Molecular Mechanism of Adaptation of *Mycobacterium tuberculosis (M.tb)* in Mesenchymal Stem Cells (MSCs)

Thesis submitted to Jawaharlal Nehru University
for the award of the degree of

DOCTOR OF PHILOSOPHY

SAMREEN FATIMA

Special Centre for Molecular Medicine
Jawaharlal Nehru University
New Delhi-110067

2019

जवाहरलाल नेहरू विश्वविद्यालय
Jawaharlal Nehru University

Date: 15th July, 2019

CERTIFICATE

This is to certify that the work embodied in this thesis entitled **"Molecular Mechanism of Adaptation of *Mycobacterium tuberculosis* (*M.tb*) in Mesenchymal Stem Cells (MSCs)"** has been done by **Ms. Samreen Fatima** at Special Centre For Molecular Medicine, Jawaharlal Nehru University, New Delhi for the degree of Doctorate of Philosophy. To the best of my knowledge, this work is original and no part of this thesis has been submitted for any other degree or diploma to any other university.

Prof. Gobardhan Das
Supervisor,
Special Centre for Molecular Medicine
Jawaharlal Nehru University
New Delhi-110067

Gobardhan Das
Professor
Special Centre for Molecular Medicine
Jawaharlal Nehru University
New Delhi-110067

Prof. Gobardhan Das
Chairperson,
Special Centre for Molecular Medicine
Jawaharlal Nehru University
New Delhi-110067

Prof. Gobardhan Das
Chairperson
Special Centre for Molecular Medicine
Jawaharlal Nehru University
New Delhi-110067

Samreen Fatima
Ph.D. student,
Special Centre for Molecular Medicine
Jawaharlal Nehru University, New Delhi- 110067

जवाहरलाल नेहरू विश्वविद्यालय
Jawaharlal Nehru University

Date: th July, 2019

CERTIFICATE

This is to certify that the work embodied in this thesis entitled **“Molecular Mechanism of Adaptation of *Mycobacterium tuberculosis* (*M.tb*) in Mesenchymal Stem Cells (MSCs)”** has been done by **Ms. Samreen Fatima** at Special Centre For Molecular Medicine, Jawaharlal Nehru University, New Delhi for the degree of Doctorate of Philosophy. To the best of my knowledge, this work is original and no part of this thesis has been submitted for any other degree or diploma to any other university.

Prof. Gobardhan Das
Supervisor,
Special Centre for Molecular Medicine
Jawaharlal Nehru University
New Delhi-110067

Prof. Gobardhan Das
Chairperson,
Special Centre for Molecular Medicine
Jawaharlal Nehru University
New Delhi-110067

Samreen Fatima
Ph.D. student,
Special Centre for Molecular Medicine
Jawaharlal Nehru University, New Delhi- 110067

जवाहरलाल नेहरू विश्वविद्यालय

Jawaharlal Nehru University

Declaration

I hereby declare that the research work embodied in this thesis entitled **"Molecular Mechanism of Adaptation of *Mycobacterium tuberculosis (M.tb)* in Mesenchymal Stem Cells (MSCs)"** has been carried out by me under the supervision of Dr. Gobardhan Das at Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi, India

Samreen Fatima
PhD student
Special Centre For Molecular Medicine
Jawaharlal Nehru University
New Delhi-110067
Date: th July 2019

Dedicated to my family..

Contents

Chapters
Page No.

