 p>0.999* p=0.989†	262.6 ± 37 p>0.999* p=0.990†
IFN-β	0	6 ± 3.4 p>0.999*	0 p>0.999* p>0.999†	12 p>0.999* p>0.999†	6 p>0.999* p>0.999†	6347 ± 302 **p<0.001*** **p<0.001†**	5513.3 ± 100.8 **p<0.001*** **p<0.001†**	4776 ± 112.2 **p<0.001*** **p<0.001†**
IFN-γ	527.3 ± 12	1014.6 ± 62.6 p=0.389*	0 p=0.287* **p<0.001†**	0 p=0.287* **p<0.001†**	0 p=0.287* **p<0.001†**	5772 ± 899.6 **p<0.001*** **p<0.001†**	4627.7 ± 522.8 **p<0.001*** **p<0.001†**	4709 ± 138.1 **p<0.001*** **p<0.001†**
IL-4	7.6 ± 4.7	517.6 ± 54.8 p=0.329*	0 p>0.999* p=0.310†	0.3 ± 0.5 p>0.999* p=0.310†	2.3 ± 2.5 p>0.999* p=0.310†	4120.6 ± 390.4 **p<0.001*** **p<0.001†**	4591 ± 715.7 **p<0.001*** **p<0.001†**	5585 ± 542.5 **p<0.001*** **p<0.001†**
IL-6	239.3 ± 19.8	2347.6 ± 315.4 **p<0.001***	0.6 ± 0.5 p=0.965* **p<0.001†**	9 ± 8.1 p=0.970* **p<0.001†**	0.6 ± 1.1 p=0.965* **p<0.001†**	6645.6 ± 538.5 **p<0.001*** **p<0.001†**	7180.3 ± 480.4 **p<0.001*** **p<0.001†**	3915.3 ± 1243 **p<0.001*** **p<0.001†**
IL-8	657 ± 19.6	9531.7 ± 572.8 **p<0.001***	889 ± 161 p=0.970* **p<0.001†**	548 ± 91.5 p>0.999 * **p<0.001†**	991.7 ± 38.2 p=0.821* **p<0.001†**	4049 ± 643.8 **p<0.001*** **p<0.001†**	4932.7 ± 52.5 **p<0.001*** **p<0.001†**	4684.3 ± 226.6 **p<0.001*** **p<0.001†**
IL-12p40	94.7 ± 6.6	254.7 ± 11.6 p=0.997*	3.7 ± 3.2 p>0.999* p=0.963†	9.3 ± 4.5 p>0.999* p=0.963†	0 p>0.999* p=0.963†	2475.7 ± 454.5 **p<0.001*** **p<0.001†**	2732 ± 421.9 **p<0.001*** **p<0.001†**	2837.7 ± 820 **p<0.001*** **p<0.001†**

*vs. Uninfected, †vs. R179

IL-12p70	16.7 ± 4.1	95.3 ± 7.5 p>0.999*	0 p>0.999* p>0.999†	0.3 ± 0.5 p>0.999* p>0.999†	0 p>0.999* p>0.999†	3419 ± 153.5 **p<0.001*** **p<0.001†**	3627.7 ± 185.2 **p<0.001*** **p<0.001†**	3172.7 ± 725.4 **p<0.001*** **p<0.001†**
IL-23	1245 ± 52.7	267.7 ± 36.6 p=0.993*	49 ± 54.5 **p<0.001*** **p<0.001†**	34 ± 7 **p<0.001*** **p<0.001†**	38.7 ± 40 **p<0.001*** **p<0.001†**	724 ± 295.7 **p<0.001*** **p<0.001†**	536.3 ± 329.8 **p<0.001*** **p<0.001†**	528.3 ± 376.6 **p<0.001*** **p<0.001†**
IL-1α	243.3 ± 9.4	267.7 ± 46.4 p>0.999*	49 ± 4.5 p=0.989* p=0.979†	34 ± 4.3 p=0.983 * p=0.969†	38.7 ± 9 p=0.985* p=0.972†	724 ± 12.5 p=0.408* p=0.478†	536.3 ± 86.6 p=0.900 * p=0.936†	4541 ± 147.1 p=0.910* p=0.945†
IL-1β	22 ± 4.3	118.3 ± 27 p>0.999*	32.3 ± 2.5 p>0.999* p>0.999†	23.3 ± 2.5 p>0.999* p>0.999†	52.7 ± 4.7 p>0.999* p>0.999†	456.7 ± 15.3 p=0.543* p=0.812†	553 ± 96.2 p=0.278* p=0.543†	372.3 ± 54.8 p=0.783* p=0.952†
TNF-α	30 ± 5.5	352.7 ± 40.7 p=0.847*	49.3 ± 13.5 p>0.999* p=0.884†	21 ± 9.5 p>0.999* p=0.827†	24.3 ± 11 p>0.999* p=0.835†	6098.7 ± 532 **p<0.001*** **p<0.001†**	6119 ± 204.4 **p<0.001*** **p<0.001†**	5411.3 ± 509.1 **p<0.001*** **p<0.001†**

*vs. Uninfected, †vs. R179

Multiplex ELISA results varied for different cytokines across the strains. Notably, three pro-inflammatory cytokines IFN-γ, IL-6 and IL-12-p40 were found to decrease upon R179 infection as compared to infection with *M. smegmatis* which might be the reason for the killing of *M. smegmatis* from 12 hours of infection. Three other cytokines namely, IL-4, IL-1β and TNF-α were observed to increase upon R179 infection as compared to infection with *M. smegmatis* at 12 hours post-infection.

Upon infection with *M. smegmatis*, six pro-inflammatory cytokines namely IDO-1, IFN-γ, IL-6, IL-8, IL-12p40 and IL-23 were found to decrease significantly upon knocking-down with *IFITs*, and increase significantly upon knocking-up with *IFITs*.

Knock-up (vector-based over-expression) and knock-down of *IFITs* in cells infected with *M. bovis* BCG at 12 hours post-infection resulted in up and down-regulation of pro-inflammatory cytokines respectively. Knocking down of *IFITs* resulted in s significant decrease in expression of IDO-1, IFN-γ, IL-4, IL-6, IL-8, IL-23 and TNF-α as compared to BCG infected cells. These cytokines were found to be significantly upregulated upon knocking up with *IFITs*. At 96 hours post BCG infection the five cytokines which were significantly up /down regulated upon knocking-up/down of *IFITs* respectively were IDO-1, IFN-γ, IL-6, IL-8, and IL-23.

In cells infected with R179 at 12 hours post-infection, seven pro-inflammatory cytokines namely IDO-1, IFN-γ, IL-6, IL-8, IL-12p70, IL-23 and TNF-α were found to increase significantly upon knocking up with *IFITs* and decrease significantly upon knocking down. Five of these cytokines namely IDO-1, IFN-γ, IL-6, IL-8, and IL-23 behaved similarly upon knocking up and knocking down *IFITs* at 96 hours post R179 infection.

An overall comparison of the results from Luminex revealed four key cytokines which dominated upon cell processing. These cytokines increased upon knock-up (vector-based over-expression) and significantly decreased ($p<0.001$) upon knocking-down *IFITs* in all three strains (*M. smegmatis*, *M. bovis* BCG and R179).

In conclusion, across all three strains, intra-cellular bacterial growth and survival measured through CFUs decreased significantly upon knocking up of *IFITs*, while we recorded an increase in CFUs upon knocking down. Using multiplex ELISA (Luminex), we found higher expression of key pro-inflammatory cytokines (i.e. IDO1, IFN-γ, IL-6, and IL-23) during knock-up (vector-based over-expression) of *IFITs* resulting in reduction of mycobacteria.

CHAPTER 4

CHAPTER 4: DISCUSSION

Discussion

This study compared the *in vitro* host-response from hMDMs to mycobacterial strains of varying pathogenicity (R179, *M. bovis* BCG and *M. smegmatis*) using AmpliSeq. The key findings were, a) we identified 19 DEGs from the AmpliSeq data (after applying stringent filtering measures) at 12 hours post-infection, b) the early host response was higher towards *M. smegmatis* as compared to BCG and R179, c) we found >50% reduction in CFUs from 12 hours to 24 hours upon infection with *M. smegmatis* d) activation of infected hMDMs with IFN-γ resulted in significant decrease in CFUs across all the strains, with *M. smegmatis* showing highest reduction, e) top canonical pathways identified by IPA showed an important role in interferon and interleukin signalling, cytokine mediated communications and pattern recognition receptors (for bacteria and viruses), f) top regulator effect networks of these DEGs have roles in activation of cell, binding of DNA and viral replication and g) *IFITs* (1,2 and 3) family found to be among the top DEGs were selected for the downstream intervention experiments, in which knock-up (vector-based over-expression) of *IFITs* resulted in a significant decrease in CFUs of all three strains, whereas knockdown increased the number of CFUs across all strains.

In the present study, for *in vitro* infection experiments, we used three different strains of mycobacteria (*M. smegmatis*, *M. bovis* BCG, *M. tb* R179) as they are the commonly used model organisms to study the *in vitro* host response towards mycobacteria (Wang et al., 2016).

Previous publications have observed similarities in RNAseq and AmpliSeq for detection of DEGs, indicating AmpliSeq as a very sensitive and cost effective technique

(Li et al., 2015). A recent publication comparing host response from alveolar macrophages and hMDMs against pathogenic H37Rv, found key genes involved in pathogenesis of TB using AmpliSeq analysis (Papp et al., 2018). In our study, we focused on the comparison of the host response from hMDMs infected with three different strains of mycobacteria, a) *M. smegmatis* (non-pathogenic), b) BCG Tokyo 172 strain (facultative pathogenic, ATCC 35737) and c) R179 Beijing genotype strain R220 (drug resistant) (Bohsali et al., 2010; Ioerger et al., 2010; Johnson et al., 2010).

In the present study, we cultured all three strains of mycobacteria in a Tween-free media. Tween 80 and other detergents used to culture *M. tuberculosis* are known to alter bacterial uptake by macrophages and subsequent innate immune response (Barrow et al., 2011). During *M. tuberculosis* infection, aerosolized bacteria enters in its native state (detergent-free) to human lung alveoli, hence we avoid using Tween 80 for bacterial culture to mimic the native state.

In this study we observed a drastic reduction in the intracellular survival of *M. smegmatis* from 12 -24 hours post-infection, which was not observed for the other two mycobacterial strains. This indicates that host can control *M. smegmatis* infection, although the reason behind this is understudied (Anes et al., 2003; Kuehnel et al., 2001). We therefore present the host transcriptome at 12 hours in response to pathogenic and non-pathogenic mycobacteria in an attempt to unravel the genes that are involved during this crucial time.

After applying stringent filtering measures to the 12 hours AmpliSeq data (including strong FDR (<0.001), strong p-value (<0.001), log counts per million (CPM), fold changes (>1.5), IPA, and biological functions), we selected 19 DEGs, which primarily includes families of interferon and interleukin related genes. The expression levels of

all 19 DEGs selected after applying stringent filters were validated through qRT-PCR. We observed a strong host response upon the non-pathogenic strain infection as compared to other two strains in AmpliSeq data. A previous study observed a strong host response in terms of higher fold changes of various DEGs upon *M. smegmatis* infection (MCGARVEY et al., 2004).

Luminex results in the present study, also showed a strong host response towards *M. smegmatis* (a mycobacterial strain which eventually gets killed inside macrophages) as compared to BCG and R179 (strains which survive inside host) at 12 hours post-infection. The levels of proteins secreted in cell supernatants at 12 hours of *M. smegmatis* infection were found to be significantly higher for important cytokines like IFN-γ, and IL-12 family (IL-12p40 and IL-12p70). Moreover, IFN-γ was found to be reduced significantly at 96 hours post R179 infection.

4.1 Activation of interferon-interleukin associated gene network

As pathogenic mycobacteria are known to survive inside macrophages by blocking phagosome maturation, the non-pathogenic strain (*M. smegmatis*) is unable to survive and eventually gets killed by the macrophages (Anes et al., 2006; Walburger et al., 2004). IFN-γ is primarily responsible for activation of macrophages and bactericidal activity (Reljic et al., 2010). It plays a key role in apoptotic induction through nitric-oxide dependent pathway in macrophages infected with mycobacteria (Denis et al., 1991; Herbst et al., 2011). We observed an increase in IL-12p40 and IL-12p70, which are the key members of IL-12 family and are primarily secreted by antigen presenting cells and macrophages (Ma et al., 2001; Vignali et al., 2012). IL-12 is known to enhance the synthesis of IFN-γ during infection (Giacomini et al., 2001).

M. smegmatis being a non-pathogenic mycobacterial strain is known to be killed within 48 hours of infection (Anes et al., 2003; Kuehnel et al., 2001). We observed a similar bacterial uptake at 4 hours post *M. smegmatis,* BCG and R179 infection. Interestingly, we observed a sudden decline in CFU counts from 12 hours to 24 hours post *M. smegmatis* infection. It has been shown by a previous publication that rapidly growing *M. smegmatis* had a sudden fall in CFUs from 12 hours to 24 hours post-infection (Anes et al., 2006). The dramatic reduction in CFUs signifies strong host response against *M. smegmatis* infection, which clearly co-relates with the AmpliSeq and the qRT-PCR data (performed in the study). Also, we observed a gradual increase in CFUs throughout BCG and R179 infection in hMDMs. A previous study also demonstrated that in host macrophages infected with *Mycobacterium tuberculosis*, there is a gradual increase in CFUs observed in the first week of infection (Pathak et al., 2012). This increment in the CFUs was also reported in later weeks post *M. tuberculosis* infection (Muñoz et al., 2005).

In the present study, we observed reduction of CFUs across all three strains in IFN-γ activated (IFN-γ^{+}) cells as compared to the wild type (IFN-γ^{0}). Previous studies have demonstrated a fall in CFUs when *M. tuberculosis* infected cells were activated with IFN-γ as compared to the un-activated cells suggesting that nitric-oxide induced apoptosis is responsible for this growth restriction (Herbst et al., 2011; McKinney et al., 2000). We observed hMDMs infected with *M. smegmatis* at 12 hours post-infection depicting highest reduction of CFUs (58% reduction) in IFN-γ^{+} cells (BCG and R179 had reduction of 17% and 20.4% respectively) as compared to IFN-γ^{0} hMDMs. Upon infection, the interaction between IFN-γ and macrophages implicate a complex mechanism reliant on both host and pathogen associated factors (Schroder et al., 2004).

The IFN-γ dependent nitric-oxide associated control of mycobacterial infection in host macrophages is well known, and the underlying mechanism is fully understood (Herbst et al., 2011).

In our study, IPA identified top canonical pathways which showed higher signalling of the interferon gene network. Differentially expressed genes identified across previous studies were found to be surprisingly discordant (Haas et al., 2016). It is noteworthy that all these previous studies have implicated the role of interferon signalling in host response towards mycobacteria (Blankley et al., 2014; Cliff et al., 2015). Also, a previous study showed that host response towards mycobacteria is associated with neutrophil-driven interferon (IFN)-inducible gene profile, consisting of both type II (IFN-γ) and type I IFN-α/β signalling (Berry et al., 2010).

Apart from interferon, the present study also detected a network of interleukin genes (IL-1β, IL-6, IL-8, IL-12β and IL-23) against mycobacterial infection. Another important IPA identified network of cytokines mediated communication between immune cells including IL-1α and IL-1β have been shown to play a key role in host response towards mycobacteria. The presence of either of these two cytokines also allows some control of acute *M. tuberculosis* infection, and IL-1 pathway is dispensable for controlling *M. bovis* BCG acute infection (Bourigault et al., 2013). Previous studies also demonstrated that *M. tuberculosis* regulates host IL-6 production to inhibit type I interferon and, consequently, disease progression (Martinez et al., 2013). Also, IL-8 has been shown to play an instrumental role in cell recruitment, since, IL-8 is the major chemokine responsible for recruiting T lymphocytes (Krupa et al., 2015). Further,

specifically, IL-12 and IL-23, have been demonstrated to confer protective cellular responses and promote survival against *M. tuberculosis* (Cooper et al., 2007).

In our study, top regulator effect networks of these 19 DEGs demonstrate an important role in cell activation, DNA binding and viral replication. With a higher consistency score predicting a unidirectional consistent gene finding directs us towards DNA activation and cellular binding. Infection with *M. tuberculosis* is known to affect DNA binding activity through IFN-γ activation (Ting et al., 1999).

4.2 Interferon-induced tetratricopeptide (*IFITs*) proteins: Their role in mycobacterial infection and survival

In our study, out of 19 selected DEGs obtained after stringent filtering of AmpliSeq data, we selected *IFIT* family (*IFIT1*, *IFIT2* and *IFIT3*) for downstream knocking-up (via vector based transfection) and knocking-down (via siRNA) experiments to decipher their role in host immune response towards mycobacteria (pathogenic/non-pathogenic). The follow-up studies were performed in THP-1 cells as hMDMs are known to share similar functionalities with THP-1 cells in response to mycobacterial infection (Mendoza-Coronel & Casta-Arreola, 2016).

IFITs are known to prevent viral replication by binding and controlling the function of viral proteins and RNAs, but their role against bacteria is not well understood. The human *IFIT* family include four members bunched on chromosome 10, namely *IFIT1 (ISG56)*, *IFIT2 (ISG54)*, *IFIT3 (ISG60* or *IFIT 4)* and *IFIT5 (ISG58)* (Fensterl & Sen, 2011).

The first discovered member of *IFIT* family was human *IFIT1* (a 56-kDa protein synthesized against stimulation of IFN), its corresponding gene is *ISG56,* was the first human *ISGs* to be cloned (Chebath et al., 1983; Kusari & Sen, 1987). *IFITs* are weakly induced in response to type III IFN (IFN-γ) and strongly in response to type I IFNs (IFN-α/β) and type III IFNs (IFN-λs) (Der et al. 1998; Kohli et al., 2012). The *IFIT* protein can distinguish between cellular and viral RNAs, and it binds to viral mRNA 5' ends whose caps lack 2'-*O*-methylation of the first ribose (Habjan et al., 2013; Kimura et al., 2013; Kumar et al., 2014)

IFIT1, *IFIT2* and *IFIT3* forms a complex and bind to 5'-ppp end of vesicular stomatitis virus (VSV), on the other hand replication of VSV was restored if gene expressing *IFITs* (1, 2 and 3) were knocked down. Similar findings were also observed for Rift valley fever virus and influenza A virus(Pichlmair et al., 2011). *IFIT1* consist of three isoforms (1, 2 and 3), *IFIT 2* has no trancription variant while *IFIT3* has two isoforms (a and b).

In the present study, we performed follow-up experiments with *IFITs* (*IFIT1*, *IFIT2* and *IFIT3*) upon infection with different mycobacterial strains (*M. smegmatis*, BCG, R179) in hMDMs. *In vitro* knocking-up of these *IFITs* resulted in a significant reduction in the mycobacterial survival. Though, knocking-up did not completely obliterate mycobacterial CFUs, but it resulted in a significant reduction in CFUs. On the other hand, knocking-down (using siRNA) *IFIT* transcripts resulted in a significant boost to mycobacterial survival (increased CFUs).

To the best of our knowledge, we do not find any literature on the role of *IFITs* in TB, thus, evaluating the role of *IFIT* family (1, 2 and 3) for boosting host response against

M. tuberculosis is novel. Intracellular bacterial growth and survival via knocking-up and knocking-down of *IFITs* was confirmed by studying mRNA expression through qRT-PCR and protein expression by Western blot. Using both techniques at mRNA and protein level, we found an increase in *IFIT* expression upon knocking-up while a significant decrease in *IFITs* expression upon knocking-down hence validating the follow-up experiments. Cell cytokines expression through Luminex was also performed as a part of follow-up studies of the *IFITs*. Luminex results revealed some key cytokines, i.e. IDO1, IFN-γ, IL-6 and IL-23, which were over-expressed ($p<0.001$) at knock-up (vector-based over-expression) and under-expressed ($p<0.001$) upon knocking-down *IFITs* in all three strains (*M. smegmatis*, BCG and R179).