Acknowledgement a

Abbreviation List c

List of Figures and Tables g

Chapter 1: Introduction 1-4

Chapter 2: Review of literature 5-48

2.1 Tuberculosis: The Global Epidemic. 6

2.2 History of tuberculosis 8
(a) Infection and inflammation
(b) NF-kB signaling pathway and its role in infection

2.3 The Pathogen: Mycobacterium tuberculosis 11

2.3.1 Morphology of *M.tb*

2.3.2 Culture and growth conditions of *M.tb*

2.3.3 *M.tb* Genome

2.3.4 Pathogenesis of *M.tb*

2.3.5 Granuloma Formation

2.4 Tuberculosis Epidemiology 19

2.4.1. Epidemiology: Global perspective

2.4.2 Epidemiology: Indian Scenario

2.5 Diagnosis of tuberculosis and treatment 23

2.5.1 Diagnosis

2.5.1.1 Radiologic studies

2.5.1.2 AFB smear microscopy and culture

2.5.1.3 Molecular methods

2.5.1.4 Drug resistant *M.tb* identification based assay

2.5.1.5 Monteux test

2.5.1.6 IFN-γ release assay

2.5.2 Treatment of tuberculosis

2.5 Challenges of tuberculosis control and cure 30

2.6 Emergence of drug resistant tuberculosis 31
2.7 Latency and Persistence in *M.tb* 32
2.8 Mesenchymal Stem Cells: Discovery and characteristics 35
(a) Discovery of MSCs
(b) Characteristics of MSCs
2.9 Immunological phenotype and function of MSCs 39
2.10 Role of stem cells in tuberculosis and emergence of drug resistance 40
2.11 Role of autophagy in tuberculosis 42
2.12 Quiescence in stem cells 44
2.13 Role of lipids in tuberculosis infection 45
2.14 Role of cytokines and chemokines in *M.tb* infection 46
Chapter 3: Rational of the Study and Objectives 49-52
Chapter 4: Materials and Methods 53-64
4.1 Ethics Statement 54
4.2 Reagents 54
4.3 Animals 54
4.4 Mesenchymal Stem Cell (MSC): Culture and Characterization 55
4.5 Bacterial Culture 55
4.6 *M.tb* infection and estimation of Colony Forming Units (CFU) 56
4.7 Flow Cytometry 56
4.8 Peripheral Blood Mononuclear Cell (PBMC) isolation from blood and differentiation to macrophages 57
4.9 Transcriptomic and data analysis 57
4.10 Real-Time PCR 58
4.11 Confocal Microscopy 58
4.12 Transmission Electron Microscopy (TEM) 59
4.13 Western Blot 59
4.14 Statistical Analysis 59
Chapter 5: Results 65-103
5.1 Culture and characterization of Mesenchymal Stem Cells (MSCs) 66
5.2 *M.tb* (H37Rv) infects MSCs and macrophages differentially 73

5.3 *M.tb* resides in different cell compartments after internalization in MSCs and macrophages 75
5.4 MSCs shield the harbored bacteria in lipid droplets by the activation of lipid synthesis pathway 78
5.5 MSCs and macrophages are differentially programmed to support dormant and active form of *M.tb* respectively 83
5.6 MSCs induce a quiescent state upon infection with *M.tb* and remain viable for an extended period 87
5.7 *M.tb* hides in lipid droplets to attain dormancy in MSCs which is reversed upon inhibition of lipid synthesis 91
5.8 *M.tb* replication inside MSCs is regulated by autophagy and *M.tb* in MSCs become non-responsive to isoniazid in a time dependent manner 93
5.9 Dormant *M.tb* in MSCs is reactivated to active replicating bacteria upon immunosuppression 97
5.10 Secretory mediators or cytokines secreted by MSCs upon infection by *M.tb* 101

Chapter 6: Discussion 104-112

Chapter 7: Summary 113-115

References 116-138

Publication 139-140

Acknowledgment

My work has come to fruition due to the liberal cooperation of several individuals. First of all, I would like to express my heartiest thanks to my supervisor Prof. Gobardhan Das, whose brilliant guidance and constant support encouraged me throughout this study. From the initial struggling days of my project till the end of my research tenure, his affectionate supervision, constant encouragement, intellectual input and critical appreciations have enriched my understanding of the subject and helped me to develop an analytical outlook. I am profoundly grateful to him.

I also owe my thanks to the Vice-chancellor of Jawaharlal Nehru University, Prof. M. Jagadesh Kumar for providing such high-quality research facilities. I express my sincere thanks to Dr. Sujata Mohanty, AIIMS, New Delhi for her kind cooperation and valuable suggestions regarding my work. I am greatly indebted to her for providing me the human mesenchymal stem cells for my research work. I would also like to thank Dr. Priyanka Pandey, NIBMG, Kolkata for helping me with the analysis of RNAseq data.

I would like to express my gratitude to the Animal House Staff of ICGEB who helped in the maintenance of mice and conducting all mice experiments.

I am especially thankful to Dr. Ved Prakash Dwivedi and Dr. Debapriya Bhattacharya for all the help and support they offered at different phases of my work. I am also grateful to all my lab mates for their great company. I would also thank my batchmates, Kirti, Pragya, Mona and Sudhir. I would also extend my thanks to my friends outside JNU, Sadia, Pallavi, Harika, Zainab, Aamir, Gaurav and Nitin for their constant support and encouragement.

This work would not have been possible without blessings of Almighty and the unconditional love and patience of my family; especially my parents, to whom this thesis is dedicated. I would like to express my heart full gratitude to my family who has always stood firmly beside me and encouraged me throughout the endeavor. I have to give a special mention for the support by my mother, Mrs. Naushaba Tauqir and my father, Mr. Asad Kamal, My Mamu, Mr. Tanveer Hasan and Mr. Khursheed Anwer for never being less than my parents in standing beside me. Without them, I would never have been where I am today. I would also thank my Khalu, Dr. Moshahid Alam Rizvi for being my mentor and guiding me at every single step.

I admit my inability to express my emotions through words for my brothers, Ashar Kamal, Fahad Kamal, Hammad Rizi and Saad Rizvi and my little sisters Zaina and Orooj, for showering me with so much of love and their concerns. Last but not the least I would also like to thank my Nani, Muani, two loving Khala and my entire family for their countless blessings. I would also like to thank my best friend, Mr. Aman Gulfam for being there with me all the time and lending me the confidence and support that I will always remember and feel blessed for.

At last but most importantly, I am indebted and grateful to the financial help from the University Grant Commission (UGC), Government of India, in form of Senior Research Fellowship.

Samreen Fatima

Abbreviations and Acronyms

AFB	Acid- Fast Bacilli
AIDS	Acquired Immunodeficiency Syndrome
APC	Allophycocyanin
APCs	Antigen presenting cells
BC	Before Christ
BCG	Bacillus Chalmette Guerin
BM	Bone marrow
BMRC	British Medical Research Council
CD 8+	Cluster of Differentiation Antigen 8
CD	Cluster of Differentiation Antigen
CD105	Cluster of Differentiation Antigen 105
CD14	Cluster of Differentiation Antigen 14
CD29	Cluster of Differentiation Antigen 29
CD34	Cluster of Differentiation Antigen 34
CD4+	Cluster of Differentiation Antigen 4
CD44	Cluster of Differentiation Antigen 44
CD45	Cluster of Differentiation Antigen 45
CD73	Cluster of Differentiation Antigen 73
CD90	Cluster of Differentiation Antigen 90
CFU	colony forming unit
CFU-F	Colony Forming Unit-Fibroblast
CT	Computed tomography
DCs	Dendritic cells
DMEM	Dulbecco Modified Essential Medium
DNA	deoxyribonucleic acid
DosR	Dormancy survival regulator
DOTS	Directly Observed Treatment Short course

E. coli	Escherichia coli
EEA1	Early endosomal autoantigen 1
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
GI	Gastrointestinal
HIV	Human Immuno-deficiency virus
HLA	Human leucocyte antigen
HSCs	Hematopoietic stem cells
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon gamma
IL-10	Interleukin-10
IL-6	Interleukin 6
IP-10	Interferon Gamma induced Protein 10
INH	Isoniazid
Inh	Isoniazid
IP-10	IFN-gamma-inducible protein 10
ISCT	International Society for Cellular Therapy
LED	Light emitting diode
LFA-1	Leukocyte function-associated antigen- 1
LM	Lipomannan
LPA	Line probe assay
M.tb	Mycobacterium tuberculosis
ManLAM	Mannose-capped lipoarabinomannan
MCM-2	Minichromosome maintenance-2
MCP-1	Monocyte chemoattractant protein 1
MDR	Multiple drug resistant
mm	Millimeter