Indoleamine 2,3-dioxygenesae-1 (IDO-1) is a heme-containing enzyme known for modulation of immunity by reducing T-cell function and engaging immune tolerance (Munn et al., 2013). IDO-1 is one of the well characterized genes in IFN-γ signalling during host response to mycobacteria. It is the first rate limiting enzyme of tryptophan metabolism along with kynurenine pathway, resulting in the formation of tryptophan metabolites, including picolinic acid and quinolinic. IDO-1 activation shown to restrict intracellular growth of *Leishmania donovanii*, *Chlamydia spec*, and *Toxoplasma gondii* by reducing formation of tryptophan (MacKenzie et al., 1999; Murray et al., 1989; Pfefferkorn et al., 1984).

It has been demonstrated that IDO-1 increased significantly during mycobacterial infection, activating the tryptophan metabolism in infected macrophages. However, IDO-1 is not essential for regulating *M. tuberculosis* infection in the mouse models (Blumenthal et al., 2012). In the present study, we found higher expression of IDO-1

after infection with different strains of mycobacteria, indicating activation of tryptophan metabolism in the infected macrophages. Also, we found increased expression of IDO-1 during knock-up (vector-based over-expression) of *IFITs* resulting in reduced CFUs, indicating the reduction in intracellular survival of *M. tuberculosis* due to knock-up (vector-based over-expression) of *IFITs* and not of higher levels of IDO-1 *in vitro* after knock-up (vector-based over-expression).

Another cytokine found to be significantly dominating during knock-up (vector-based over-expression) of *IFITs* is IFN-γ. It has been proven that IFN-γ activated macrophages kill mycobacteria via nitric oxide induced apoptosis independent of autophagy (Herbst et al., 2011). Also, IFN-γ believed to act directly on activated T cells (CD4+) during BCG infection by a) activating CD4+ T cells apoptosis molecules and b) by inducing cell-extrinsic apoptosis signals that kill CD4+ cells (Li et al., 2007). Further, it has been proven that pathogenic *M. tuberculosis* evade host immune response from macrophages by hindering IFN-γ transcription without affecting Janus kinase-STAT pathway (Ting et al., 1999).

It is well established that *M. tuberculosis* interferes with host signalling pathways activated with IFN-γ for its survival inside macrophages (Casanova et al., 2002; Fortune et al., 2004; Pai et al., 2003). In the present study, we found higher levels of IFN-γ after hMDMs were infected with *M. smegmatis*, BCG and R179. It is noteworthy, the levels of IFN-γ increased further (even significantly higher than usual infection) after vector-based knock-up (vector-based over-expression) of *IFITs*. On the other hand, *IFITs* knock-down via siRNA completely eliminated IFN-γ expression. Thus, establishing the

importance of *IFITs* in mounting IFN-γ based defence mechanism against killing of mycobacteria in human macrophages.

Similar to IFN-γ, higher levels of IL-6 were also found after *IFITs* knock-up (vector-based over-expression). IL-6 has been shown to be differentially expressed as part of a protective immune response in *M. tuberculosis* infected mice (Martinez et al., 2013). But similar to IDO-1, IL-6 is not essential for anti-mycobacterial mechanisms (Sodenkamp et al., 2012). It has been demonstrated that *M. tuberculosis* utilizes IL-6 as one of the pathway molecules to inhibit IFN-γ (Pai et al., 2003). Also, the downregulation of IL-6 in BMDMs infected with attended or virulent *M. tuberculosis* induces transcription of IFN-γ related genes (Martinez et al., 2013). Further, IL-6 appears to be a major cytokine in mycobacteria-infected *in vitro* murine peritoneal macrophages and can be used as a potent biomarker of *M. tuberculosis* infection (Singh & Goyal, 2013). Here we found increased IL-6 levels after infection with all three strains of *M. tuberculosis* and these levels increased further after the knock-up (vector-based over-expression) and decrease upon knock-down of *IFITs*. However, these results of increased IFN-γ and IL-6 at the same time are confounding, but increased IFN-γ levels (higher than IL-6) can compensate for the inhibitory effects of IL-6 and thus result in intracellular killing of mycobacteria inside the macrophages (reduced CFUs).

The fourth highly significant cytokine found in the present study is IL-23. It has been found to induce production of IL-17 from *M. tuberculosis* infected CD4+ cells (Th17) (Rodriguez et al., 2007). Also, IL-23 was found to induce IL-17 levels from T helper cells in healthy tuberculin reactors (Paidipally et al., 2009). In turns, IL-17 was found

to be antagonized by production of Th1 and Th2 cell related cytokines such as IL-12, IFN-γ and IL-4 (Laurence et al., 2007; Sadlack et al., 1993). Further, it has been demonstrated that IL-23 (and subsequently IL-17) mRNA expression is reduced in PBMCs of TB patients before and after stimulation with purified protein derivatives (PPD) as compared to PBMCs of healthy controls, indicating higher levels of IL-23 confer protection against *M. tuberculosis* in healthy individuals (Heidarnezhad et al., 2016). In the present study we found increased expression of IL-23 mRNA and cytokine levels post *IFITs* knock-up (vector-based over-expression) as compared to infection alone, indicating that increased IL-23 levels help in reducing intracellular survival of *M. tuberculosis* (reduced CFUs). This can be attributed to induction of IL-17 and IFN-γ cytokines through IL-23.

The above result shows that *IFITs* play an important role in mycobacterial killing. Knocking up of *IFITs* inside macrophages causes a significant increase in key pro-inflammatory cytokines (IDO-1, IL-6, IL-23 and IFN-γ) resulting in mycobacterial killing. Knocking down of *IFITs* inside macrophages causes a significant decrease in key pro-inflammatory cytokines (IDO-1, IL-6, IL-23 and IFN-γ) resulting in mycobacterial survival.

4.3 Limitations of the study

There are a few limitations in the study, 1) we selected only three different strains of pathogenic and non-pathogenic mycobacteria to study the host response. The number and types of strains can be increased in future studies in order to investigate a broader response of host towards the mycobacteria. 2) There were only two time points selected for AmpliSeq analysis, and the major emphasis was on 12 hours only as all the DEGs

did not show up at 96 hours. This was a part of the design of the current study in selecting DEGs, and time points can be increased in future studies to investigate the host response in detail. 3) Macrophage polarity was not determined upon infection with mycobacteria which will be a part of our future study. 4) Comparison of the three *IFITs* as individual and combinational therapy using *IFIT1*, *IFIT2* and *IFIT3* to study a synergistic effect in cells upon knocking-up and knocking-down was not performed in our study. This will be a part of a new project in our laboratory to carry out detailed analysis of the effect of this gene *in vitro* as well as in animal models (*in-vivo*).

4.4 Conclusion

We compared *M. smegmatis* with *M. bovis* BCG and R179. Host response towards *M. smegmatis* was higher in AmpliSeq data as compared to other two strains. This data was co-relating with qRT-PCR and Multiplex ELISA data, hence proving that the increased host response causes killing of *M. smegmatis* from 12 hours post-infection. All 19 DEGs selected in our data showed a higher response towards *M. smegmatis* hence proving our hypothesis of comparing the host response towards pathogenic and non-pathogenic mycobacteria.

Based upon the pathogenicity of native form of mycobacteria (mycobacteria grown in detergent-free medium), we found differed host responses in hMDMs. Activation of infected hMDMs using IFN-γ resulted in a reduction of CFUs, indicating importance of interferon in mycobacterial survival. mRNA and protein level comparisons at different time points, before and after infection, demonstrated strong role for the connected network of interferon and interleukin family. This network was able to successfully counter *M. smegmatis* but succumb to *M. bovis* BCG and *M. tb* R179.

Based on our results of the present study, mycobacterial strain for setting up *in vitro* infection experiments should be carefully selected.

Our follow-up studies used knocking up and knocking down of *IFITs* in hMDMs infected with different strains of mycobacteria. Interestingly, intra-cellular bacterial growth and survival measured through CFUs, decreased significantly upon knocking up *of IFITs*, while CFUs increased significantly upon knocking down *IFIT1, IFIT2* and *IFIT3* in the host macrophages. Luminex result revealed some key cytokines, i.e. IDO1, IFN-γ, IL-6 and IL-23, which dominated upon cell processing. Figure 4.1 depicts the schematic diagram for the knocking-up and knocking-down experiments conducted in the present study. These cytokines increased upon knock-up (vector-based over-expression) and decreased ($p<0.001$) upon knocking-down *IFITs* in all three strains (*M. smegmatis*, BCG and R179).

This knowledge will broaden the scope for host drug targets that will pave the path for the development of host-based bacteriostatic immuno-therapy adjunct to current chemotherapy, to which the pathogenic mycobacteria cannot develop resistance.

Based on our results we have filed a patent for the role of *IFIT*s as potential therapeutic target in TB (Patent pending Ref No. SU-DISC-2019-0021 New TB Disclosure).

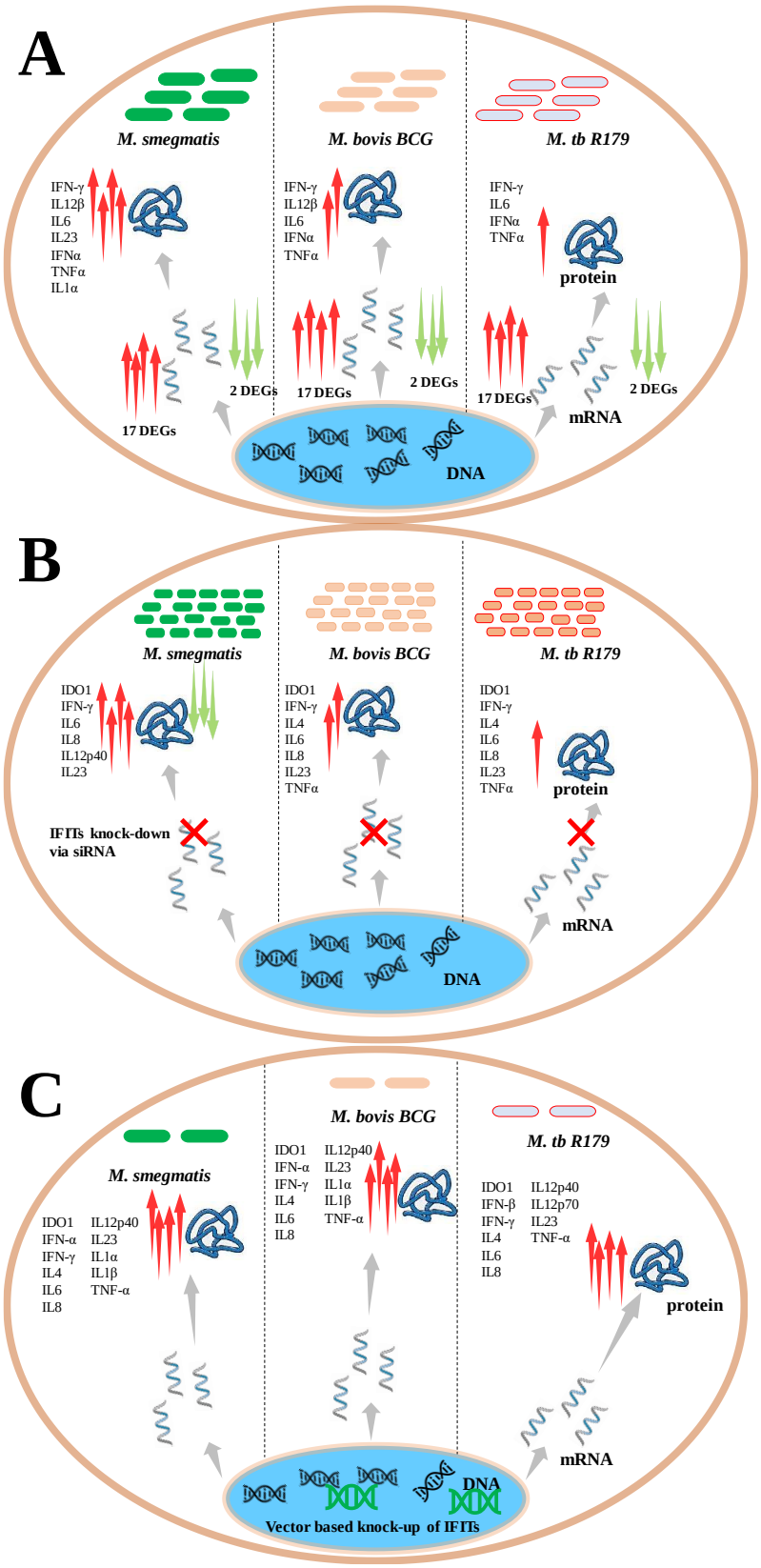


Figure 4.1: Schematic representation of the host response towards mycobacteria. A) Human macrophage response upon infection with different strains of mycobacteria. 17 up-regulated and 2 down-regulated genes were observed at mRNA level along with various cytokines up-regulated at protein level. B) Infected macrophage response upon knocking down of *IFITs* resulting in increase in bacterial counts. C) Infected macrophage response upon knocking up of *IFITs* gene family resulting in decrease in bacterial counts.

Bibliography

1. Abate, G., & Hoft, D. F. (2016). Immunotherapy for tuberculosis: future prospects. *ImmunoTargets and Therapy*, *5*, 37–45. https://doi.org/10.2147/ITT.S81892
2. Acosta-Rodriguez, E. V., Rivino, L., Geginat, J., Jarrossay, D., Gattorno, M., Lanzavecchia, A., … Napolitani, G. (2007). Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nature Immunology*, *8*(6), 639–646. https://doi.org/10.1038/ni1467
3. Aguilar-Ayala, D. A., Tilleman, L., Nieuwerburgh, F. V., Deforce, D., Palomino, J. C., Vandamme, P., … Martin, A. (2017). The transcriptome of Mycobacterium tuberculosis in a lipid-rich dormancy model through RNAseq analysis. *Scientific Reports*, *7*(1), 17665. https://doi.org/10.1038/s41598-017-17751-x
4. Anes, E., Kühnel, M. P., Bos, E., Moniz-Pereira, J., Habermann, A., & Griffiths, G. (2003). Selected lipids activate phagosome actin assembly and maturation resulting in killing of pathogenic mycobacteria. *Nature Cell Biology*, *5*(9), 793–802. https://doi.org/10.1038/ncb1036
5. Ayrapetyan, M., Williams, T. C., & Oliver, J. D. (2015). Bridging the gap between viable but non-culturable and antibiotic persistent bacteria. *Trends in Microbiology*, *23*(1), 7–13. https://doi.org/10.1016/j.tim.2014.09.004
6. Barnes, P. F., Bloch, A. B., Davidson, P. T., & Snider, D. E. (1991). Tuberculosis in patients with human immunodeficiency virus infection. *The New England Journal of Medicine*, *324*(23), 1644–1650. https://doi.org/10.1056/NEJM199106063242307
7. Barry, C. E., Lee, R. E., Mdluli, K., Sampson, A. E., Schroeder, B. G., Slayden, R. A., & Yuan, Y. (1998). Mycolic acids: structure, biosynthesis and physiological functions. *Progress in Lipid Research*, *37*(2–3), 143–179.
8. Başoğlu, O. K., Bacakoğlu, F., Cok, G., Sayiner, A., & Ateş, M. (1999). The oral

glucose tolerance test in patients with respiratory infections. *Monaldi Archives for Chest Disease = Archivio Monaldi Per Le Malattie Del Torace*, *54*(4), 307–310.

9. Betts, J. C., Lukey, P. T., Robb, L. C., McAdam, R. A., & Duncan, K. (2002). Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. *Molecular Microbiology*, *43*(3), 717–731.
10. Blumenthal, A., Nagalingam, G., Huch, J. H., Walker, L., Guillemin, G. J., Smythe, G. A., … Saunders, B. M. (2012). M. tuberculosis induces potent activation of IDO-1, but this is not essential for the immunological control of infection. *PloS One*, *7*(5), e37314. https://doi.org/10.1371/journal.pone.0037314
11. Bohsali, A., Abdalla, H., Velmurugan, K., & Briken, V. (2010). The non-pathogenic mycobacteria M. smegmatis and M. fortuitum induce rapid host cell apoptosis via a caspase-3 and TNF dependent pathway. *BMC Microbiology*, *10*, 237. https://doi.org/10.1186/1471-2180-10-237
12. Brady, N. J., Chuntova, P., & Schwertfeger, K. L. (2016). Macrophages: Regulators of the Inflammatory Microenvironment during Mammary Gland Development and Breast Cancer. *Mediators of Inflammation*, *2016*, 4549676. https://doi.org/10.1155/2016/4549676
13. Brennan, M. J., Delogu, G., Chen, Y., Bardarov, S., Kriakov, J., Alavi, M., & Jacobs, W. R. (2001). Evidence that mycobacterial PE_PGRS proteins are cell surface constituents that influence interactions with other cells. *Infection and Immunity*, *69*(12), 7326–7333. https://doi.org/10.1128/IAI.69.12.7326-7333.2001
14. Byron, S. A., Van Keuren-Jensen, K. R., Engelthaler, D. M., Carpten, J. D., & Craig, D. W. (2016). Translating RNA sequencing into clinical diagnostics: opportunities and challenges. *Nature Reviews. Genetics*, *17*(5), 257–271. https://doi.org/10.1038/nrg.2016.10

15. Caron, E., & Hall, A. (1998). Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. *Science (New York, N.Y.)*, *282*(5394), 1717–1721.
16. Casamassimi, A., Federico, A., Rienzo, M., Esposito, S., & Ciccodicola, A. (2017). Transcriptome Profiling in Human Diseases: New Advances and Perspectives. *International Journal of Molecular Sciences*, *18*(8). https://doi.org/10.3390/ijms18081652
17. Casanova, J.-L., & Abel, L. (2002). Genetic dissection of immunity to mycobacteria: the human model. *Annual Review of Immunology*, *20*, 581–620. https://doi.org/10.1146/annurev.immunol.20.081501.125851
18. Chebath, J., Merlin, G., Metz, R., Benech, P., & Revel, M. (1983). Interferon-induced 56,000 Mr protein and its mRNA in human cells: molecular cloning and partial sequence of the cDNA. *Nucleic Acids Research*, *11*(5), 1213–1226.
19. Christoforidis, S., Miaczynska, M., Ashman, K., Wilm, M., Zhao, L., Yip, S. C., … Zerial, M. (1999). Phosphatidylinositol-3-OH kinases are Rab5 effectors. *Nature Cell Biology*, *1*(4), 249–252. https://doi.org/10.1038/12075
20. Costa, V., Aprile, M., Esposito, R., & Ciccodicola, A. (2013). RNA-Seq and human complex diseases: recent accomplishments and future perspectives. *European Journal of Human Genetics: EJHG*, *21*(2), 134–142. https://doi.org/10.1038/ejhg.2012.129
21. Cywes, C., Hoppe, H. C., Daffé, M., & Ehlers, M. R. (1997). Nonopsonic binding of Mycobacterium tuberculosis to complement receptor type 3 is mediated by capsular polysaccharides and is strain dependent. *Infection and Immunity*, *65*(10), 4258–4266.
22. Decker, T., Stockinger, S., Karaghiosoff, M., Müller, M., & Kovarik, P. (2002). IFNs and STATs in innate immunity to microorganisms. *The Journal of Clinical Investigation*, *109*(10), 1271–1277. https://doi.org/10.1172/JCI15770

23. Delogu, G., Sanguinetti, M., Pusceddu, C., Bua, A., Brennan, M. J., Zanetti, S., & Fadda, G. (2006). PE_PGRS proteins are differentially expressed by Mycobacterium tuberculosis in host tissues. *Microbes and Infection*, *8*(8), 2061–2067. https://doi.org/10.1016/j.micinf.2006.03.015

24. Der, S. D., Zhou, A., Williams, B. R., & Silverman, R. H. (1998). Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(26), 15623–15628.