μm	Micrometer
MOI	Multiplicity of infection
MSCs	Mesenchymal Stem Cells
MTD	Mycobacterium tuberculosis Direct Test
mTOR	Mammalian target of rapamycin
NAA test	Nucleic acid amplification testing
NF-kB	Nuclear factor kappa B
NK	Natural killer cells
NLRs	Nucleotide-binding oligomerization domain (NOD) like receptors
NS	Non-significant
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PC	phosphatidylcholine
PCNA	Proliferating cell nuclear antigen
PE	Phosphatidylethanolamine
PE	Phycoerythrin
PE	Proline glutamate
PECAM-1	Platelet/endothelial cell adhesion molecule
PPARγ2	Peroxisome proliferation-activated receptor 2
PPE	Proline-proline-glutamate
qPCR	Real time polymerase chain reaction
RNA	Ribonucleic acid
RNAseq	RNA sequencing
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RR-TB	Rifampin resistant tuborculosis
S.E.M	Standard Error Mean

SKP2	S-phase kinase 2
SNPs	Single Nucleotide Polymorphism
T7SS	Type VII secretion system
TAG	Triacylglycerol
TAM	Tyro3/Axl/Mer
TB	Tuberculosis
TDR	Totally drug-resistant
TGFβ	Transforming growth factor
tgs1	Triacylglycerol synthase 1
Th1	T helper cells-1
Th2	T helper cells-2
TLRs	Toll-like receptors
TNF-KO	Tumor Necrosis Factor Knock out
TNF-α.	Tumor necrosis factor alpha
USA	United States of America
VCAM-1	Vascular cell adhesion molecule -1
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
XDR	Extensively drug resistant
XXDR	Extensively extremely drug-resistant

List of Figures and Table

List Of Figures

Chapter 2

Figure 1:	Predictable rates of TB cases worldwide	7
Figure 2:	Scanning Electron Micrograph (SEM) of *Mycobacterium tuberculosis*	12
Figure 3:	The diagrammatic representation of the *M.tb* genome	14
Figure 4:	Pathogenesis of tuberculosis	17
Figure 5:	Representation of the percentage of new TB cases with MDR-TB in 2012	22
Figure 6:	The Venn-diagram indicating 30 countries with huge TB burden.	22
Figure 7:	Diagrammatic representation of the Cornell model of latent tuberculosis	34

Chapter 4

Figure 1:	Image of Bone Marrow derived Mesenchymal Stem Cells (BM-MSCs) at passage 3 adhered to the surface of 100mm culture dish	70
Figure 2:	CFU-F assay for the estimation of self-renewal capacity of BM-MSCs.	70
Figure 3:	Positive expression of mesenchymal stem cell markers (CD105, CD 73, CD90 and CD 29) and negative expression of hematopoietic markers (CD34/45, HLA-I and HLA-II) in BM-MSC at passage 3.	71
Figure 4:	Image showing the differentiation of hMSCs into osteocytes (Alizarin Red Staining).	71
Figure 5:	Image showing the differentiation of hMSCs into chondrocytes (Alcian Blue Staining).	72
Figure 6:	Image showing the differentiation of hMSCs into adipocytes (Oil Red O Staining).	72
Figure 7:	Growth Kinetics of *M.tb* in BM-MSCs and PBMC derived macrophages at different time points in a time-course experiment.	74
Figure 8:	Confocal microscopy images of macrophage and MSCs infected with *M.tb*-GFP at 72 hours post infection	74
Figure 9:	Confocal microscopy images of M.tb-GFP infected macrophages with M.tb localizing in the early endosome immediately after infection (0 hour).	76

Figure 10:	Confocal microscopy images of *M.tb*-GFP infected MSCs with *M.tb* localizing in the cytosol immediately after infection	76
Figure 11:	Graphical representation of percentage colocalisation of M.tb with endosomes (Rab5) and cytosol (Phalloidin) in both MSCs and macrophages (THP-1)	77
Figure 12:	Heat map showing differential regulation of genes involved in sphingolipid synthesis pathway.	80
Figure 13:	Confocal microscopy images showing co-localization of M.tb-GFP with lipid bodies stained with LipidTox in macrophages (THP-1) and MSCs at 72 hrs post infection.	80
Figure 14:	Graphical representations of the mean intensity of lipid bodies stained with LipidTox in macrophages (THP-1) and MSCs post infection with M.tb-GFP at 72 hours	81
Figure 15:	Graphical representation of the percentage co-localization of M.tb-GFP with lipid bodies in both macrophages (THP-1) and MSCs	81
Figure 16:	Graphical representation depicting increasing mean intensity of lipid bodies stained with LipidTox in MSC from 0 hours upto 120 hours.	82
Figure 17:	Transmission Electron Microscopy (TEM) images of MSCs infected with M.tb, 72 hrs post-infection	82
Figure 18:	Graphical Representation depicting relative expression of dormancy genes of *M.tb* within MSCs and macrophages	85
Figure 19:	Graphical representation depicting relative expression of replicative genes of *M.tb* in MSCs and macrophages	85
Figure 20:	Graphical representation depicting relative expression of dormancy genes of M.tb in CD45-Sca1+ MSCs sorted from bone marrow of infected mice as compared to log phase bacteria	86
Figure 21:	Graphical representation depicting relative expression of replicative genes of M.tb in CD45+CD11b+ macrophages sorted from lungs of infected mice as compared to MSCs from the bone marrow	86
Figure 22:	Heat map showing the relative expression fold of cell proliferation and quiescence genes in uninfected and *M.tb* infected MSCs at 48 and 96 hrs post infection.	88

Figure 23:	Graphical representation of validation of relative expression of cell proliferation and quiescence genes in MSCs as compared to uninfected control at 72 hours.	89
Figure 24:	Validation of relative expression of cell proliferation and quiescence genes in macrophages as compared to uninfected control at 72 hours	89
Figure 25:	Western blots showing forkhead signaling pathway from uninfected and M.tb infected MSCs at 96 hours.	90
Figure 26:	Graphical Representation of densitometry data of the western blot of forkhead signaling pathway.	90
Figure 27:	Graphical representation of the relative expression of dormancy genes of M.tb in infected MSCs treated with or without lipid inhibitor, Triacsin C (0.05 μM) at 72 hrs post infection	92
Figure 28:	Graphical representation of the relative expression of replicative genes of M.tb inside MSCs treated with or without Triacsin C (0.05 μM) compared to macrophages at 72 hours post infection	92
Figure 29:	Heat map showing the relative expression of autophagy pathway genes in uninfected and M.tb infected MSCs at 48 hrs and 96 hrs post infection.	94
Figure 30:	Growth kinetics of M.tb in macrophages (PBMC-derived) either infected alone with M.tb and/or treated with rapamycin or isoniazid (10 μg/ml) and isoniazid + rapamycin	95
Figure 31:	Growth kinetics of M.tb in MSCs either infected alone and/or treated with rapamycin (1 μM for 3 hrs before infection), isoniazid (10 μg/ml) and isoniazid + rapamycin.	95
Figure 32:	M.tb burden in lungs isolated from mice treated with or without isoniazid, rapamycin or isoniazid+rapamycin.	96
Figure 33:	Schematic representation of the reactivation experiment	96
Figure 34:	*M.tb* reactivation in lungs isolated from mice treated with isoniazid and isoniazid+rapamycin followed by dexamethasone treatment	97
Figure 35:	Graphical representation depicting relative expression of replicative genes of *M.tb* from bone marrow of isoniazid-treated mice compared to infected control	98

Figure 36:	Graphical representation depicting relative expression fold of dormancy genes of *M.tb* from bone marrow of isoniazid-treated mice compared to infected control	99
Figure 37:	Graphical representation depicting relative expression of replicative genes of *M.tb* from lungs of isoniazid-treated mice compared to infected control	99
Figure 38:	Graphical representation depicting relative expression of dormancy genes of *M.tb* from lung of isoniazid-treated mice compared to infected control	100
Figure 39:	Graphical representation depicting relative expression of replicative genes of *M.tb* from bone marrow of dexamethasone treated mice compared to INH control	100
Figure 40:	Graphical representation depicting relative expression of dormancy genes of *M.tb* from bone marrow of dexamethasone treated mice compared to INH control.	101
Figure 41:	Cytokines secreted upon *M.tb* infection in MSCs compared to macrophages (THP-1) cells.	103