25. Dheenadhayalan, V., Delogu, G., Sanguinetti, M., Fadda, G., & Brennan, M. J. (2006). Variable Expression Patterns of Mycobacterium tuberculosis PE_PGRS Genes: Evidence that PE_PGRS16 and PE_PGRS26 Are Inversely Regulated In Vivo. *Journal of Bacteriology*, *188*(10), 3721–3725. https://doi.org/10.1128/JB.188.10.3721-3725.2006

26. Dubnau, E., Fontán, P., Manganelli, R., Soares-Appel, S., & Smith, I. (2002). Mycobacterium tuberculosis genes induced during infection of human macrophages. *Infection and Immunity*, *70*(6), 2787–2795.

27. Dubos, R. J., & Davis, B. D. (1946). FACTORS AFFECTING THE GROWTH OF TUBERCLE BACILLI IN LIQUID MEDIA. *The Journal of Experimental Medicine*, *83*(5), 409–423.

28. Dye, C. (2006). Global epidemiology of tuberculosis. *Lancet (London, England)*, *367*(9514), 938–940. https://doi.org/10.1016/S0140-6736(06)68384-0

29. Ernst, J. D. (1998). Macrophage Receptors for Mycobacterium tuberculosis. *Infection and Immunity*, *66*(4), 1277–1281.

30. Fensterl, V., & Sen, G. C. (2011). The ISG56/IFIT1 gene family. *Journal of Interferon & Cytokine Research: The Official Journal of the International Society for Interferon*

and Cytokine Research, *31*(1), 71–78. https://doi.org/10.1089/jir.2010.0101

31. Fenton, M. J. (1998). Macrophages and tuberculosis. *Current Opinion in Hematology*, *5*(1), 72–78.
32. Fisher, M. A., Plikaytis, B. B., & Shinnick, T. M. (2002). Microarray analysis of the Mycobacterium tuberculosis transcriptional response to the acidic conditions found in phagosomes. *Journal of Bacteriology*, *184*(14), 4025–4032.
33. Fortune, S. M., Solache, A., Jaeger, A., Hill, P. J., Belisle, J. T., Bloom, B. R., … Ernst, J. D. (2004). Mycobacterium tuberculosis inhibits macrophage responses to IFN-gamma through myeloid differentiation factor 88-dependent and -independent mechanisms. *Journal of Immunology (Baltimore, Md.: 1950)*, *172*(10), 6272–6280.
34. Habjan, M., Hubel, P., Lacerda, L., Benda, C., Holze, C., Eberl, C. H., … Pichlmair, A. (2013). Sequestration by IFIT1 impairs translation of 2'O-unmethylated capped RNA. *PLoS Pathogens*, *9*(10), e1003663. https://doi.org/10.1371/journal.ppat.1003663
35. Harshey, R. M., & Ramakrishnan, T. (1977). Rate of ribonucleic acid chain growth in Mycobacterium tuberculosis H37Rv. *Journal of Bacteriology*, *129*(2), 616–622.
36. Heidarnezhad, F., Asnaashari, A., Rezaee, S. A., Ghezelsofla, R., Ghazvini, K., Valizadeh, N., … Rafatpanah, H. (2016). Evaluation of Interleukin17and Interleukin 23 expression in patients with active and latent tuberculosis infection. *Iranian Journal of Basic Medical Sciences*, *19*(8), 844–850.
37. Hellwig, S. M. M., van Oirschot, H. F. L. M., Hazenbos, W. L. W., van Spriel, A. B., Mooi, F. R., & van de Winkel, J. G. J. (2001). Targeting to Fcγ Receptors, But Not CR3 (CD11b/CD18), Increases Clearance of Bordetella pertussis. *The Journal of Infectious Diseases*, *183*(6), 871–879. https://doi.org/10.1086/319266
38. Herbst, S., Schaible, U. E., & Schneider, B. E. (2011). Interferon Gamma Activated Macrophages Kill Mycobacteria by Nitric Oxide Induced Apoptosis. *PLOS ONE*, *6*(5),

e19105. https://doi.org/10.1371/journal.pone.0019105

39. Hussain, S., Zwilling, B. S., & Lafuse, W. P. (1999). Mycobacterium avium infection of mouse macrophages inhibits IFN-gamma Janus kinase-STAT signaling and gene induction by down-regulation of the IFN-gamma receptor. *Journal of Immunology (Baltimore, Md.: 1950)*, *163*(4), 2041–2048.
40. Im, J. G., Itoh, H., Shim, Y. S., Lee, J. H., Ahn, J., Han, M. C., & Noma, S. (1993). Pulmonary tuberculosis: CT findings--early active disease and sequential change with antituberculous therapy. *Radiology*, *186*(3), 653–660. https://doi.org/10.1148/radiology.186.3.8430169
41. Ioerger, T. R., Feng, Y., Chen, X., Dobos, K. M., Victor, T. C., Streicher, E. M., … Sacchettini, J. C. (2010). The non-clonality of drug resistance in Beijing-genotype isolates of Mycobacterium tuberculosis from the Western Cape of South Africa. *BMC Genomics*, *11*, 670. https://doi.org/10.1186/1471-2164-11-670
42. Jaconi, M. E., Lew, D. P., Carpentier, J. L., Magnusson, K. E., Sjögren, M., & Stendahl, O. (1990). Cytosolic free calcium elevation mediates the phagosome-lysosome fusion during phagocytosis in human neutrophils. *The Journal of Cell Biology*, *110*(5), 1555–1564.
43. Jacquier, A. (2009). The complex eukaryotic transcriptome: unexpected pervasive transcription and novel small RNAs. *Nature Reviews. Genetics*, *10*(12), 833–844. https://doi.org/10.1038/nrg2683
44. Jang, J. C., & Nair, M. G. (2013). Alternatively Activated Macrophages Revisited: New Insights into the Regulation of Immunity, Inflammation and Metabolic Function following Parasite Infection. *Current Immunology Reviews*, *9*(3), 147–156. https://doi.org/10.2174/15733955096661312102325 48
45. Jeon, C. Y., & Murray, M. B. (2008). Diabetes mellitus increases the risk of active

tuberculosis: a systematic review of 13 observational studies. *PLoS Medicine*, *5*(7), e152. https://doi.org/10.1371/journal.pmed.0050152

46. Johnson, G. L., & Lapadat, R. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science (New York, N.Y.)*, *298*(5600), 1911–1912. https://doi.org/10.1126/science.1072682
47. Johnson, R., Warren, R., Strauss, O. J., Jordaan, A. M., Falmer, A. A., Beyers, N., … Victor, T. C. (2006). An outbreak of drug-resistant tuberculosis caused by a Beijing strain in the western Cape, South Africa. *The International Journal of Tuberculosis and Lung Disease: The Official Journal of the International Union Against Tuberculosis and Lung Disease*, *10*(12), 1412–1414.
48. Katibah, G. E., Lee, H. J., Huizar, J. P., Vogan, J. M., Alber, T., & Collins, K. (2013). tRNA binding, structure, and localization of the human interferon-induced protein IFIT5. *Molecular Cell*, *49*(4), 743–750. https://doi.org/10.1016/j.molcel.2012.12.015
49. Kimura, T., Katoh, H., Kayama, H., Saiga, H., Okuyama, M., Okamoto, T., … Takeda, K. (2013). Ifit1 inhibits Japanese encephalitis virus replication through binding to 5' capped 2'-O unmethylated RNA. *Journal of Virology*, *87*(18), 9997–10003. https://doi.org/10.1128/JVI.00883-13
50. Koch, R. (2010). *Die Ätiologie der Tuberkulose*. http://dx.doi.org/10.25646/5088
51. Kohli, A., Zhang, X., Yang, J., Russell, R. S., Donnelly, R. P., Sheikh, F., … Kottilil, S. (2012). Distinct and overlapping genomic profiles and antiviral effects of Interferon-λ and -α on HCV-infected and non-infected hepatoma cells. *Journal of Viral Hepatitis*, *19*(12), 843–853. https://doi.org/10.1111/j.1365-2893.2012.01610.x
52. Koul, A., Herget, T., Klebl, B., & Ullrich, A. (2004). Interplay between mycobacteria and host signalling pathways. *Nature Reviews. Microbiology*, *2*(3), 189–202. https://doi.org/10.1038/nrmicro840

53. Kuehnel, M. P., Goethe, R., Habermann, A., Mueller, E., Rohde, M., Griffiths, G., & Valentin-Weigand, P. (2001). Characterization of the intracellular survival of Mycobacterium avium ssp. paratuberculosis: phagosomal pH and fusogenicity in J774 macrophages compared with other mycobacteria. *Cellular Microbiology*, *3*(8), 551–566. https://doi.org/10.1046/j.1462-5822.2001.00139.x

54. Kumar, P., Sweeney, T. R., Skabkin, M. A., Skabkina, O. V., Hellen, C. U. T., & Pestova, T. V. (2014). Inhibition of translation by IFIT family members is determined by their ability to interact selectively with the 5'-terminal regions of cap0-, cap1- and 5'ppp- mRNAs. *Nucleic Acids Research*, *42*(5), 3228–3245. https://doi.org/10.1093/nar/gkt1321

55. Kusari, J., & Sen, G. C. (1987). Transcriptional analyses of interferon-inducible mRNAs. *Molecular and Cellular Biology*, *7*(1), 528–531.

56. Laurence, A., Tato, C. M., Davidson, T. S., Kanno, Y., Chen, Z., Yao, Z., … O'shea, J. J. (2007). Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity*, *26*(3), 371–381. https://doi.org/10.1016/j.immuni.2007.02.009

57. Lee, K. S., Song, K. S., Lim, T. H., Kim, P. N., Kim, I. Y., & Lee, B. H. (1993). Adult-onset pulmonary tuberculosis: findings on chest radiographs and CT scans. *AJR. American Journal of Roentgenology*, *160*(4), 753–758. https://doi.org/10.2214/ajr.160.4.8456658

58. Leisching, G., Pietersen, R.-D., Wiid, I., & Baker, B. (2016). Virulence, biochemistry, morphology and host-interacting properties of detergent-free cultured mycobacteria: An update. *Tuberculosis (Edinburgh, Scotland)*, *100*, 53–60. https://doi.org/10.1016/j.tube.2016.07.002

59. Leisching, Gina, Pietersen, R.-D., Mpongoshe, V., van Heerden, C., van Helden, P., Wiid, I., & Baker, B. (2016). The Host Response to a Clinical MDR Mycobacterial

Strain Cultured in a Detergent-Free Environment: A Global Transcriptomics Approach. *PLoS ONE*, *11*(4). https://doi.org/10.1371/journal.pone.0153079

60. Leisching, Gina, Pietersen, R.-D., van Heerden, C., van Helden, P., Wiid, I., & Baker, B. (2017). RNAseq reveals hypervirulence-specific host responses to M. tuberculosis infection. *Virulence*, *8*(6), 848–858. https://doi.org/10.1080/21505594.2016.1250994
61. Li, W., Turner, A., Aggarwal, P., Matter, A., Storvick, E., Arnett, D. K., & Broeckel, U. (2015). Comprehensive evaluation of AmpliSeq transcriptome, a novel targeted whole transcriptome RNA sequencing methodology for global gene expression analysis. *BMC Genomics*, *16*. https://doi.org/10.1186/s12864-015-2270-1
62. Li, X., McKinstry, K. K., Swain, S. L., & Dalton, D. K. (2007). IFN-gamma acts directly on activated CD4+ T cells during mycobacterial infection to promote apoptosis by inducing components of the intracellular apoptosis machinery and by inducing extracellular proapoptotic signals. *Journal of Immunology (Baltimore, Md.: 1950)*, *179*(2), 939–949.
63. Liu, Y.-C., Zou, X.-B., Chai, Y.-F., & Yao, Y.-M. (2014). Macrophage polarization in inflammatory diseases. *International Journal of Biological Sciences*, *10*(5), 520–529. https://doi.org/10.7150/ijbs.8879
64. Lowe, R., Shirley, N., Bleackley, M., Dolan, S., & Shafee, T. (2017). Transcriptomics technologies. *PLoS Computational Biology*, *13*(5). https://doi.org/10.1371/journal.pcbi.1005457
65. MacKenzie, C. R., Langen, R., Takikawa, O., & Däubener, W. (1999). Inhibition of indoleamine 2,3-dioxygenase in human macrophages inhibits interferon-gamma-induced bacteriostasis but does not abrogate toxoplasmastasis. *European Journal of Immunology*, *29*(10), 3254–3261. https://doi.org/10.1002/(SICI)1521-4141(199910)29:10<3254::AID-IMMU3254>3.0.CO;2-0

66. Madonna, G. S., Ledney, G. D., Elliott, T. B., Brook, I., Ulrich, J. T., Myers, K. R., … Walker, R. I. (1989). Trehalose dimycolate enhances resistance to infection in neutropenic animals. *Infection and Immunity*, *57*(8), 2495–2501.

67. Manabe, Y. C., & Bishai, W. R. (2000). Latent Mycobacterium tuberculosis-persistence, patience, and winning by waiting. *Nature Medicine*, *6*(12), 1327–1329. https://doi.org/10.1038/82139

68. Mariotti, S., Teloni, R., Iona, E., Fattorini, L., Giannoni, F., Romagnoli, G., … Nisini, R. (2002). Mycobacterium tuberculosis subverts the differentiation of human monocytes into dendritic cells. *European Journal of Immunology*, *32*(11), 3050–3058. https://doi.org/10.1002/1521-4141(200211)32:11<3050::AID-IMMU3050>3.0.CO;2-K

69. Martinez, A. N., Mehra, S., & Kaushal, D. (2013). Role of interleukin 6 in innate immunity to Mycobacterium tuberculosis infection. *The Journal of Infectious Diseases*, *207*(8), 1253–1261. https://doi.org/10.1093/infdis/jit037

70. McGrath, K. E., Frame, J. M., & Palis, J. (2015). Early hematopoiesis and macrophage development. *Seminars in Immunology*, *27*(6), 379–387. https://doi.org/10.1016/j.smim.2016.03.013

71. McHale, C. M., Zhang, L., Thomas, R., & Smith, M. T. (2013). Analysis of the transcriptome in molecular epidemiology studies. *Environmental and Molecular Mutagenesis*, *54*(7), 500–517. https://doi.org/10.1002/em.21798

72. Melo, M. D., Catchpole, I. R., Haggar, G., & Stokes, R. W. (2000). Utilization of CD11b knockout mice to characterize the role of complement receptor 3 (CR3, CD11b/CD18) in the growth of Mycobacterium tuberculosis in macrophages. *Cellular Immunology*, *205*(1), 13–23. https://doi.org/10.1006/cimm.2000.1710

73. Menozzi, F. D., Rouse, J. H., Alavi, M., Laude-Sharp, M., Muller, J., Bischoff, R., …

Locht, C. (1996). Identification of a heparin-binding hemagglutinin present in mycobacteria. *The Journal of Experimental Medicine*, *184*(3), 993–1001.

74. Millington, K. A., Gooding, S., Hinks, T. S. C., Reynolds, D. J. M., & Lalvani, A. (2010). Mycobacterium tuberculosis-specific cellular immune profiles suggest bacillary persistence decades after spontaneous cure in untreated tuberculosis. *The Journal of Infectious Diseases*, *202*(11), 1685–1689. https://doi.org/10.1086/656772
75. Mosser, D. M., & Edwards, J. P. (2008). Exploring the full spectrum of macrophage activation. *Nature Reviews. Immunology*, *8*(12), 958. https://doi.org/10.1038/nri2448
76. Munn, D. H., & Mellor, A. L. (2013). Indoleamine 2,3 dioxygenase and metabolic control of immune responses. *Trends in Immunology*, *34*(3), 137–143. https://doi.org/10.1016/j.it.2012.10.001
77. Murray, H. W., Szuro-Sudol, A., Wellner, D., Oca, M. J., Granger, A. M., Libby, D. M., … Rubin, B. Y. (1989). Role of tryptophan degradation in respiratory burst-independent antimicrobial activity of gamma interferon-stimulated human macrophages. *Infection and Immunity*, *57*(3), 845–849.
78. Naidoo, P., Theron, G., Rangaka, M. X., Chihota, V. N., Vaughan, L., Brey, Z. O., & Pillay, Y. (2017). The South African Tuberculosis Care Cascade: Estimated Losses and Methodological Challenges. *The Journal of Infectious Diseases*, *216*(Suppl 7), S702–S713. https://doi.org/10.1093/infdis/jix335
79. Oluboyo, P. O., & Erasmus, R. T. (1990). The significance of glucose intolerance in pulmonary tuberculosis. *Tubercle*, *71*(2), 135–138.
80. Ottmani, S.-E., Murray, M. B., Jeon, C. Y., Baker, M. A., Kapur, A., Lönnroth, K., & Harries, A. D. (2010). Consultation meeting on tuberculosis and diabetes mellitus: meeting summary and recommendations. *The International Journal of Tuberculosis and Lung Disease: The Official Journal of the International Union Against*

Tuberculosis and Lung Disease, *14*(12), 1513–1517.

81. Pai, R. K., Convery, M., Hamilton, T. A., Boom, W. H., & Harding, C. V. (2003). Inhibition of IFN-gamma-induced class II transactivator expression by a 19-kDa lipoprotein from Mycobacterium tuberculosis: a potential mechanism for immune evasion. *Journal of Immunology (Baltimore, Md.: 1950)*, *171*(1), 175–184.
82. Paidipally, P., Periasamy, S., Barnes, P. F., Dhiman, R., Indramohan, M., Griffith, D. E., … Vankayalapati, R. (2009). NKG2D-dependent IL-17 production by human T cells in response to an intracellular pathogen. *Journal of Immunology (Baltimore, Md.: 1950)*, *183*(3), 1940–1945. https://doi.org/10.4049/jimmunol.0803578
83. Pancholi, P., Mirza, A., Bhardwaj, N., & Steinman, R. M. (1993). Sequestration from immune CD4+ T cells of mycobacteria growing in human macrophages. *Science (New York, N.Y.)*, *260*(5110), 984–986.
84. Papp, A. C., Azad, A. K., Pietrzak, M., Williams, A., Handelman, S. K., Jr, R. P. I., … Sadee, W. (2018). AmpliSeq transcriptome analysis of human alveolar and monocyte-derived macrophages over time in response to Mycobacterium tuberculosis infection. *PLOS ONE*, *13*(5), e0198221. https://doi.org/10.1371/journal.pone.0198221
85. Peto, H. M., Pratt, R. H., Harrington, T. A., LoBue, P. A., & Armstrong, L. R. (2009). Epidemiology of extrapulmonary tuberculosis in the United States, 1993-2006. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *49*(9), 1350–1357. https://doi.org/10.1086/605559
86. Pfefferkorn, E. R. (1984). Interferon gamma blocks the growth of Toxoplasma gondii in human fibroblasts by inducing the host cells to degrade tryptophan. *Proceedings of the National Academy of Sciences of the United States of America*, *81*(3), 908–912.
87. Pichlmair, A., Lassnig, C., Eberle, C.-A., Górna, M. W., Baumann, C. L., Burkard, T. R., … Superti-Furga, G. (2011). IFIT1 is an antiviral protein that recognizes 5’-

triphosphate RNA. *Nature Immunology*, *12*(7), 624–630. https://doi.org/10.1038/ni.2048

88. Piétu, G., Mariage-Samson, R., Fayein, N.-A., Matingou, C., Eveno, E., Houlgatte, R., … Auffray, C. (1999). The Genexpress IMAGE Knowledge Base of the Human Brain Transcriptome: A Prototype Integrated Resource for Functional and Computational Genomics. *Genome Research*, *9*(2), 195–209.
89. Poulsen, A. (1950). Some clinical features of tuberculosis. 1. Incubation period. *Acta Tuberculosea Scandinavica*, *24*(3–4), 311–346.
90. Rao, M., Ligeiro, D., & Maeurer, M. (2019). Precision medicine in the clinical management of respiratory tract infections including multidrug-resistant tuberculosis: learning from innovations in immuno-oncology. *Current Opinion in Pulmonary Medicine*, *25*(3), 233–241. https://doi.org/10.1097/MCP.0000000000000575
91. Rodriguez, G. M., Voskuil, M. I., Gold, B., Schoolnik, G. K., & Smith, I. (2002). ideR, An essential gene in mycobacterium tuberculosis: role of IdeR in iron-dependent gene expression, iron metabolism, and oxidative stress response. *Infection and Immunity*, *70*(7), 3371–3381.
92. Ronacher, K., Joosten, S. A., van Crevel, R., Dockrell, H. M., Walzl, G., & Ottenhoff, T. H. M. (2015). Acquired immunodeficiencies and tuberculosis: focus on HIV/AIDS and diabetes mellitus. *Immunological Reviews*, *264*(1), 121–137. https://doi.org/10.1111/imr.12257
93. Roy, S., Schmeier, S., Kaczkowski, B., Arner, E., Alam, T., Ozturk, M., … Suzuki, H. (2018). Transcriptional landscape of Mycobacterium tuberculosis infection in macrophages. *Scientific Reports*, *8*(1), 6758. https://doi.org/10.1038/s41598-018-24509-6
94. Sadlack, B., Merz, H., Schorle, H., Schimpl, A., Feller, A. C., & Horak, I. (1993).

Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell*, *75*(2), 253–261.

95. Sakamoto, K. (2012). The pathology of Mycobacterium tuberculosis infection. *Veterinary Pathology*, *49*(3), 423–439. https://doi.org/10.1177/0300985811429313
96. Sani, M., Houben, E. N. G., Geurtsen, J., Pierson, J., de Punder, K., van Zon, M., … Peters, P. J. (2010). Direct visualization by cryo-EM of the mycobacterial capsular layer: a labile structure containing ESX-1-secreted proteins. *PLoS Pathogens*, *6*(3), e1000794. https://doi.org/10.1371/journal.ppat.1000794
97. Schlesinger, L. S., Bellinger-Kawahara, C. G., Payne, N. R., & Horwitz, M. A. (1990). Phagocytosis of Mycobacterium tuberculosis is mediated by human monocyte complement receptors and complement component C3. *Journal of Immunology (Baltimore, Md.: 1950)*, *144*(7), 2771–2780.
98. Schnappinger, D., Ehrt, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., … Schoolnik, G. K. (2003). Transcriptional Adaptation of Mycobacterium tuberculosis within Macrophages: Insights into the Phagosomal Environment. *The Journal of Experimental Medicine*, *198*(5), 693–704. https://doi.org/10.1084/jem.20030846
99. Schüller, S., Neefjes, J., Ottenhoff, T., Thole, J., & Young, D. (2001). Coronin is involved in uptake of Mycobacterium bovis BCG in human macrophages but not in phagosome maintenance. *Cellular Microbiology*, *3*(12), 785–793.
100. Sia, I. G., & Wieland, M. L. (2011). Current Concepts in the Management of Tuberculosis. *Mayo Clinic Proceedings*, *86*(4), 348–361. https://doi.org/10.4065/mcp.2010.0820
101. Singh, P. P., & Goyal, A. (2013). Interleukin-6: a potent biomarker of mycobacterial infection. *SpringerPlus*, *2*. https://doi.org/10.1186/2193-1801-2-686
102. Smith, I. (2003). Mycobacterium tuberculosis pathogenesis and molecular

determinants of virulence. *Clinical Microbiology Reviews*, *16*(3), 463–496.

103. Sodenkamp, J., Waetzig, G. H., Scheller, J., Seegert, D., Grötzinger, J., Rose-John, S., … Hölscher, C. (2012). Therapeutic targeting of interleukin-6 trans-signaling does not affect the outcome of experimental tuberculosis. *Immunobiology*, *217*(10), 996–1004. https://doi.org/10.1016/j.imbio.2012.01.015
104. Steyn, A. J. C., Collins, D. M., Hondalus, M. K., Jacobs, W. R., Kawakami, R. P., & Bloom, B. R. (2002). Mycobacterium tuberculosis WhiB3 interacts with RpoV to affect host survival but is dispensable for in vivo growth. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(5), 3147–3152. https://doi.org/10.1073/pnas.052705399
105. Stokes, R. W., Norris-Jones, R., Brooks, D. E., Beveridge, T. J., Doxsee, D., & Thorson, L. M. (2004). The glycan-rich outer layer of the cell wall of Mycobacterium tuberculosis acts as an antiphagocytic capsule limiting the association of the bacterium with macrophages. *Infection and Immunity*, *72*(10), 5676–5686. https://doi.org/10.1128/IAI.72.10.5676-5686.2004
106. STREPTOMYCIN treatment of tuberculous meningitis. (1948). *Lancet (London, England)*, *1*(6503), 582–596.
107. Sturgill-Koszycki, S., Schlesinger, P. H., Chakraborty, P., Haddix, P. L., Collins, H. L., Fok, A. K., … Russell, D. G. (1994). Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. *Science (New York, N.Y.)*, *263*(5147), 678–681.
108. TB Statistics for South Africa | National & provincial. (n.d.). Retrieved October 8, 2018, from TB Facts | TB, tests, drugs, statistics website: https://www.tbfacts.org/tb-statistics-south-africa/
109. The non-pathogenic mycobacteria M. smegmatis and M. fortuitum induce rapid host

cell apoptosis via a caspase-3 and TNF dependent pathway. - PubMed - NCBI. (n.d.). Retrieved April 18, 2019, from https://www.ncbi.nlm.nih.gov/pubmed/20831789

110. Ting, L.-M., Kim, A. C., Cattamanchi, A., & Ernst, J. D. (1999). Mycobacterium tuberculosis Inhibits IFN-γ Transcriptional Responses Without Inhibiting Activation of STAT1. *The Journal of Immunology*, *163*(7), 3898–3906.
111. Trosko, J. E. (2008). Human Adult Stem Cells as the Target Cells for the Initiation of Carcinogenesis and for the Generation of "Cancer Stem Cells." *International Journal of Stem Cells*, *1*(1), 8–26.
112. Uhlin, M., Andersson, J., Zumla, A., & Maeurer, M. (2012). Adjunct immunotherapies for tuberculosis. *The Journal of Infectious Diseases*, *205 Suppl 2*, S325-334. https://doi.org/10.1093/infdis/jis197
113. Velculescu, V. E., Zhang, L., Zhou, W., Vogelstein, J., Basrai, M. A., Bassett, D. E., … Kinzler, K. W. (1997). Characterization of the yeast transcriptome. *Cell*, *88*(2), 243–251.
114. Venters, B. J., & Pugh, B. F. (2009). How eukaryotic genes are transcribed. *Critical Reviews in Biochemistry and Molecular Biology*, *44*(2–3), 117–141. https://doi.org/10.1080/10409230902858785
115. Vickers, T. A., Lima, W. F., Wu, H., Nichols, J. G., Linsley, P. S., & Crooke, S. T. (2009). Off-target and a portion of target-specific siRNA mediated mRNA degradation is Ago2 "Slicer" independent and can be mediated by Ago1. *Nucleic Acids Research*, *37*(20), 6927–6941. https://doi.org/10.1093/nar/gkp735
116. Vidya Vijayan, K. K., Karthigeyan, K. P., Tripathi, S. P., & Hanna, L. E. (2017). Pathophysiology of CD4+ T-Cell Depletion in HIV-1 and HIV-2 Infections. *Frontiers in Immunology*, *8*. https://doi.org/10.3389/fimmu.2017.00580
117. Voskuil, M. I., Visconti, K. C., & Schoolnik, G. K. (2004). Mycobacterium

tuberculosis gene expression during adaptation to stationary phase and low-oxygen dormancy. *Tuberculosis (Edinburgh, Scotland)*, *84*(3–4), 218–227. https://doi.org/10.1016/j.tube.2004.02.003

118. Wang, C., Mahrous, E. A., Lee, R. E., Vestling, M. M., & Takayama, K. (2011). Novel Polyoxyethylene-Containing Glycolipids Are Synthesized in Corynebacterium matruchotii and Mycobacterium smegmatis Cultured in the Presence of Tween 80. *Journal of Lipids*, *2011*, 676535. https://doi.org/10.1155/2011/676535
119. WHO. (n.d.). *Global Tuberculosis Report 2018, WHO*. Retrieved from https://www.who.int/tb/publications/global_report/en/
120. WHO | *Global report on diabetes*. (n.d.). Retrieved April 1, 2019, from WHO website: http://www.who.int/diabetes/global-report/en/
121. WHO | WHO End TB Strategy. (n.d.). Retrieved April 2, 2019, from WHO website: https://www.who.int/tb/post2015_strategy/en/
122. Wojciechowski, W., DeSanctis, J., Skamene, E., & Radzioch, D. (1999). Attenuation of MHC class II expression in macrophages infected with Mycobacterium bovis bacillus Calmette-Guérin involves class II transactivator and depends on the Nramp1 gene. *Journal of Immunology (Baltimore, Md.: 1950)*, *163*(5), 2688–2696.
123. Wurmser, A. E., Gary, J. D., & Emr, S. D. (1999). Phosphoinositide 3-kinases and their FYVE domain-containing effectors as regulators of vacuolar/lysosomal membrane trafficking pathways. *The Journal of Biological Chemistry*, *274*(14), 9129–9132.
124. Yang, J., Zhang, L., Yu, C., Yang, X.-F., & Wang, H. (2014). Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomarker Research*, *2*, 1. https://doi.org/10.1186/2050-7771-2-1

125. Yarkoni, E., & Bekierkunst, A. (1976). Nonspecific resistance against infection with Salmonella typhi and Salmonella typhimurium induced in mice by cord factor (trehalose-6,6'-dimycolate) and its analogues. *Infection and Immunity*, *14*(5), 1125–1129.

126. Zhai, W., Wu, F., Zhang, Y., Fu, Y., & Liu, Z. (2019). The Immune Escape Mechanisms of Mycobacterium Tuberculosis. *International Journal of Molecular Sciences*, *20*(2). https://doi.org/10.3390/ijms20020340.

APPENDIX

Health Research Ethics Committee (HREC)

Approval Notice

New Application

07/12/2017

Project ID:1210

HREC Reference # S17/10/211

Title: Global transcriptomic investigation of the human macrophage response to infection with Mycobacteria

Dear Ms Abhilasha Mishra,

The **New Application** received on 03/11/2017 was reviewed by members of **Health Research Ethics Committee** via **expedited** review procedures on 07/12/2017 and was approved.

Please note the following information about your approved research protocol:

Protocol Approval Period: 07-**Dec-2017 – 06-Dec-2018**

Please remember to use your Project ID **[1210]** on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review

Please note you can submit your progress report through the online ethics application process, available at: https://applyethics.sun.ac.za/Project/Index/1293 and the application should be submitted to the Committee before the year has expired. Please see Forms and Instructions on our HREC website for guidance on how to submit a progress report.

The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Translation of the consent document(s) to the language(s) applicable to your study participants should now be submitted to the HREC.

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility, permission must still be obtained from the relevant authorities (Western Cape Departement of Health and/or City Health) to conduct the research as stated in the protocol. Please consult the Western Cape Government website for access to the online Health Research Approval Process, see: https://www.westerncape.gov.za/general-publication/health-researchapproval-process. Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and instructions, please visit: Forms and Instructions on our

HREC website Links Application Form Direct Link If you have any questions or need

further assistance, please contact the HREC office at 021 938 9677.

Yours sincerely,

Francis Masiye,

HREC Coordinator,

Health Research Ethics Committee 2 (HREC 2).

Federal Wide Assurance Number: 00001372

Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No. 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki and the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles, Structures and Processes 2015 (Departement of Health).

UNIVERSITEIT
STELLENBOSCH
UNIVERSITY

Progress Report Approval Letter
31/10/2018

Project Reference #: 1210

Ethics Reference #: S17/10/211

Title: Global transcriptomic investigation of the human macrophage

response to infection with Mycobacteria Dear Miss Abhilasha

Mishra,

Your request for extension/annual renewal of ethics approval dated 17/10/2018 10:58 refers.

The Health Research Ethics Committee reviewed and approved the annual progress report you submitted through an expedited review process.

The approval of this project is extended for a further year.

Approval date: 31 October 2018

Expiry date: 30 October 2019

Kindly be reminded to submit progress reports two (2) months before expiry date.

Where to submit any documentation

Kindly note that the HREC uses an electronic ethics review management system, *Infonetica*, to manage ethics applications and ethics review process. To submit any documentation to HREC, please click on the following link: https://applyethics.sun.ac.za.

Please remember to use your **Project ID** [1210] and Ethics Reference Number S17/10/211 on any documents or correspondence with the HREC concerning your research protocol.

National Health Research Ethics Council (NHREC) Registration Numbers: REC-130408-012 for HREC1 and REC-230208-010 for HREC2

Federal Wide Assurance Number: 00001372

Institutional Review Board (IRB) Number: IRB0005240 for HREC1

Institutional Review Board (IRB) Number: IRB0005239 for HREC2

The Health Research Ethics Committee complies with the SA National Health Act No. 61 of 2003 as it pertains to health research and the United

States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki and the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles, Structures and Processes 2015 (Department of Health).

Yours sincerely,

Mrs. Ashleen Fortuin

Health Research Ethics Committee

Healthy study participants needed with Black and White Ethnicity

The study participants should have the following;

1. Between age of 18-40 years
2. No cough, fever, weight loss and insomnia
3. No chronic disease or underwent any recent surgery
4. Not on steroids or any strong medication
5. Clear Chest X-ray and HIV negative
6. No habit of smoking or alcohol intake
7. Feeling fit and have a healthy lifestyle with normal sleeping habits

If interested to participate in the study, please contact abhilasha@sun.ac.za or call on 021 938 9406, mobile 0642537028

80 mL of intra-venous blood will be drawn. **A compensation of R250 will be provided to the consented participants.**
The participation is completely voluntary.

Checklist for screening of healthy participants

GT MDM (2017)	
HC (Study Code)	
Name & Surname	
Contact No.	
Email ID	
Age (years)	
BMI (kg/m^2)	
Ethnicity	
Gender (female/male)	
QUANTIferon/IGRA Test (positive/negative)	
Cheat X-ray	
HIV (positive/negative)	
Any chronic disease (Y/N)	
On any strong medication/steroids (Y/N)	
Underwent any surgery (Y/N)	
Cough/Fever (Y/N)	
Weight loss (Y/N)	
Insomnia (Y/N)	
Normal sleeping and eating habits (Y/N)	
Healthy lifestyle (Y/N)	
Smoking/Alcohol (Y/N)	
Informed consent (Y/N)	
Fit to Criteria (Y/N)	

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM Adult consent form

TITLE OF THE RESEARCH PROJECT:

Global transcriptomic investigation of the human macrophage response to infection with pathogenic and non-pathogenic Mycobacteria

REFERENCE NUMBER:

PRINCIPAL INVESTIGATOR:	Dr. Bienyameen Baker
ADDRESS:	F446 Fisan Building Tygerberg Medical Campus Francie van Zijl Drive Cape Town
CONTACT NUMBER: Dear Sir / Madam:	021 938 9402

We will investigate the effect of *M. tuberculosis* bacteria on specific blood cells of healthy individuals. We hope the crucial information from this study will help in understanding the mechanism of TB infection. This will help in formulating new strategy to combat this disease.

Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research is about and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way. You are also free to withdraw from the study at any point, even if you have started to take part.

This study has been approved by the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study about?

The purpose of this study is to enumerate important biomolecule in specific immune cells (macrophages) after infection with different strains of TB bacteria. These immune cells will be isolated from blood of healthy human adults. This *in vitro* (within lab) experiment will be carried out at two time points, after infection of immune cells with different strains of TB bacteria i.e. 12 and 96 hours.
Tuberculosis is one of the biggest public health hazards around the globe. It claims millions of lives every year. About one third of the global population is infected with TB bacteria, but infection translates to active disease in 10% of the infected individuals. It means, human immunity does

have unexplained mechanism to counter these TB bacteria's. Studying the effect of TB bacteria on the levels of biomolecules altered/secreted from immune cells of healthy individuals will help in understanding these enigmas.
In the present study, healthy volunteers (male / female) over the age of 18 who have no symptoms of TB can participate. The study participants should satisfy the inclusion criteria of the study. Upto 20 adults will be taking part between May 2017 and May 2019.

The current study is being done at the Faculty of Medicine and Health Sciences, Tygerberg Campus, Cape Town, SA. If you do decide to take part in this study you will be asked to provide about 80mL of blood.

We will also ask you to give us some medical information and information about you, so that we can analyse our data from your blood cells. We will also ask you to have a chest radiograph, and have an HIV test if you don't know your status or at least two months has passed since your last test (if you do not want to be tested for HIV you can still participate in the study).

Why have you been invited to participate?

As per your medical and radiological records you are in sound health and satisfy all the inclusion criteria of our study

Will you benefit from taking part in this research?

The results of the study will not directly benefit you or change the way that TB is being treated. However, these results may help us understand the interaction of tuberculosis bacteria with our immune cells within a healthy control.

Who will have access to your medical records?

All personal and medical information collected will be treated as strictly confidential. If you consent to take part, the staff working on the study and ethics committee would be able to see your medical records to ensure that the study is being carried out correctly. Details about you stored on a secure database/computer will be identified by a unique study number and your initials but will not include your name or other identifying details. The information that will be collected will include characteristics of your health status, result from chest X-rays and blood investigations, antibiotics you were taking, and how you were feeling at the particular study visit (coughing, fever).

All study papers about you will be kept at the study site or in secure storage locations (like those used by doctors or lawyers) for a minimum of 5 years after study completion i.e. publication, and then destroyed (shredded).

The results of the study may be published in international medical journals so that doctors and other health care workers may benefit and learn from this work. You will however not be identified in these publications.

YOU CAN EXPECT THAT:

1. your name or any identifying information will not be shared in any publications or reports;
2. your personal information will not be given to anyone outside the study staff;

Are there any risks involved in your taking part in this research?

Phlebotomy will be performed by trained research nurse and there are no risks involved. But, as caution, you are not allowed to participate if:

- You are pregnant
- Smoker or alcoholic
- Diabetic or Hypertensive
- Fever or common cold (running nose)
- You previously had a collapsed lung or pulmonary haemorrhage (severe bleeding of the lung) in the last 10 years
- Has any infectious diseases or using antibiotics
- Coughing ever caused you to become dizzy and faint
- You previously had a brain haemorrhage (bleeding)
- You spend sleepless nights

We will maintain confidentiality of your name and details of your laboratory examination. But, if you need urgent medical care, we would like to tell more about your medical history to the health care worker looking after you, which would help him/her to decide on the medical care you need.

This is regarded as a safe procedure and is routinely performed in many settings where healthy individuals volunteer for research studies.
If you feel uncomfortable and disagree to participate, you can withdraw at any time point of study.

What will your responsibilities be?