List Of Tables

Table 1	List of primers used in the study	60
Table 2	List of materials used in the study	62

CHAPTER 1
Introduction

Tuberculosis (TB) is a fatal disease which has been inflicting humankind since time immemorial. This deadly disease is spread by the bacilli, known as *Mycobacterium tuberculosis* (*M.tb)* and is transmitted from one person to the other by aerosol droplets containing the bacterium, which are inhaled by a healthy person and reach the alveoli of the lungs. TB causes nearly two million deaths annually throughout the world (WHO, 2017). Of the individuals infected by TB, only 5-10% develops active TB and the remaining, 90-95% of the individuals mount an effective immune response and successfully eliminate the majority of the replicating bacteria. However, a minor population of bacteria which evades host immune response survives in an asymptomatic condition known as dormant or latent tuberculosis (Flynn and Chan, 2001). Upon the advent of perturbation of the immune response such as HIV co-infection, diabetes or malnutrition, the individuals may become prone to reactivation of the disease (Gomez and McKinney, 2004). Only 5-10 percent of latently infected individuals develop active disease during their lifetime whereas 30 percent of HIV co-infected individuals develop active TB (Gomez and McKinney, 2004). One- third of the living population is surviving with the latent form of TB which is an enormous reservoir, waiting for the catastrophe that impairs immunity and may lead to a pandemic. Directly Observed Treatment Short course (DOTS) therapy, the only treatment of TB available, consists of multiple antibiotics, is lengthy and possesses severe toxicity and side effects (Shah et al., 2007). This treatment involves the administration of isoniazid (INH), rifampicin, pyrazinamide, and ethambutol for the initial two months, followed by an additional four months treatment with isoniazid and rifampicin for regular TB. However, treatment of drug-resistant TB takes even longer time, often upto 24 months.

Interestingly, DOTS treatment eliminates the majority of the bacteria within a month of treatment, and patients become free of clinical symptoms. Once patients, especially in less fortunate and underdeveloped countries, start feeling better, they discontinue the medications which are the major cause of generation of drug-resistant TB (Caminero et al., 2010). Incomplete treatment may also leave persistent bacteria that may be drug sensitive but unresponsive to drugs (Velayati, 2006), which may also convert to drug-resistant forms in due course of time. Almost all countries, irrespective of their socio-economic status, are under threat from different drug-resistant forms of TB (Caminero et al., 2010). There are still no substantial explanations for the unresponsiveness of these persisting organisms to antibiotics. Current antibiotic therapy is mostly focused on eliminating replicating *M.tb* organisms while dormant bacteria which are metabolically inactive and non-replicating continue to persist by being unresponsive to drugs. Macrophages are the natural host for *M.tb*, where they replicate and survive by employing a variety of host-evasion mechanisms that include inhibition of phagolysosomal fusion (Ponpuak et al., 2010; Armstrong et al., 1971; Gomez, J.E. et al., 2004; Fabri et al., 2011), translocation to the cytosolic compartment (Rahman et al., 2014; van der Wel et al., 2007; Jamwal et al., 2016) and preventing acidification of lysosomal compartments (Sturgill-Koszycki et al., 1994). These replicating bacteria are sensitive to antibiotics and are readily eliminated within a short period of treatment. On the other hand, non-replicating bacteria survive within granulomatous structures and persist therein in latent non-replicating form until the advent of a compromised immune state. Recently, we and others have shown that mesenchymal stem cells (MSCs) make a niche of fine balance of latent TB and host immune response in the granulomatous lesion

(Raghuvanshi et al., 2010). The shield of MSCs which play a dual role in *M.tb* pathogenesis, eliminating the majority of the *M.tb* organism by releasing nitric oxide (NO) intermediates and at the same time protecting the bacteria from infiltrating T-cells by suppressing the T-cell response. It is well documented that MSCs have immunomodulatory characteristics and secrete cytokines which regulate the microenvironment in the host tissue. (Sordi et al., 2005). Following the work of Raghuvanshi et al, 2010, other groups elucidated the importance of MSCs in TB control and in the emergence of drug resistance and persistence (Das et al., 2013; Garhyan et al., 2015). However, it is still not well discerned how *M.tb* bacteria shield itself from the host immune response using MSCs as harboring sites. Moreover, the mechanism employed by MSCs in favoring the survival of *M.tb* and establishment of the persisting dormant population which are unresponsive to drugs is largely unknown.

Here, we have tried to study the mechanism of molecular adaptation of *M.tb* in MSCs. We establish that MSCs are the natural host for dormant *M.tb*. Upon uptake by MSCs, *M.tb* induces the expression of dormancy regulon genes in MSCs which drives the bacteria towards dormancy and simultaneously induces quiescence in the host cells. In contrast, *M.tb* residing in macrophages continues to replicate and causes macrophage necrosis. MSCs employ the process of autophagy to eliminate the latent bacteria residing in the cell and a combination of conventional drugs of the DOTS therapy and known mTOR inhibitor, rapamycin, which induces autophagy, may help in the elimination of both active and latent TB populations and prevent reactivation of the disease.

CHAPTER 2

Review of literature

2.1 Tuberculosis: The Global Epidemic

Tuberculosis (TB) is a deadly human infectious disease caused by obligate intracellular pathogen *Mycobacterium tuberculosis.* This disease principally affects the lungs (Bloom, 1994; Zaman, 2010). Despite its existence from more than 70000 years it still is the cause of high mortality in the modern world (Mac Donald et al., 2015). According to the World Health Organization (WHO) report, 10.4 million new cases and 1.8 million deaths have been registered from TB each year (WHO report, 2017). Most of the new cases (approximately 3 million) are not reported, and a major number is devoid of proper government programs and treatments (Bloom, 2017). Based on the site of infection, TB is characterized into two classes- pulmonary TB and extrapulmonary TB. Pulmonary TB is a contagious infection that primarily infects lungs while extrapulmonary TB involves organs other than the lung. The organ systems most commonly affected by extra-pulmonary TB are the "gastrointestinal (GI) system", "central nervous system", skin, liver, and bone. Most TB infections generally do not result in symptomatic disease outcome rather they remain asymptomatic causing latent TB. Out of the total individuals infected only one-tenth develop active TB disease. The remaining 90% of individuals successfully hold the infection either by the elimination of bacteria or by inducing the dormant state of *M.tb*. One of the major challenges of TB is that the bacteria may persist in individuals in a latent state for several years and can be reactivated to active disease upon induction of immune-compromised conditions like HIV co-infection. Symptoms of pulmonary TB include chronic cough, blood containing sputum, fever, night sweats and weight loss (Houban et al., 2016). People who are suffering from HIV/AIDS or those who smoke are more likely to get active TB owing to their weak immune system. Due to

the complex mechanism of infection and progression, TB has always been a health burden.