If you decide to take part, you will agree to provide 80mL of your blood at one time point. It is however very important that you carefully follow the instruction of the research nurse and answer his/her queries about your health status and medical history.
Will you be paid to take part in this study and are there any costs involved? You will receive few amount as compensation or refreshment for each of your visits. There will be few costs (R250) involved for you, if you do take part.

Future use of specimens
We will store any the specimens or their derivatives that are collected from you during your participation in the study. We may use these to investigate other research questions. This will be stored without any personal identifiers.

What will happen in the unlikely event of some form of injury occurring as a direct result of your taking part in this research study?

If you are injured as a result of participation in this study, the study clinic will give you immediate necessary treatment for your injuries. The cost of this treatment will not be charged to you. You will then be told where you may receive additional treatment.

Is there anything else that you should know or do?

- You can contact the study PI (contact details above)
- You can contact the Health Research Ethics Committee at 021 938 9406 if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I (name and surname)……..……. agree to take part in a research study entitled "Global transcriptomic investigation of the human macrophage response to infection with pathogenic and non-pathogenic *Mycobacterium tuberculosis*".

I declare that:

- I have read this information and consent form that is written in a language which I am fluent in and comfortable to read. / it was read to me.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

I allow the staff working on the study to collect information about my health status, medical records and HIV status (if available) and enter this into the study database.

□ **Yes**, I allow the staff working on the study to collect information about my HIV status and enter this information into the study database

□ **No**, I don't allow that my HIV status is collected for study, but I still want to take part in this study

Signed at (*place*) .....…..…….. on (*date*) ……...…….. .

.....

Signature of participant

Declaration by the investigator or delegated person obtaining consent

I *(name and surname)* ……..…… declare that:

- I explained the information in this document to the above participant
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use an interpreter. (*If an interpreter was used then the interpreter must sign the declaration below.)*

Signed at (*place*) .....…..…….. on (*date*) ……...…….. .

.....
Signature of the person obtaining consent

<u>Declaration by interpreter</u> (if applicable)

I *(name and surname)* ……..…… declare that:

- I assisted the person obtaining consent to explain the information in this document to the above participant in his / her preferred language.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .....…..…….. on (*date*) ……...……..

.....
Signature of interpreter

<u>Declaration by witness</u> (if applicable)

I *(name and surname)* ……..…… declare that:

□ Verbal informed consent was obtained by the above participant. The participant has been informed about the risks and the benefits of the research, understands such risks and benefits and is able to give consent to participation, without coercion, undue influence or inappropriate incentives.

Signed at (*place*) .....…..…….. on (*date*) ……...……..

.....
Signature of witness

Table Appendix V: List of quantification of RNA specimen of the study participants and determination of RIN score

Tube #	Sample name	[RNA]	RIN	[DNA]	DNA:RNA
1	A1	55	10	5.08	0.092
2	A2	57	10	4.24	0.074
3	A3	57	10	5.14	0.090
4	A4	57	9.8	4.9	0.086
5	A5	63	9.7	5.98	0.095
6	A6	66	10	7.54	0.114
7	A7	77	9.9	5.82	0.076
8	B1	77	10	8.34	0.108
9	B2	95	10	9	0.095
10	B3	86	10	7.54	0.088
11	B4	96	10	8.38	0.087
12	B5	121	10	15.4	0.127
13	B6	64	10	14.9	0.233
14	B7	70	10	14.5	0.207
15	C1	51	10	6.32	0.124
16	C2	34	10	4.94	0.145
17	C3	35	10	4.58	0.131
18	C4	28	10	2.9	0.104
19	C5	81	10	20.6	0.254
20	C6	93	10	23.2	0.249
21	C7	86	10	21.8	0.253
22	D1	29	10	2.88	0.099

23	D2	33	10	3.26	0.099
24	D3	21	10	1.84	0.088
25	D4	50	9.9	2.62	0.052
26	D5	89	10	11.8	0.133
27	D6	90	10	7.2	0.080
28	D7	110	10	15.7	0.143
29	E1	59	10	3.7	0.063
30	E2	88	10	6.36	0.072
31	E3	93	10	6.22	0.067
32	E4	73	10	4.52	0.062
33	E5	111	10	10.4	0.094
34	E6	111	10	10.4	0.094
35	E7	111	10	11.6	0.105
36	F1	105	10	10	0.095
37	F2	84	10	10.8	0.129
38	F3	100	10	13	0.130
39	F4	82	10	9.8	0.120
40	F5	110	10	18	0.164
41	F6	100	10	12.8	0.128
42	F7	121	10	15.8	0.131
43	G1	53	10	3.9	0.074
44	G2	68	9.6	4.1	0.060
45	G3	53	10	4.16	0.078
46	G4	68	9.9	5	0.074
47	G5	113	10	13	0.115

Tube #	Sample name	[RNA]	RIN	[DNA]	DNA:RNA
48	G6	74	10	10.4	0.141
49	G7	107	10	10.2	0.095
50	H1	66	10	3.38	0.051
51	H2	72	9.8	3.2	0.044
52	H3	44	9	1.5	0.034
53	H4	61	9.6	2.9	0.048
54	H5	149	10	15.9	0.107
55	H6	117	10	10.8	0.092
56	H7	132	10	14	0.106
57	I1	84	10	5.94	0.071
58	I2	82	10	5.34	0.065
59	I3	88	10	6.4	0.073
60	I4	74	10	3.62	0.049
61	I5	137	10	15.5	0.113
62	I6	117	10	11	0.094
63	I7	121	10	10.7	0.088
64	J1	42	10	1.88	0.045
65	J2	35	10	2.32	0.066
66	J3	57	10	2.6	0.046
67	J4	62	9	3.1	0.050
68	J5	68	10	2.32	0.034
69	J6	51	10	3.14	0.062
70	J7	67	10	1.86	0.028
71	K1	42	10	2.12	0.050

Tube #	Sample name	[RNA]	RIN	[DNA]	DNA:RNA
72	K2	49	10	1.98	0.040
73	K3	35	10	1.72	0.049
74	K4	27	10	1.7	0.063
75	K5	34	10	1.71	0.050
76	K6	86	10	13	0.151
77	K7	82	10	12.1	0.148
78	L1	31	10	1.61	0.052
79	L2	27	9.9	1.52	0.056
80	L3	39	10	2.18	0.056
81	L4	40	10	2.46	0.062
82	L5	49	9.9	3.32	0.068
83	L6	38	10	2.8	0.074
84	L7	47	10	3.6	0.077

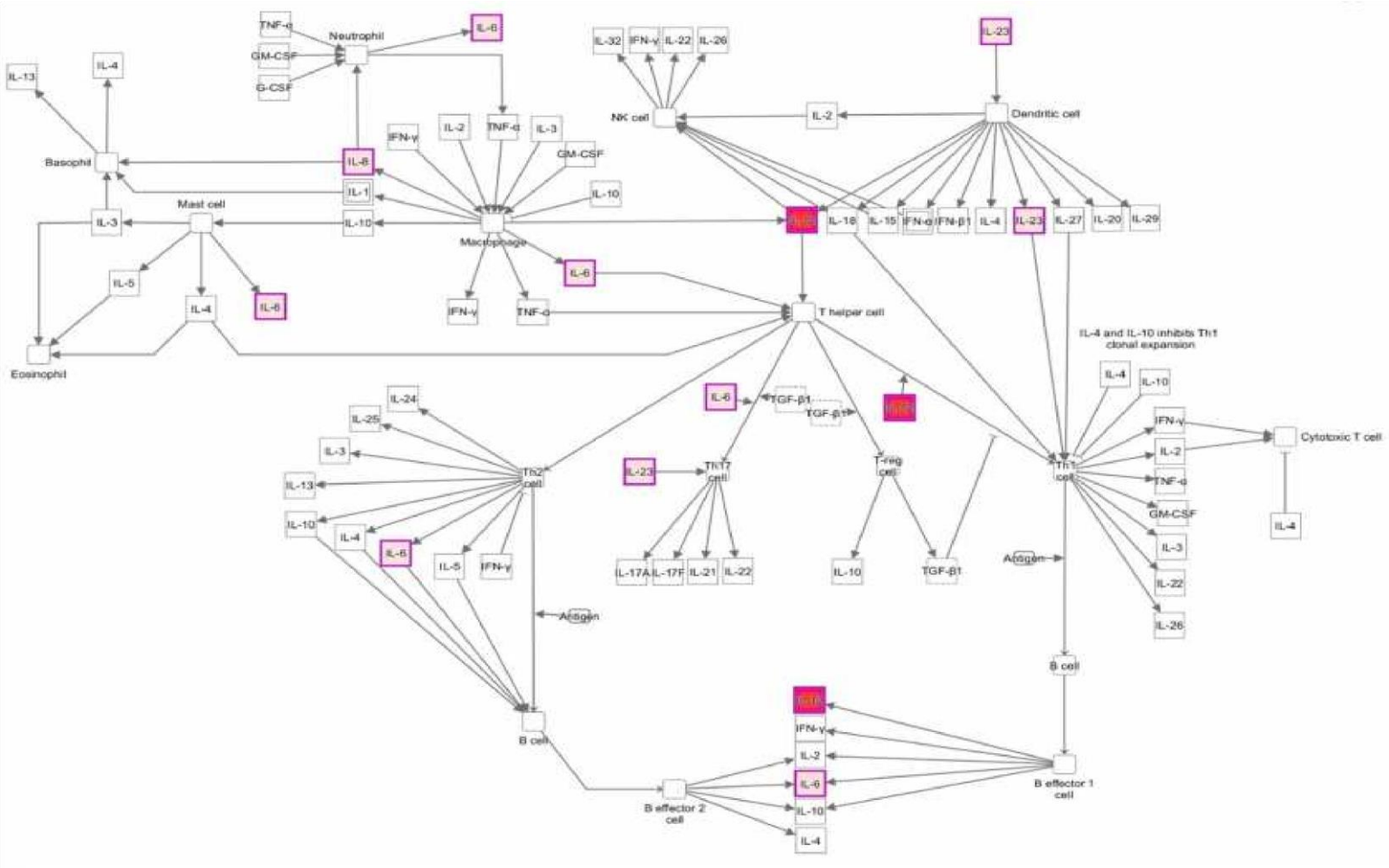


Figure Appendix VIa: Role of cytokines in mediating communication between immune cells. Red indicates mRNA up-regulation, pink as moderate expression and white as no expression. Intensity of colour is proportional to log2 differential expression ratio (DER) of average samples.

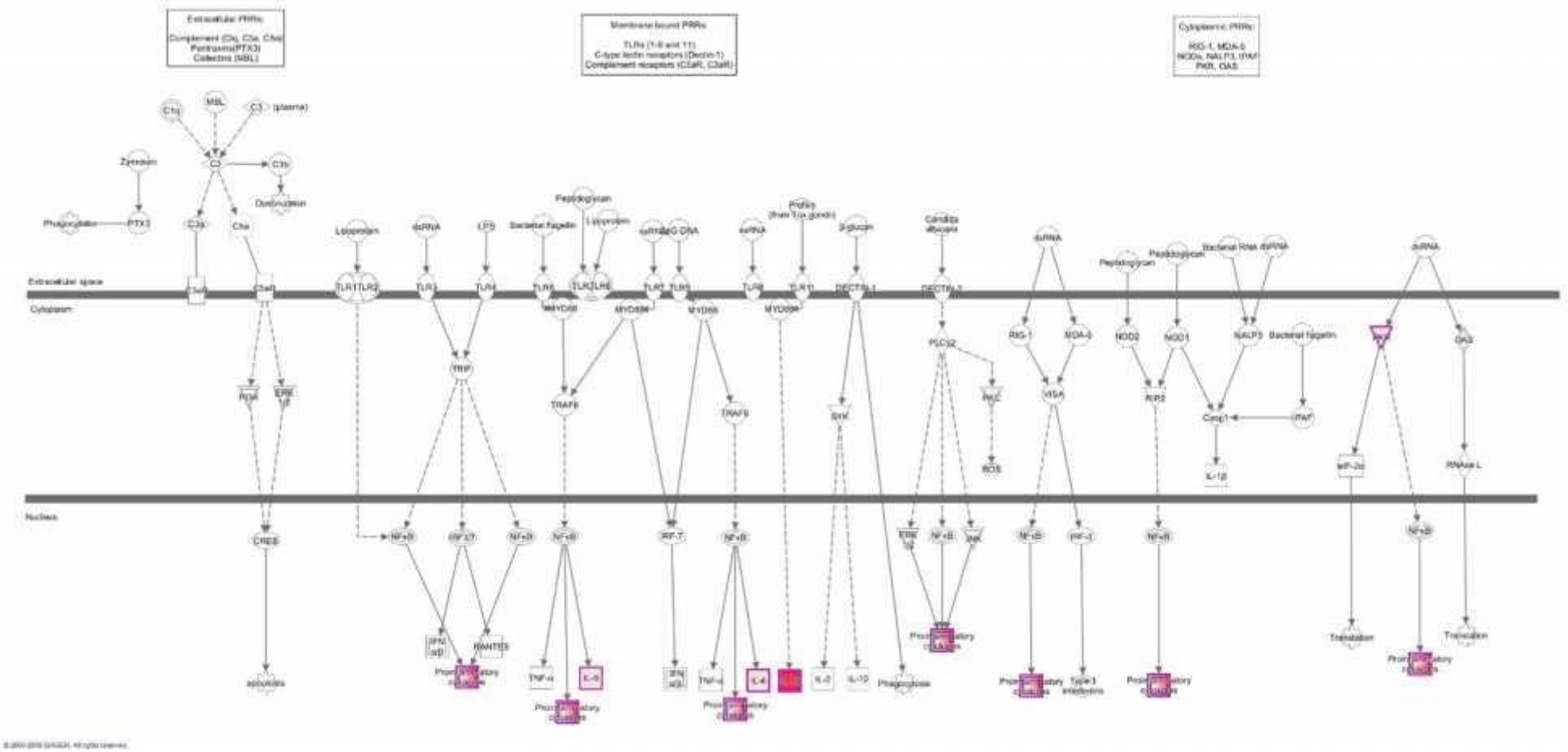


Figure Appendix VIb: Role of Pattern Recognition Receptors (PRRs) in recognition of bacteria and viruses. Red indicates mRNA upregulation, pink as moderate expression and white as no expression. Intensity of colour is proportional to log2 differential expression ratio (DER) of average samples.

Table Appendix VII: Expression of early response cytokines from hMDMs before and after infection with BCG and R179 measured at 24 and 72 hours post-infection

Cytokine	Uninfected	BCG	R179
at 24 hours			
IDO-1	1423.6 ± 338.6	1547.4 ± 670.8 0.807*	1571.5 ± 378.1 0.738* 0.991‡
IFN-γ	1832.9 ± 1609.9	3145.3 ± 1758.5 0.108*	2464.1 ± 1206.9 0.580* 0.532‡
IL1-β	22.1 ± 11.6	92 ± 106.3 0.124*	98.3 ± 101.2 0.086* 0.982‡
IL-12p70	93.6 ± 122.4	132.8 ± 118 0.669*	94 ± 92.5 0.999* 0.674‡
IL-12p40	56.7 ± 43.2	2583.1 ± 2100.3 **0.0003***	1441.5 ± 1241.8 0.055* 0.131‡
IL-4	597.6 ± 449.1	811.5 ± 355.7 0.399*	790.8 ± 389.8 0.471* 0.991‡
IL-6	1930.8 ± 628.5	6964.4 ± 2861.1 <**0.0001***	5483.9 ± 1463.8 **0.0002*** 0.149‡
TNF-α	358 ± 178.4	11422.3 ± 22764 0.234*	6857.5 ± 6715.4 0.596* 0.773‡
IL-23	1351.6 ± 373	1523.3 ± 396.6 0.433*	1463.4 ± 209.5 0.697* 0.901‡
IFN-α	38.4 ± 6.1	81.8 ± 10.3 <**0.0001***	80.3 ± 14.1 <**0.0001*** 0.936‡
IFN-β	0 ± 0	0 ± 0 0.999*	0 ± 0 0.999* 0.999‡
IL-1α	256.4 ± 31.7	271.3 ± 15.5 0.237*	271 ± 14.7 0.252* 0.999‡

IL-8	11225.2 ± 3829	12430.2 ± 2242.4 0.622*	13118 ± 3190 0.318* 0.855‡
Cytokine	**Uninfected**	**BCG**	**R179**
at 72 hours			
IDO-1	1085.9 ± 356.6	1167.6 ± 212.2 0.748*	1202.3 ± 233.5 0.559* 0.948‡
IFN-γ	325.7 ± 263.2	1259.6 ± 598.6 **0.0002***	1213.1 ± 563.9 **0.0003*** 0.971‡
IL1-β	3.2 ± 3.2	27.8 ± 23.5 **0.0005***	7.2 ± 6.7 0.774* **0.003**‡
IL-12p70	47.9 ± 63.3	60.7 ± 66.7 0.879*	57 ± 64.2 0.937* 0.989‡
IL-12p40	161.4 ± 294.1	232.5 ± 175.5 0.698*	225.9 ± 143.7 0.743* 0.996‡
IL-4	390.2 ± 506.9	714.1 ± 566.5 0.294*	582.8 ± 488.1 0.641* 0.812‡
IL-6	611.1 ± 239	2972 ± 1182.7 <**0.0001***	2459.2 ± 959.8 <**0.0001*** 0.346‡
TNF-α	102.7 ± 93.5	187.6 ± 83.1 **0.044***	173.1 ± 70.3 0.109* 0.904‡
IL-23	1082.5 ± 455.5	1067 ± 493.5 0.995*	1082.5 ± 274.6 0.999* 0.995‡

IFN-α	38.5 ± 31.6	59.3 ± 18.2 <**0.0001***	54.9 ± 16.3 <**0.0001*** 0.773‡
IFN-β	0 ± 0	0 ± 0 0.999*	0 ± 0 0.999* 0.999‡
IL-1α	170.9 ± 50	239.1 ± 38.5 **0.001***	223.6 ± 36.3 **0.011*** 0.645‡
IL-8	5630.1 ± 3898.3	10276 ± 3583 **0.006***	8112.9 ± 2705.1 0.194* 0.284‡

*= Uninfected v/s Test (*M. smegmatis*, BCG, R179), †= M.smegmatis v/s BCG/R179, ‡= BCG v/s R179. Data represented as mean ± Standard Deviation

(Under peer review, Virulence)

Distinct host-immune response towards species related intracellular mycobacterial killing: A transcriptomic study

Abhilasha Madhvi[1*†], Hridesh Mishra[1*], Novel N. Chegou[1], Carel J. Van Heerden[2], Gerard Tromp[1], R.D Pietersen[1], Gina Leisching[1], Bienyameen Baker[1†]

Affiliations:

[1]DST-NRF Centre of Excellence for Biomedical Tuberculosis Research; South African Medical Research Council Centre for Tuberculosis Research; Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town.

[2]Central Analytical Facility (CAF), DNA sequencing unit, Stellenbosch University, Stellenbosch, South Africa.

[†]**Corresponding author**: abhilasha@sun.ac.za , brubaker@sun.ac.za

*Abhilasha Madhvi and Hridesh Mishra contributed equally to this work

Running title: *In vitro* host response of human macrophages to mycobacteria

Keywords: Pathogenic, non-pathogenic, host response, *in vitro*, interferon-interleukin network, differentially expressed genes (DEGs), tween-free media

Data Availability Statement: AmpliSeq data have been deposited in the NCBI Gene Expression Omnibus (GEO) database with experiment series accession number [GSE122619].

(Published in CIMID, copy attached)

Comparison of human monocyte derived macrophages and THP1-like macrophages as *in vitro* models for *M. tuberculosis* infection.

Abhilasha Madhvi*[1], Hridesh Mishra*[1], Leisching GR[1], Mahlobo PZ[1], Baker B[1].

[1] DST-NRF Centre of Excellence for Biomedical Tuberculosis Research; South African Medical Research Council Centre for Tuberculosis Research; Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa.

Corresponding author: abhilasha@sun.ac.za , brubaker@sun.ac.za

*Abhilasha Madhvi and Hridesh Mishra contributed equally to this work.

Short title: THP-1cells and hMDMs as suitable *in vitro* models for *M. tuberculosis* infection.

ORCID ID: abhilasha@sun.ac.za

Figure 1.1: permission

Mishra, AM, Mej [abhilasha@sun.ac.za]

From:	permissions@who.int
Sent:	Tuesday, July 2, 2019 5:38 PM
To:	Mishra, AM, Mej [abhilasha@sun.ac.za]
Cc:	permissions@who.int
Subject:	ID: 293056 Permission authorization for WHO copyrighted material

Dear Mrs Mishra

Thank you for your request for permission to reproduce, reprint or translate certain WHO copyrighted material. On behalf of the World Health Organization, we are pleased to authorize your request to reproduce the WHO

materials as detailed in the form below, subject to the terms and conditions of the non-exclusive licence below.

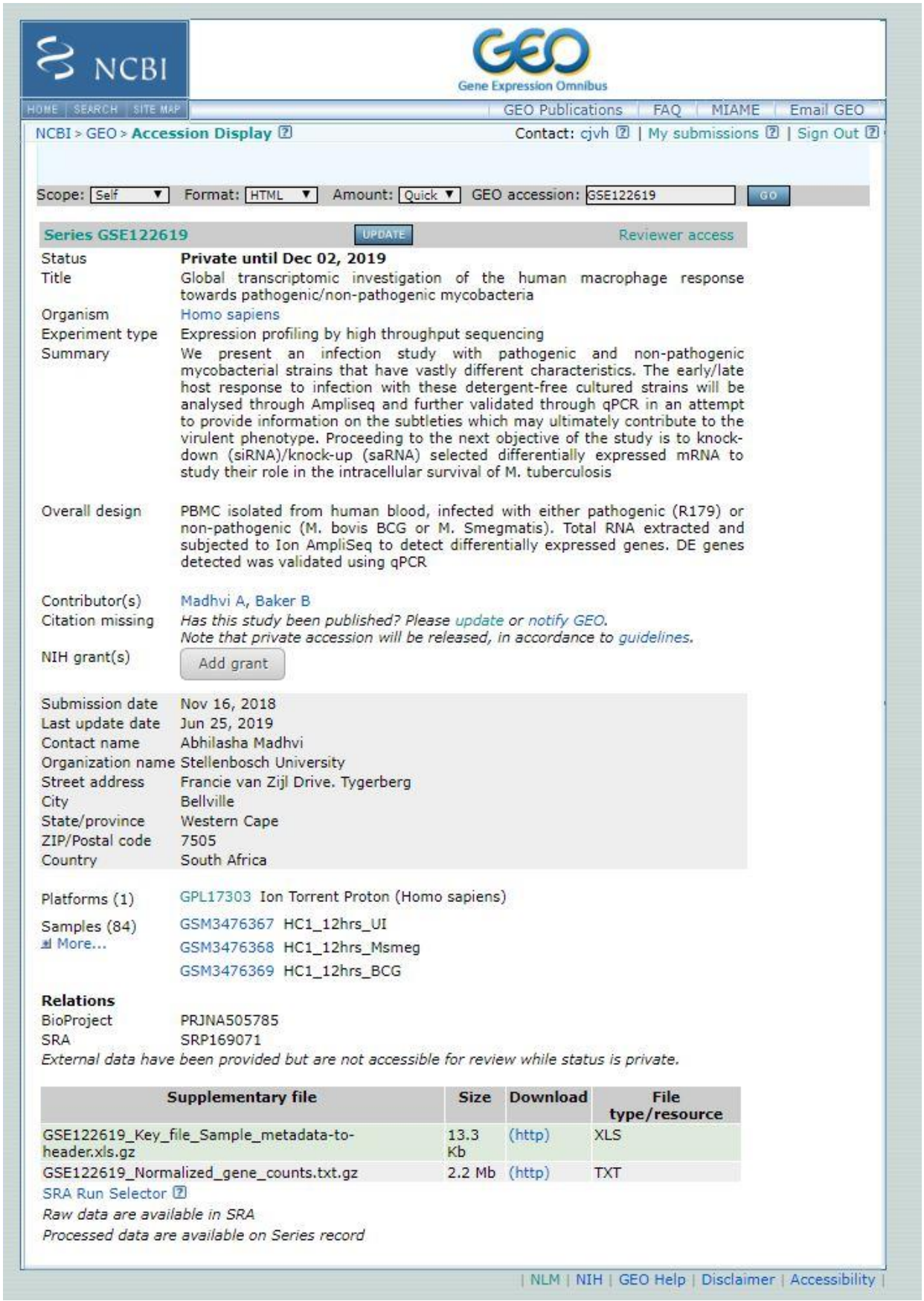
If you have questions regarding this authorization, please contact permissions@who.int.

We thank you for your interest in WHO published materials.

Kind regards,

WHO Permissions team

The Gene Expression Omnibus Database-NCBI-NIH



NCBI

Gene Expression Omnibus

HOME | SEARCH | SITE MAP | GEO Publications | FAQ | MIAME | Email GEO

NCBI > GEO > **Accession Display** | Contact: cjvh | My submissions | Sign Out

Scope: Self | Format: HTML | Amount: Quick | GEO accession: GSE122619 | GO

Series GSE122619 | UPDATE | Reviewer access

Status	**Private until Dec 02, 2019**
Title	Global transcriptomic investigation of the human macrophage response towards pathogenic/non-pathogenic mycobacteria
Organism	Homo sapiens
Experiment type	Expression profiling by high throughput sequencing
Summary	We present an infection study with pathogenic and non-pathogenic mycobacterial strains that have vastly different characteristics. The early/late host response to infection with these detergent-free cultured strains will be analysed through Ampliseq and further validated through qPCR in an attempt to provide information on the subtleties which may ultimately contribute to the virulent phenotype. Proceeding to the next objective of the study is to knock-down (siRNA)/knock-up (saRNA) selected differentially expressed mRNA to study their role in the intracellular survival of M. tuberculosis
Overall design	PBMC isolated from human blood, infected with either pathogenic (R179) or non-pathogenic (M. bovis BCG or M. Smegmatis). Total RNA extracted and subjected to Ion AmpliSeq to detect differentially expressed genes. DE genes detected was validated using qPCR
Contributor(s)	Madhvi A, Baker B
Citation missing	*Has this study been published? Please update or notify GEO.* *Note that private accession will be released, in accordance to guidelines.*
NIH grant(s)	Add grant
Submission date	Nov 16, 2018
Last update date	Jun 25, 2019
Contact name	Abhilasha Madhvi
Organization name	Stellenbosch University
Street address	Francie van Zijl Drive. Tygerberg
City	Bellville
State/province	Western Cape
ZIP/Postal code	7505
Country	South Africa
Platforms (1)	GPL17303 Ion Torrent Proton (Homo sapiens)
Samples (84)	GSM3476367 HC1_12hrs_UI
More...	GSM3476368 HC1_12hrs_Msmeg
	GSM3476369 HC1_12hrs_BCG

Relations

BioProject	PRJNA505785
SRA	SRP169071

External data have been provided but are not accessible for review while status is private.

Supplementary file	Size	Download	File type/resource
GSE122619_Key_file_Sample_metadata-to-header.xls.gz	13.3 Kb	(http)	XLS
GSE122619_Normalized_gene_counts.txt.gz	2.2 Mb	(http)	TXT

SRA Run Selector

Raw data are available in SRA

Processed data are available on Series record

| NLM | NIH | GEO Help | Disclaimer | Accessibility |

Plagiarism report

Abhi July 2019

ORIGINALITY REPORT

22%	17%	7%	10%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	scholar.sun.ac.za Internet Source	2%
2	ici2013.org Internet Source	1%
3	Submitted to University of Stellenbosch, South Africa Student Paper	1%
4	www.biochem.mpg.de Internet Source	1%
5	Leisching, Gina, Ray-Dean Pietersen, Vuyiseka Mpongoshe, Carel van Heerden, Paul van Helden, Ian Wiid, and Bienyameen Baker. "The Host Response to a Clinical MDR Mycobacterial Strain Cultured in a Detergent-Free Environment: A Global Transcriptomics Approach", PLoS ONE, 2016. Publication	1%
6	pdfs.semanticscholar.org Internet Source	1%

Comparative Immunology, Microbiology and Infectious Diseases 67 (2019) 101355

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Comparison of human monocyte derived macrophages and THP1-like macrophages as *in vitro* models for *M. tuberculosis* infection

Abhilasha Madhvi[1,*], Hridesh Mishra[1], GR Leisching, PZ Mahlobo, B Baker[*]

DST-NRF Centre of Excellence for Biomedical Tuberculosis Research, South African Medical Research Council Centre for Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa

ARTICLE INFO

Keywords:
M. Tuberculosis
Host-pathogen interaction
Host-Response
Drug-susceptible
Drug-resistant

ABSTRACT

Macrophages are the preferential cell types to study various aspects of mycobacterial infection. Commonly used infection models for *in-vitro* studies are primary macrophages such as human monocyte derived macrophages (hMDMs) and macrophage like cell lines (THP-1). It is not clear if commercially available THP-1 cells can be used as hMDMs alternative for *in-vitro M.tb* infection experiments. We conducted a detailed investigation of the hMDM and THP-1 response to mycobacterial infection on a comparative basis and assess the most crucial aspects of infection which are most commonly studied. We assessed mycobacterial uptake and intracellular growth over time of a pathogenic drug-resistant and drug-susceptible *M.tb* strains (R179 and H37Rv) through colony forming units (CFUs). Both strains depicted similar uptake and intracellular growth in hMDMs and THP-1 macrophages over time (R179, $p = 0.954$) (H37Rv, $p = 0.922$). Cytotoxicity assays revealed a consistent viability up to day 16 post-infection across the strains in both THP-1 and hMDMs (R179, $p = 0.271$) (H37Rv, $p = 0.068$). Interestingly, both cell lines showed similar mycobacterial uptake and cellular viability in both susceptible as well as resistant *M.tb* strains. Cytokine/chemokine mRNA analysis through qPCR found no difference between cell types. Further, cytokine secretion measured through Luminex revealed no difference across the strains. Also, cytokine secretion analysis showed no difference in both cell lines across strains. In conclusion, our study shows that THP-1 and hMDMs bacterial uptake, viability and host response to drug-susceptible and drug-resistant mycobacterial infections are similar. Therefore, present study demonstrate that THP-1 cells are suitable substitutes for hMDMs for *in-vitro M.tb* infection experiments.

1. Introduction

Macrophages are the first line of defense against any invading pathogen [1] and play a key role in the elimination of mycobacteria. In susceptible individuals, macrophages provide a niche for its replication [2], and are therefore studied in-depth in an attempt to unravel the events at the host-pathogen interface early post-infection. Macrophages as models for mycobacterial infection studies are thus central to the advancement of the current understanding of host-pathogen interactions and are widely used amongst researchers in the field.

There are various macrophage models in use which include primary macrophages as well as macrophage cell lines to study *M. tuberculosis* (*M.tb*) infection. Primary macrophages include human monocyte derived macrophages (hMDMs) and mouse bone marrow-derived macrophages (BMDMs). There are a number of cell lines depicting macrophage models such as the human THP-1 and U937 and the murine RAW264.7 and J774 cell lines [3]. The advantage of using macrophages from *Mus musculus* is that there is substantially lower variability between mice when compared to the variability observed in *Homo sapiens* [4]. *Ex vivo* human macrophages as models for infection are however preferred, with variability between individuals being controlled for best by increasing sample size. hMDMs are isolated from the natural host and are comparatively easy to differentiate *in vitro* from human blood monocytes. However, to draw blood from humans, ethical permission is required which is often a lengthy process. In contrast, cell lines are homogenous, easy to proliferate and easier to maintain in the laboratory. There is the disadvantage that cell lines derived from transformed or immortalized cells have a tendency to be genetically unstable and may exhibit uncharacteristic/aberrant signaling mechanisms [5].

As mentioned previously, hMDMs are considered to be the first line of defense against mycobacterial infection due to their extensive role in stimulating immune response and playing an important role in

* Corresponding authors.
E-mail addresses: abhilasha@sun.ac.za (A. Madhvi), brubaker@sun.ac.za (B. Baker).
[1] Joint first authors.

https://doi.org/10.1016/j.cimid.2019.101355
Received 23 July 2019; Received in revised form 22 September 2019; Accepted 23 September 2019

activation of the adaptive immune response and tissue homeostasis [6]. THP-1 is a monocyte leukemia cell line from humans which differentiate into active macrophages after treatment with phorbol 12-myristate 13-acetate (PMA). This cell line has been used extensively to study monocyte/macrophage mechanisms, their regulatory functions, nutrient transport and signaling pathways [7].

Therefore, research comparing infection of *M.tb* to both cells (hMDMs and THP-1) are scarce. Also, whether they can be used as each other's alternative is unclear. Hence, in the present study, we are comparing hMDM and THP-1 response at various levels to mycobacterial infection. Here, for the first time we conduct a head to head comparison of both cell types using a drug-susceptible (H37Rv) and a drug-resistant strain (R179) of *M.tb* through bacterial uptake, host cell viability, mRNA expression level as well as cytokines secretion upon infection.

2. Methodology

2.1. Cells and culture medium

Human macrophage-like cells, THP-1 (ATCC-88081201), were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (Biochrome, Germany). The cells were incubated at 37 °C in a 5% CO_2 incubator. THP-1 cells were treated with a final concentration of 100 nM Phorbol 12-Myristate 13-Acetate (PMA; Sigma Aldrich, USA) for 48 h. In the present study, commercially available THP-1 like macrophages were used, experiments were carried out within 5 passages to avoid change of cellular properties [8]. Cells were in a healthy and stable morphological state. The morphology of cells was frequently examined under a microscope. Cytotoxicity analysis was also performed to study host cell viability.

For human monocyte derived macrophage cells, phlebotomy was performed on three healthy individuals (80 ml blood) after their written consent (in accordance with declaration of Helsinki) in Na-Heparin vacutainers. Ethical permission for the same had been obtained from the Ethics Committee, Stellenbosch University, Tygerberg campus, Cape Town (HREC Reference #S17/10/211). Healthy individuals were selected based on inclusion criteria such as no symptoms of tuberculosis including clear Chest X-Ray, night sweats and fever. Individuals on strong medication or any recent past surgery, pregnancy, anemia and insomnia were among the exclusion criteria. Whole blood from the recruited participants were further diluted with PBS (Sigma Aldrich, USA) in 1:1 ratio. This diluted ratio of blood and PBS was gently poured over the Histopaque (Sigma Aldrich, USA) layer and centrifuged at 804x*g* for 20 min. The buffy coat layer that appears between the blood plasma and the RBCs, containing the peripheral blood mononuclear cells (PBMCs) was collected in different tubes and washed twice with PBS. Total cells obtained was counted and proceeded for monocyte derived macrophages (MDM) isolation and differentiation.

Cells were cultured from human blood in Teflon jars using RPMI-1640 (Sigma Aldrich, USA) medium supplemented with 20% heparinized plasma and incubated at 37^{0}C, 5% CO_2 for 5 days. This allows the differentiation of monocytes into macrophages. The lids of the Teflon jars were tightened and placed on ice for 30 min. Cells were collected from Teflon jars using a Pasteur pipette. Each Teflon jar was washed and pooled into the same tube with cold RPMI-HEPES (Sigma Aldrich, USA) (4 ml per wash). The tubes were spun at 130×*g* for 10 min at 4 ºC with no brake. The pellets were re-suspended in RPMI-glut and counted. The required volume of heparinized plasma (20% final), 10% human serum and RPMI-Glut was added to the cell suspension and plated in 24 well culture plates. The cells were incubated for 2 h at 37 ºC with 5% CO_2 for adherence of MDMs.

2.2. Bacterial strains and infection conditions

Two pathogenic *M.tb* strains R179 (drug-resistant Beijing 220 clinical isolate) and H37Rv (drug-susceptible *M.tb* strain) were selected for infection. Mycobacteria were cultured in Middlebrook 7H9 (with 10% OADC and 0.5% glycerol) without Tween 80, as the detergent is known to affect macrophage uptake and the host response to *M.tb* [9]. For infection experiments, human macrophages as well as THP-1 cells were seeded in 24-well ultralow attachment surface culture plates at 0.35×10^6 cells per well. Both cell types were infected with the two pathogenic mycobacterial strains at a MOI = 2 using the "syringe settle filtrate" (SSF) method [10] and incubated four hours for bacterial uptake. A similar uptake (measured by CFUs) of the two strains was observed in both hMDMs and THP-1 cells. The cells were then washed with phosphate buffered saline (PBS) three times to remove any extracellular mycobacteria. Cells were incubated for an additional 20/92 h in complete RPMI medium (24/96 h in total depicting early and late response to infection). Uninfected hMDMs as well as THP-1 cells served as the control/uninfected samples.

2.3. RNA extraction

Total RNA from human macrophages and THP-1 cells were extracted using the RNeasy Plus Mini Kit (QIAGEN, USA) as per the manufacturer's instructions. The extraction was performed immediately following the 24- and 96 hs infection period. The 'gDNA eliminator' column included in this kit was used to remove genomic DNA in all samples. For each experiment, RNA quantity and quality were measured using Agilent 2100 Bioanalyzer. The RNA with a high RNA integrity Number (RIN) (≥9) was used for cDNA preparation prior to quantitative real time PCR experiments.

2.4. Quantitative qPCR

For cDNA preparation 0.5 µg RNA was converted using the Quantitect ^{R} Reverse Transcription Kit (QIAGEN, USA). To ensure the removal of genomic DNA, 'gDNA wipe-out buffer' was added to RNA (included in the kit) prior to the RNA conversion step. qPCR amplification was run on a LightCycler ^{R} 96 system (Roche, Germany). LightCycler^R 480 SYBR Green I Master was used for various differentially expressed genes using QuantiTect^R primer assays with 10 µl of reaction volume. The reference genes (hsUBC and hsGAPDH) were selected conferring to stable expression levels of known cytokines. The amplification process involves 45 cycles of 95 º C for 10 s followed by 60 º C for 10 s and finally 72 °C for 10 s. Gene expression fold-changes was computed for pathogenic infected and uninfected macrophages using calibrated normalized relative quantities using the equation $N = N_0 \times 2^{Cp}$. All qPCRs were done on RNA extracted from six different experiments. All biological replicates having a positive control and a non-reverse transcription control was run in triplicate (along with calibrator) as per the MIQE Guidelines [10].

2.5. Determination of bacterial uptake and viability

Infected cells were lysed using 0.1% Triton X-100. Bacterial uptake was determined by serial dilution (10^{-1}–10^{-4}) and plating out of mycobacteria onto 7H11 agar plates. The agar plates were incubated at 37 °C for 5 weeks and CFUs/ml was determined. Bacterial survival within the infected cells was monitored at 4 h and day 1 to day 16 post-infection respectively. Fresh media was replaced after every four days of the culture.

2.6. Cytotoxicity analysis

Both hMDMs and THP-1 like-macrophages were seeded in a 24 well plate with 0.35×10^6 cells/well. Cells were maintained for 16 days post-infection. Every set of cells including uninfected and infected cells were processed at 4, 8, 12, 14, and 16 days post-infection respectively. Fresh media was replaced at every four days of culture. We did not have

a concern about cellular confluence as THP-1 cells treated with PMA leads to maturation, differentiation and a very low rate of proliferation, hence we did not experience any issue with the cellular confluence during the 16 day experiment.

Cell cytotoxicity was tested with Roche WST-1 Cell Cytotoxicity Reagent (Roche, USA) in 1:10 dilution of WST-1 reagent to RPMI complete media. Cells were incubated for 1 h at 37 ° C and 5% CO_2. Absorbance was measured at 450 and 630 nm. The difference between the two absorbance readings was plotted in Excel as percentage values.

2.7. Luminex assay

Sample levels were evaluated using ThermoFisher Luminex kits 12plex PPX-12 (ThermoFisher Custom Procarta-12 Plex) on Bioplex platform (Bioplex™, BioRad Laboratories). Luminex assay was performed following ThermoFisher protocol and instructions. The assay was performed by a single technician where all samples were evaluated in duplicate. All analyte levels included in the kits which are a part of quality control reagents were within the expected ranges. Co-efficient of variation of these samples for duplicate runs did not vary a lot between analytes for both inter and intra plate. The variation range for duplicate runs was below 20% (5.2%–19.6% range). The standard curve for all samples ranged from 3.6 to 10000 pg/ml. Bioplex Manager Software version 4.1.1 was used for data analysis.

2.8. Statistical analysis

Real time qPCR data was analyzed using Light Cycler 96 SW 1.1 Software and Graph-pad Prism V7. Relative Expression of the cytokines was measured through the software in response to the Calibrator and non-transcription control. The relative expression data of the cytokines was further analyzed through Graph-pad prism to generate the p-values through One-Way ANOVA. The *p*-values were finally generated through Multiple Testing using Tukey corrections. The data (in triplicate) was finally plotted in histograms with respective mean and standard deviations. Cytotoxicity graphs and CFUs were plotted with an average of the technical triplicates leading to the mean of all the Biological replicates. Statistical analysis was performed through Graph-pad Prism V7 software where the percentage of every expressing cell was generated, and p value was calculated using One-Way ANOVA. Luminex data was analyzed by One-Way ANOVA using Graph-pad Prism V7 for Windows (Graph-pad Software, San Diego California, USA).

3. Results

3.1. Determination of colony forming units

Intracellular growth rates were determined by colony forming units per ml (CFUs/ml) over a period of 16 days (Fig. 1A and B) at 4 h post-infection, THP-1 cells were infected with 122,000 CFUs/ml of R179 and 131,000 CFUs/ml of H37Rv. Also, at 4 h post-infection, hMDMs were infected with 124,000 CFUs/ml of R179 and 132,000 CFUs/ml of H37Rv. CFUs were measured at day 0, 1, 4, 8, 12, 14- and 16-days post-infection. No difference in the mycobacterial uptake (measured by CFUs) was observed between the two cell types using R179 ($p = 0.954$) and H37Rv ($p = 0.922$). Also, there was no difference observed in mycobacterial uptake between drug-resistant R179 and drug-susceptible H37Rv *M.tb* pathogenic strains for both THP-1 ($p = 0.894$) and hMDMs ($p = 0.949$).

3.2. THP-1 and hMDM viability over-time post-infection

Cell viability was tested in both cell types which included uninfected and infected cells (Fig. 2). THP-1 cells had 90 and 88% viability at day 1 and 4 post-infection with R179, whereas had 93 and 91% viability at day 1 and 4 post-infection with H37Rv. Cell viability

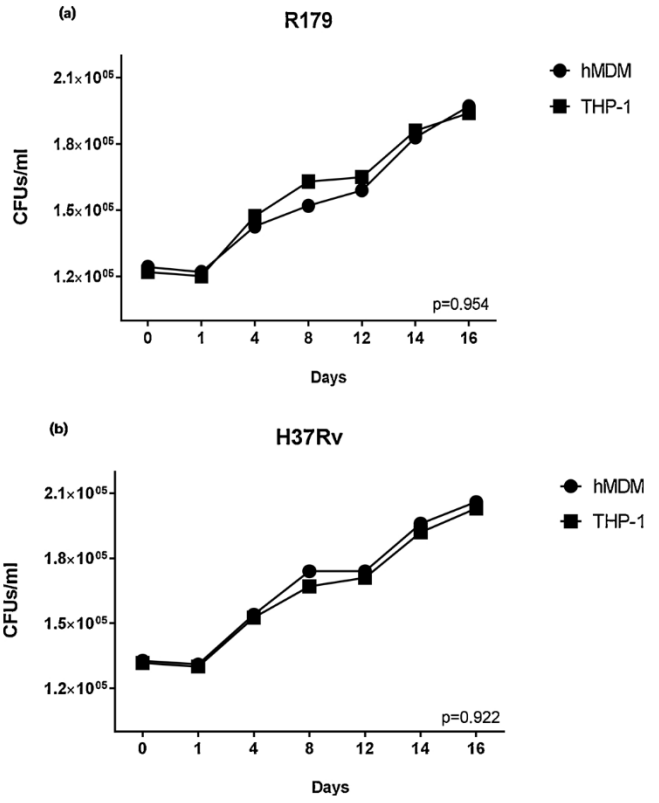


Fig. 1. Colony counts of M.tb from day 0 (at 4 h post infection) up-to day 16 post infection in hMDM and THP-1 cells (Fig. 1A and B). THP-1 cells and hMDMs are depicted with two different markers at different time points. Both culture models represent a similar trend with a gradual increase in CFUs/ml up-to day 16. The data represents the means of results from three different experiments and standard deviation of the means was calculated.

decreased slightly over time. On the other hand, hMDMs had 89 and 87% viability at day 1 and 4 post-infection with R179 and had 91 and 90% viability at day 1 and 4 post-infection with H37Rv, and like THP-1 cells, viability in hMDMs also decreased slightly over time. Thus, no significant difference in the cell viability was observed after infection with drug-susceptible and drug-resistant *M.tb* strains. It is noteworthy, that the cell viability was found similar across both cell types, indicating their life-span *in vitro* post-infection is similar, for both THP-1 and hMDMs (R179, $p = 0.271$) (H37Rv, $p = 0.168$) respectively. There was also no difference observed in viability between the susceptible H37Rv and resistant R179 *M.tb* strains for both THP-1 ($p = 0.221$) and hMDMs ($p = 0.647$).

3.3. Determination of cytokine/chemokine mRNA levels in THP-1 and hMDMs at 24 and 96 h post-infection

We assessed gene expression of the typical pro- and anti-inflammatory cytokines and chemokines induced after infection with M.tb. We did this through qPCR and assessed relative expression of the genes in uninfected and infected samples (Fig. 3a and b).

On studying relative expression of IL-6, we found that upon infection with R179, THP-1 and hMDMs had no difference at their mRNA level at both 24- and 96 hs post-infection. Upon infection with H37Rv, THP-1 cells showed higher expression at 24 h post-infection ($p = 0.036$), but no difference at 96 h post-infection.

IL-12 mRNA levels were similarly expressed at 24 h post-infection with R179 and H37Rv for both THP-1 cells and hMDMs. However, after 96 h of infection, hMDM showed relatively higher expression as compared to THP-1 cells. Upon infection with R179, hMDM had higher mRNA level expression ($p = 0.044$). hMDMs showed higher mRNA

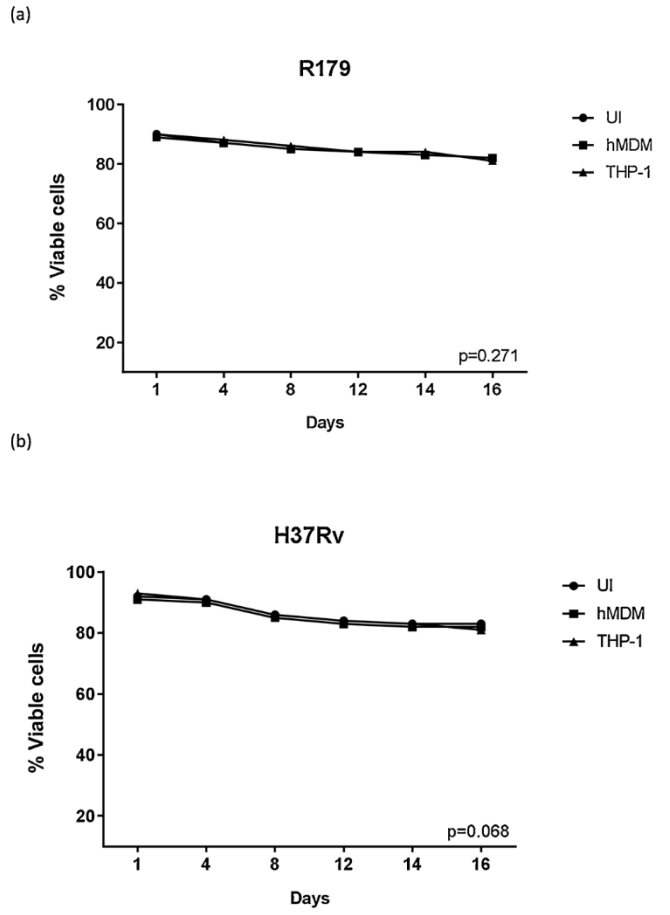


Fig. 2. Host cell viability upon infection with *M.tb* strains was measured through cytotoxicity assay from day 1 to day 16 post-infection. Histogram depicts an average of the uninfected hMDMs and THP-1 s along with infected hMDMs and THP1 cells. Fig. 2(A): Cytotoxicity analysis of cells infected with R179. Fig. 2(B): Cytotoxicity analysis of cells infected with H37Rv. The data represents the means of results from three different experiments and the bars represent standard deviation of the means.

expression than THP-1 cells upon infection with H37Rv ($p = 0.001$).

Next, we assessed the expression of the chemokines CCL2 and CCL5 upon infection. CCL2 showed no difference upon R179 infection at 24- and 96 hs post-infection. Relative expression of CCL2 in hMDMs infected with H37Rv was significantly high when compared to that of THP-1 cells at both 24- and 96 -hs post-infection (p-value < 0.001). The relative expression of CCL5 on the other hand had no difference between THP-1 cells and hMDMs after infection with both pathogenic strains at 24- and 96 -hs post-infection.

THP-1 cells and hMDMs had no difference in IL-1β expression at both time points across the strains. Interestingly, both cell types showed a strain-specific response after infection with H37Rv through significantly upregulating IL-1β expression. The trend of IL-1β mRNA expression was consistent in both cell types.

Both TNF-α and IFN-γ had increased expression after infection in both cell lines at both time points but did not show any significant differences and followed a similar level of expression. Finally, we measured the relative expression of IL-10 and observed that THP-1 cells and hMDMs had elevated levels of the mRNA throughout the 96-h infection period, whereas the THP-1 cells appeared to have an early response to infection after infecting with R179. Thereafter, expression remained similar. The relative expression of IL-10 which is a known anti-inflammatory cytokine had no major difference in the uninfected samples rather showed a significant difference in hMDM cells infected with H37Rv at 24 h when compared to THP-1 cells ($p = 0.024$). After studying relative expressions, we can conclude that there is no major difference observed in cytokines/chemokines mRNA levels between infected THP-1 and hMDMs at 24 and 96 h post infection. Although there were significant inter-strain differences in mRNA levels over-time, this did not contribute to overall significance.

3.4. Secreted cytokine levels between infected THP-1 and hMDMs at 24 and 96 h post-infection

Secreted cytokines measured using cell supernatant did not show any significant difference across the strains between the two cell lines. Luminex assay was performed and the secretory cytokines/chemokines was measured against their standards in duplicates for 24- and 96 -hs post-infection (Fig. 4). Five different cytokines including IL-6, IL-12, IL-1β, IFN-γ and TNF-α were measured through Luminex and the data was analyzed using Graph-pad Prism. The cytokines secretion had no difference when compared across the strains. It was interesting to note that all five cytokines did not show any significant difference at 24 h post-infection across the strains for THP-1 cells and hMDMs (R179, $p = 0.861$) (H37Rv, $p = 0.986$). The cytokines were also measured at 96 h post-infection, and still did not show any significant difference as late expressions across the strains for THP-1 and hMDMs (R179, $p = 0.765$) (H37Rv, $p = 0.826$). There was also no difference observed in cytokines secretion between the susceptible H37Rv and resistant R179 *M.tb* strains for both THP-1 ($p = 0.168$) and hMDM ($p = 0.272$) at 24 h post-infection and THP-1 ($p = 0.324$) and hMDM ($p = 0.296$) at 96 h post-infection.

4. Discussion

In the present study, we have used two types of macrophage cells (hMDMs and THP-1) to study host response towards *M.tb* infection, both these cell types are widely used to study associations at the host-pathogen interface [12]. We also focused on comparing a drug-susceptible and a drug-resistant *M.tb* strain (H37Rv and R179), hence to give a clear picture for type of infection and host response. We carefully selected six cytokines and two chemokines for monitoring mRNA expression level through qPCR (IL-6, IL-12, IL1β, IFN-γ, TNFα, IL-10, CCL2 and CCL5). We also measured five signature cytokines (IL-6, IL-12, IL1β, IFN-γ and TNFα) among the above eight for studying protein secretion through Luminex. The selected panel of cytokines and chemokines were reported to play specific roles in context of *M.tb* infection [13]. Importantly, in the present study, we have avoided any external stimulation, including cell isolation through bead separation, LPS or any cytokine stimulation such as IFN-γ, hence minimizing any alteration in surface receptors or cytokines expression, which was lacking in previous studies. There are studies which include activation factors for cellular differentiation and isolation. Bead separation or magnetic activated cell sorting methods for macrophage isolation and other chemicals-supplementation for macrophage differentiation and intracellular growth are used while reviewing variants of *M.tb* [14,15].

The main findings of the present study is as follow: 1) we found similar uptake of bacteria in both cell types (hMDMs and THP-1) at various time points (up to 16 days) post-infection, both cell types had similar response with drug-susceptible (H37Rv) and drug-resistant (R179) pathogenic *M.tb* strains, 2) the viability of both cell types were found to be similar at various time points (up to day 16) post-infection, 3) comparison of the mRNA expression level between the two cell types before and after infection (24 and 96 h), revealed no difference in signature cytokines (IFN-γ, IL-1β and TNF-α) and chemokine (CCL5), though some of the cytokines/chemokines differed across both cell types and 4) comparison of secreted cytokines levels before and after infection of both cell types, were found to be similar at both time points (24 and 96 h) across the strains.

Previous studies investigated the comparison of primary and secondary cell lines including blood macrophages and THP-1 like

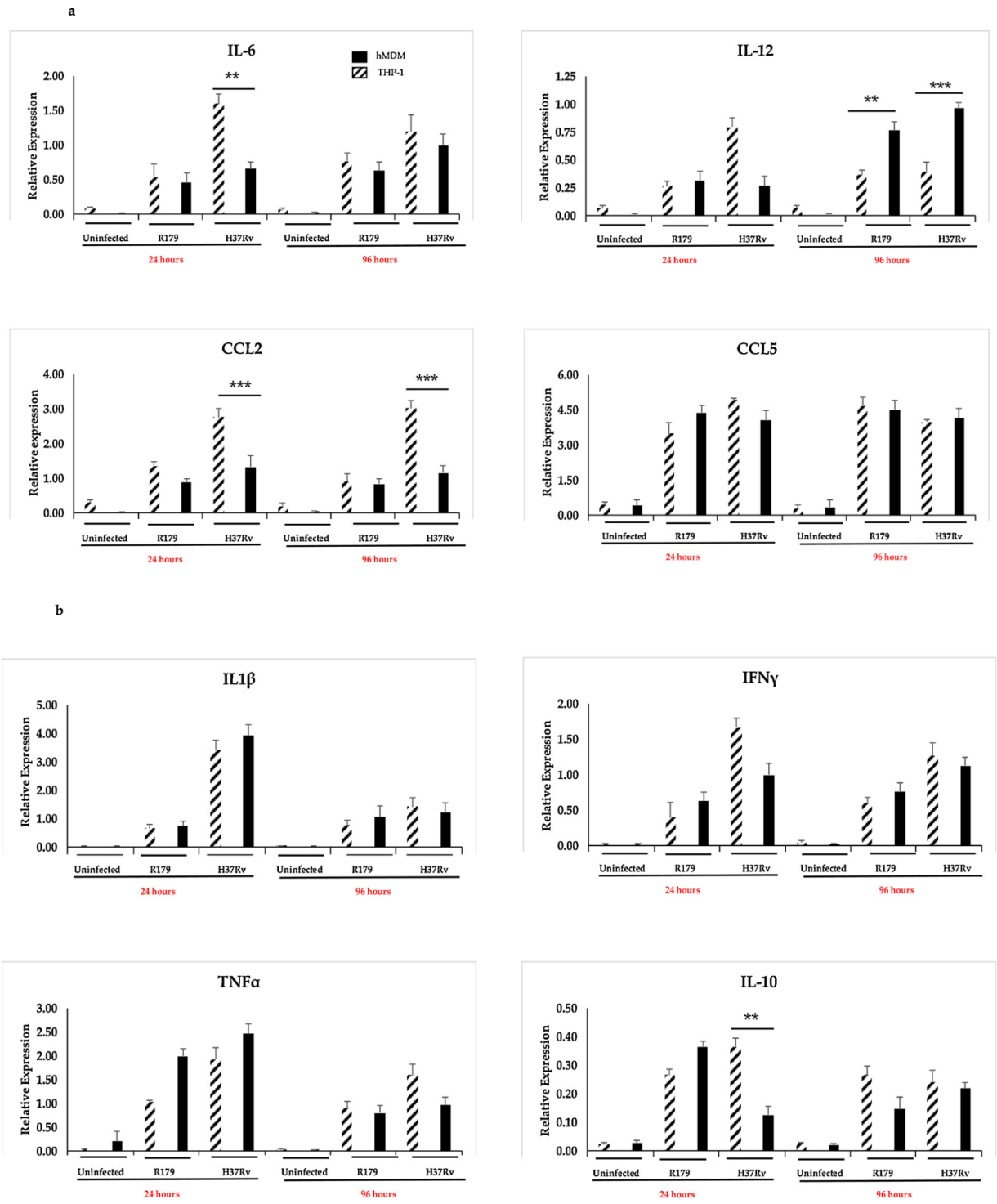


Fig. 3. (a&b): qPCR-based analysis of pro- and anti-inflammatory cytokines and chemokines in hMDM and THP-I cells after 24 and 96 h of infection with *M.tb*. Relative mRNA expression (fold change) of various cytokines and chemokines induced by human cells following infection with two *M.tb* strains (R179 and H37Rv) as analyzed through qPCR (n = 3). GAPDH and UBC used as reference genes. Standard deviation is shown by error bars. One-way-ANOVA with Tukey correction for multiple comparisons was used to determine p values depicted as (*) where ** = $p < 0.05$, *** = $p < 0.001$.

macrophages for bacterial uptake measured by colony forming units. They showed both primary and secondary macrophages CFUs were similar at resting phase *i.e.* without any activation or stimulation [1]. Another study showed no difference in bacterial uptake in THP-1 and hMDMs in a control state. But, upon stimulation with p19 (19-kDa *M. tuberculosis* glycoprotein), a significant reduction in CFU recovery was noted. This reduction was still similar in both the cell types indicating that there is minimal or no significant difference in *M.tb* infected hMDMs and THP-1 cells [16]. In the present study, we avoided any kind of stimulations that could alter surface receptors. Moreover, we performed head-to-head comparison of CFUs for hMDMs and THP-1 which showed similar CFUs at various time points (up to day 16 post-infection) for both susceptible and resistant mycobacterial strains (R179, p = 0.954 and H37Rv, p = 0.922). Results indicated, both cell types had similar bacterial uptake (similar CFUs), this has useful implications, as THP-1 cells can be used as an alternate to human blood macrophages

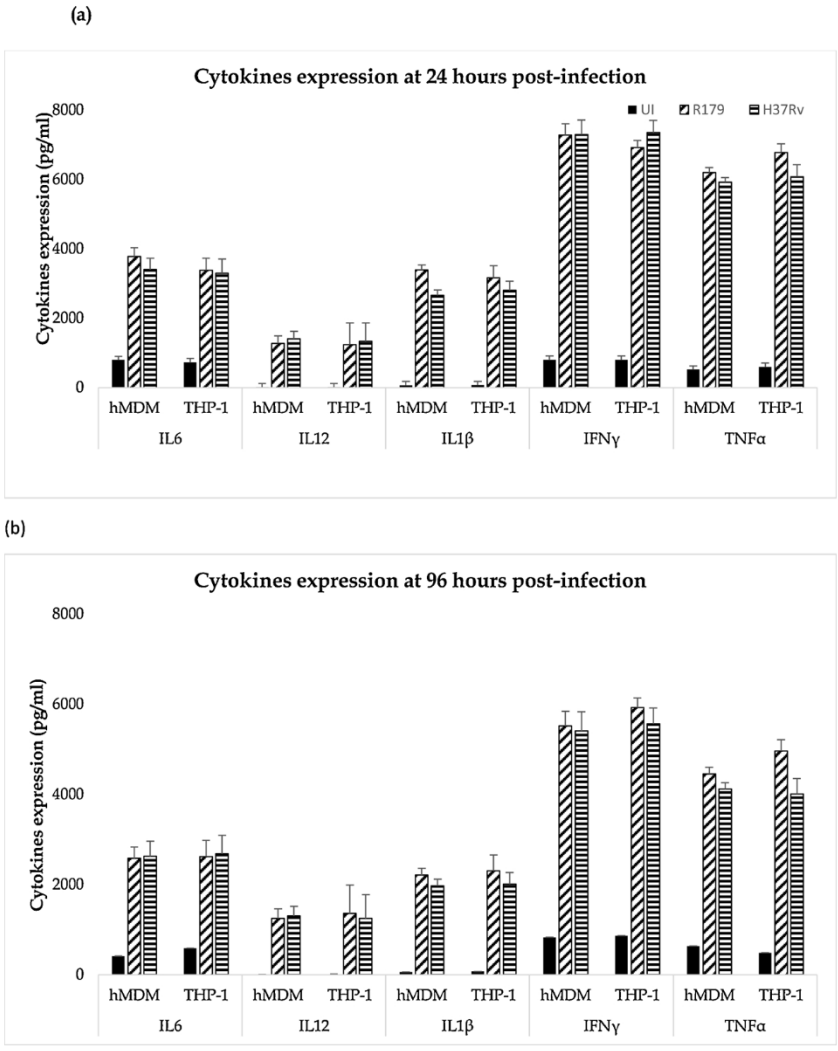


Fig. 4. (a&b): Cytokines secretion through Luminex. Luminex was performed in both cell lines hMDMs and THP-1 s upon 24 and 96 h post-infection with H37Rv and R179 *M.tb* strains. The data represents the means of results from three different experiments and the bars represent standard deviation of the means.

in *in-vitro* experiments encompassing *M.tb* infection (either susceptible or resistant).

As tissue macrophages originating from circulating mononuclear monocytes do not need to self-renew, hence the hMDMs do not proliferate *in-vitro* [16]. Similarly, treating THP-1 cells with PMA leads to maturation, differentiation and a very low proliferation rate [17–19]. In the present study, we cultured hMDMs and THP-1 cells up-to day 16 post-infection to study the intra-cellular bacterial growth and the host cell viability. During the 16 days of the experiments no extracellular bacterial growth was detected by regular visual inspections using the microscope. Fresh media was replaced after every 4 days of culture. Luminex assay was performed at 24- and 96 -hs post-infection (without any media replacement), hence not effecting the cytokine secretion into the cell supernatant during this period.

In the present study, host cell viability of both cell types analyzed by a cell cytotoxicity assay proved to be informative for *in-vitro M.tb* infection experiments. It was essential to study the uninfected and infected cell viability over time post-infection. Both cell types at MOI of 2 had $\leq$85% viability up to day 8 post-infection and gradually decreased by day 16 following the same trend. Previous study showed difference in cell viability in the two cell lines but at higher MOI (10 and 100). They reported that upon *M.tb* infection, hMDMs had higher cell death compared to THP-1 at MOI of 10 and 100. But, they also showed that at MOI of 1, there was no significant change in the cytotoxicity between the two cell lines [21]. Present study agrees with the results of the previous study, and the viability of both cell types are similar over 16 days post-infection, indicating both cell types have similar survivability under infection.

We determined the host response of both cell types by quantifying mRNA expression by qPCR and determination of secreted levels of cytokines and chemokines using Luminex. qPCR is considered to have high sensitivity and accuracy for gene expression quantification [22]. We carefully selected a panel of six cytokines and two chemokines which are known to play significant role in *M.tb* infection.

We found three cytokines (IFN-γ, IL1β, and TNFα) and one chemokine (CCL5) to be non-significant between the two cell types at two time points (24 and 96 h post-infection) across both strains. IFN-γ, is a known signature cytokine in *M.tb* infection and regarded as crucial cytokine to fight against *M.tb* [23,24]. IFN-γ showed a similar gene expression by qPCR in both cell types after infection with both susceptible and resistant strains. Also, we found similar level of secreted IFN-γ (detected by Luminex assay) from both cell types across susceptible and resistant strains, hence, confirming the qPCR findings. It is important to note that IFN-γ consistently remained similar in both cell types, indicating a similar host response from both cell types.

IL-1β did show an early response at 24 h in both the cell types upon H37Rv infection, though similar in both cell types. The expression reduced at later time point (96 h post-infection) and consistently remained similar in both cell types. The secreted levels of IL-1β, determined by luminex assay was found to be similar in both cell types at

24- and 96 hs post-infection. IL-1β is regarded as crucial cytokine for survival against *M.tb* infection, previous studies have shown that IL-1β knock-out mice were more susceptible to *M.tb* infection [25,26]. Other important cytokine was TNF-α, which is believed to be responsible in apoptosis of *M.tb* infected cells *in-vitro* [27,28]. Further, secreted levels and mRNA expression of TNF-α was also found to be similar in both cell types at 24 and 96 h, these results are in agreement with a the previously published study [1].

We also measured IL-6, which was shown to be regulated by *M.tb* to inhibit type I interferon signaling and, consequently, disease progression in TB [29]. It was also reported that IL-6 secreted after infection of macrophages with *M.tb* inhibits the responses of uninfected macrophages to IFN-γ [30]. In the present study, the mRNA levels of IL-6 was found to be significantly higher in THP-1 cells as compared to hMDMs at 24 h post-infection with H37Rv, though the expression level were found to be similar after 96 h. This indicated an early response in THP-1 cells after infection with susceptible *M.tb* (H37Rv). The expression level of IL-6 was found to be similar in both cell types at 24 and 96 h after infection with R179. Since, the secreted levels of IL-6 measured through luminex was found to be similar at both time points across both *M.tb* strains, this indicated that even though the mRNA levels were higher in THP-1 cells at 24 h, the secreted levels of IL-6 were still found to be similar.

We measured IL-12 which is known to have agonist and protective role in mycobacteria infection [31,32]. We found significantly higher IL-12 in hMDMs when compared with THP-1 cells across the two pathogenic strains at 96 h. On the other hand, mRNA levels of IL-12 was found to be similar at 24 h. IL-12 is known as an essential marker for survival after *M.tb* infection [13]. But, alike IL-6, we found similar secreted levels of IL-12 from both cell types at 24 and 96 h post infection with susceptible and resistant *M.tb* strains, indicating similar host response of both cell types.

Previous study shows the relation of IL-10 with *M.tb* infection. *M.tb* infected THP-1 cells is known to induce IL-10 gene expression [33]. We, therefore measured IL-10, a known anti-inflammatory cytokine where THP-1 had higher expression upon H37Rv infection at 24 h when compared with hMDMs, however, there was no difference at later time point (at 96 h). The cytokine secretion of IL-10 was found to be similar for both cell types at 24 and 96 h after infection with R179.

A known chemokine CCL2 showed higher expressions in THP-1 cells at both early (24 h) and late (96 h) post H37Rv infection. However, the expression level of CCL2 after infection with R179 was found to be similar for both cell types at 24 and 96 h. CCL2 is known to maximize and organize early macrophages in the lungs, which was strongly depicted by THP-1 cells as compared to hMDMs. Chemokine CCL5 showed similar mRNA expression throughout (24 and 96 h post-infection) in both the cell types across the two strains. CCL5 is speculated to enhance macrophage *M.tb* killing and facilitate early dendritic cell accumulation in the lymph node [13].

An overall similar host response (measured by cytokines/chemokines) at mRNA levels across the two pathogenic strains in both cell types was observed. There are some minor variations in mRNA levels, particularly after infection with susceptible strain (H37Rv), but this did not result in any variation observed in mycobacterial uptake, cellular viability as well as host response towards cytokines secretion. Host response towards mRNA expression was found to be consistently similar after infection with resistance strain R179 as compared to the susceptible H37Rv.

Overall, we observed a similar trend in both the cell types without any notable significant differences between the two. The results therefore validate the utility of THP-1 cells to study *M.tb* infection and are comparable to the hMDMs response to infection, *i.e.* THP-1 cells behave like native human monocyte derived macrophages with regards to the parameters measured in this study. As an in-depth study to relate the physiological functions exhibited by these cells, THP-1 cells prove to be a valuable model exploring macrophage specific genes. Due to their similarity and relatively similar behavior with native cells, they have proven to be a valuable model for macrophage differentiation mechanisms.

Our study has some limitations, we only used two pathogenic *M.tb* strains. Though our motive was to compare both susceptible and resistant strains, we will compare more strains in future studies. Also, we have measured the CFUs and viability of cells up to day 16, thus, we cannot conclude what happens to bacterial uptake, viability and host response at later time points. Moreover, we are fully aware that we should investigate other host response signatures (with broad panel of cytokines and chemokines).

We therefore conclude that both cell types, *i.e.* hMDMs and THP-1 have shown similar bacterial uptake (measured by CFUs), cellular viability and similar host response signature biomarkers to both drug-susceptible (H37Rv) and drug-resistant (R179) mycobacterial infection.

Authors contributions

AM, HM and BB designed the experiments. AM, GL and ZM performed the experiments. AM and HM analyzed the data. AM, HM, BB and GL wrote and edited the manuscript.

Declaration of Competing Interest

The authors declare that no conflict of interest exists.

Acknowledgements

We thank Carine Kunsevi-Kilola for her assistance in hMDM differentiation and isolation.

This work was supported by DST-NRF Centre of Excellence for Biomedical Tuberculosis Research; South African Medical Research Council Centre for Tuberculosis Research; Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa.

References

[1] E. Mendoza-Coronel, M. Castañón-Arreola, Comparative evaluation of in vitro human macrophage models for mycobacterial infection study, Pathog. Dis. 74 (2016), https://doi.org/10.1093/femspd/ftw052.

[2] H. Clay, J.M. Davis, D. Beery, A. Huttenlocher, S. Lyons, L. Ramakrishnan, Dichotomous role of the macrophage in early Mycobacterium marinum infection of the zebrafish, Cell Host Microbe. 2 (2007) 29–39, https://doi.org/10.1016/j.chom.2007.06.004.

[3] B.K. Johnson, R.B. Abramovitch, Macrophage infection models for Mycobacterium tuberculosis, Methods Mol. Biol. Clifton NJ. 1285 (2015) 329–341, https://doi.org/10.1007/978-1-4939-2450-9_20.

[4] Michael A. Grippi, Jack A. Elias, Jay A. Fishman, Robert M. Kotloff, Allan I. Pack, Robert M. Senior, et al., Fishman's Pulmonary Diseases and Disorders, Fifth, AccessMedicine, McGraw-Hill Medical (Internet), 2008 (cited 2018 Mar 27). Available from: https://accessmedicine.mhmedical.com/book.aspx?bookID=1344.

[5] A. Frattini, M. Fabbri, R. Valli, E. De Paoli, G. Montalbano, L. Gribaldo, et al., High variability of genomic instability and gene expression profiling in different HeLa clones, Sci. Rep. 5 (2015) 15377, https://doi.org/10.1038/srep15377.

[6] J. Jantsch, K.J. Binger, D.N. Müller, J. Titze, Macrophages in homeostatic immune function, Front. Physiol. 5 (2014), https://doi.org/10.3389/fphys.2014.00146.

[7] W. Chanput, J.J. Mes, H.J. Wichers, THP-1 cell line: an in vitro cell model for immune modulation approach, Int. Immunopharmacol. 23 (2014) 37–45, https://doi.org/10.1016/j.intimp.2014.08.002.

[8] R.W. Stokes, D. Doxsee, The receptor-mediated uptake, survival, replication, and drug sensitivity of Mycobacterium tuberculosis within the macrophage-like cell line THP-1: a comparison with human monocyte-derived macrophages, Cell. Immunol. 197 (1999) 1–9, https://doi.org/10.1006/cimm.1999.1554.

[9] G. Leisching, R.-D. Pietersen, I. Wiid, B. Baker, Virulence, biochemistry, morphology and host-interacting properties of detergent-free cultured mycobacteria: an update, Tuberc. Edinb. Scotl. 100 (2016) 53–60, https://doi.org/10.1016/j.tube.2016.07.002.

[10] G. Leisching, R.-D. Pietersen, V. Mpongoshe, C. van Heerden, P. van Helden, I. Wiid, et al., The host response to a clinical MDR mycobacterial strain cultured in a detergent-free environment: a global transcriptomics approach, PLoS One 11 (2016), https://doi.org/10.1371/journal.pone.0153079.

[12] S. Dühring, J. Ewald, S. Germerodt, C. Kaleta, T. Dandekar, S. Schuster, Modelling the host–pathogen interactions of macrophages and Candida albicans using Game

Theory and dynamic optimization, J. R. Soc. Interface 14 (2017), https://doi.org/10.1098/rsif.2017.0095.
[13] R. Domingo-Gonzalez, O. Prince, A. Cooper, S.A. Khader, Cytokines and chemokines in Mycobacterium tuberculosis infection, Microbiol. Spectr. 4 (2016), https://doi.org/10.1128/microbiolspec.TBTB2-0018-2016.
[14] K. Menck, D. Behme, M. Pantke, N. Reiling, C. Binder, T. Pukrop, et al., Isolation of human monocytes by double gradient centrifugation and their differentiation to macrophages in teflon-coated cell culture bags, J. Vis. Exp. JoVE. (2014), https://doi.org/10.3791/51554.
[15] M.J. Fultz, S.A. Barber, C.W. Dieffenbach, S.N. Vogel, Induction of IFN-gamma in macrophages by lipopolysaccharide, Int. Immunol. 5 (1993) 1383–1392.
[16] M. López, L.M. Sly, Y. Luu, D. Young, H. Cooper, N.E. Reiner, The 19-kDa Mycobacterium tuberculosis protein induces macrophage apoptosis through toll-like Receptor-2, J. Immunol. 170 (2003) 2409–2416, https://doi.org/10.4049/jimmunol.170.5.2409.
[17] F.K. Swirski, I. Hilgendorf, C.S. Robbins, From proliferation to proliferation: monocyte lineage comes full circle, Semin. Immunopathol. 36 (2014) 137–148, https://doi.org/10.1007/s00281-013-0409-1.
[18] H. Schwende, E. Fitzke, P. Ambs, P. Dieter, Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3, J. Leukoc. Biol. 59 (1996) 555–561.
[19] T. Starr, T.J. Bauler, P. Malik-Kale, O. Steele-Mortimer, The phorbol 12-myristate-13-acetate differentiation protocol is critical to the interaction of THP-1 macrophages with Salmonella typhimurium, PLoS One 13 (2018) e0193601, https://doi.org/10.1371/journal.pone.0193601.
[21] P. Bettencourt, N. Carmo, D. Pires, P. Timóteo, E. Anes, Mycobacterial Infection of Macrophages: The Effect of the Multiplicity of Infection, in: A. Méndez-Vilas (Ed.), Antimicrobial Research: Novel Bioknowledge and Educational Programs, Formatex Research Center, Badajoz, Spain, 2017, pp. 651–664.
[22] R.D. Canales, Y. Luo, J.C. Willey, B. Austermiller, C.C. Barbacioru, C. Boysen, et al., Evaluation of DNA microarray results with quantitative gene expression platforms, Nat. Biotechnol. 24 (2006) 1115–1122, https://doi.org/10.1038/nbt1236.
[23] J. Lee, H. Kornfeld, Interferon-γ regulates the death of M. Tuberculosis-infected macrophages, J. Cell Death 3 (2010) 1–11.
[24] I.Y. Nikitina, A.V. Panteleev, E.V. Sosunova, N.L. Karpina, T.R. Bagdasarian, I.A. Burmistrova, et al., Antigen-specific IFN-γ responses correlate with the activity of M. Tuberculosis infection but are not associated with the severity of tuberculosis disease, J. Immunol. Res. 2016 (2016) 7249369, https://doi.org/10.1155/2016/7249369.
[25] K.D. Mayer-Barber, B.B. Andrade, D.L. Barber, S. Hieny, C.G. Feng, P. Caspar, et al., Innate and adaptive interferons suppress IL-1α and IL-1β production by distinct pulmonary myeloid subsets during Mycobacterium tuberculosis infection, Immunity 35 (2011) 1023–1034, https://doi.org/10.1016/j.immuni.2011.12.002.
[26] K.D. Mayer-Barber, D.L. Barber, K. Shenderov, S.D. White, M.S. Wilson, A. Cheever, et al., Caspase-1 independent IL-1beta production is critical for host resistance to mycobacterium tuberculosis and does not require TLR signaling in vivo, J. Immunol. 2010 (184) (1950) 3326–3330, https://doi.org/10.4049/jimmunol.0904189.
[27] J. Keane, M.K. Balcewicz-Sablinska, H.G. Remold, G.L. Chupp, B.B. Meek, M.J. Fenton, et al., Infection by Mycobacterium tuberculosis promotes human alveolar macrophage apoptosis, Infect. Immun. 65 (1997) 298–304.
[28] J. Keane, H.G. Remold, H. Kornfeld, Virulent Mycobacterium tuberculosis strains evade apoptosis of infected alveolar macrophages, J. Immunol. 1950 (164) (2000) 2016–2020.
[29] A.N. Martinez, S. Mehra, D. Kaushal, Role of interleukin 6 in innate immunity to Mycobacterium tuberculosis infection, J. Infect. Dis. 207 (2013) 1253–1261, https://doi.org/10.1093/infdis/jit037.
[30] V. Nagabhushanam, A. Solache, L.-M. Ting, C.J. Escaron, J.Y. Zhang, J.D. Ernst, Innate inhibition of adaptive immunity: mycobacterium tuberculosis-induced IL-6 inhibits macrophage responses to IFN-gamma, J. Immunol. 2003 (171) (1950) 4750–4757.
[31] A.M. Cooper, A.D. Roberts, E.R. Rhoades, J.E. Callahan, D.M. Getzy, I.M. Orme, The role of interleukin-12 in acquired immunity to Mycobacterium tuberculosis infection, Immunology 84 (1995) 423–432.
[32] C. Hölscher, R.A. Atkinson, B. Arendse, N. Brown, E. Myburgh, G. Alber, et al., A protective and agonistic function of IL-12p40 in mycobacterial infection, J. Immunol. 2001 (167) (1950) 6957–6966.
[33] X. Wang, Y. Wu, J. Jiao, Q. Huang, Mycobacterium tuberculosis infection induces IL-10 gene expression by disturbing histone deacetylase 6 and histonedeacetylase 11 equilibrium in macrophages, Tuberculosis 108 (2018) 118–123, https://doi.org/10.1016/j.tube.2017.11.008.