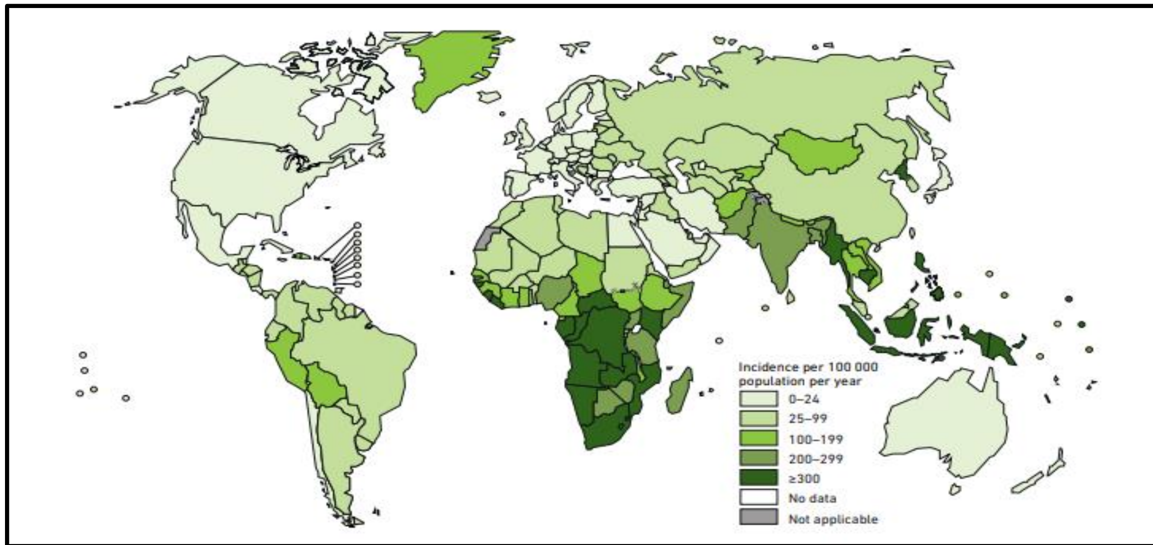

Figure 1. Predictable rates of TB cases worldwide (WHO Report, 2018).

The major concern with the prevalence of TB is that it affects poor and developing countries of South East Asia and Africa including some parts of Latin America (Frieden et al., 2003). In 2017, the proportion of an annual number of incident TB cases compared to the total strength of the population was different in the countries. Less than 10 cases for each 100 000 of the population has been reported in developed countries, between 150 to 400 in the countries with high rates of TB infection **(Figure 1)**, which crosses 500 individuals in the underdeveloped countries including South Africa (WHO Report, 2017). After the emergence of HIV-TB coinfection and drug resistance, the cases of TB re-emergence have increased the threat due to TB many folds, which mostly affects the developing nations.

2.2 History of tuberculosis

Tuberculosis has been known to infect mankind since ancient times but the word tuberculosis was first coined in 1839 by J. L. Schöenlein. The "genus" Mycobacterium is assumed to have evolved in the history of the world approximately 150 million years before (Daniel, 2006). Early ancestors of Mycobacterium tuberculosis (*M.tb*) may have evolved simultaneously with primitive humans around 3 million years ago mostly in the region of East Africa. The individual species belonging to the "*M.tb* complex" are the descendants of a common *M.tb* progenitor population which supposedly evolved 15000-30000 years back (Gutierrez et al., 2005). The remains of pre-historic human population and mummies of Egypt also have been reported to contain the DNA of *M.tb* bacilli (Zink et al., 2003). Due to preserving nature of hard tissues like bone, bone TB could be identified by observing bone deformities in individual who died 4000 years ago. Before modernization and the industrial revolution, it was a common belief that tuberculosis is associated with vampires; as the death of a member due to TB led to others also losing their health. The domestication of cattle which started 25000 years back also may have contributed to the infection of mycobacterium from animals to the human-beings (Stead et al., 1997*)* It was hypothesized that *M.tb* may have evolved from *Mycobacterium bovis (M.bovis)* which is behind the emergence of TB in livestock. Later, the genetic study of Mycobacterium tuberculosis complex infecting humans and in animals suggested that although they are evolutionarily connected but are not transferred from cattle to the human population. Members of Mycobacterium species share 99.9% similarity but are differentiated by the presence of rare single nucleotide polymorphisms (SNPs). SNPs study also suggested that *M.tb* and *M.bovis* have evolved simultaneously and that *M.tb*

and *M.bovis* may have evolved from originator species which may be similar to *M.canetti* (Mostowy et al., 2005).

The *M.tb* bacilli were initially described in 1882 by the German scientist, Robert Koch, who later in 1905 received the noble prize in physiology and medicine for his breakthrough study. To memorate Koch's discovery, 24th March is celebrated as world Tuberculosis Days worldwide (Koch, 1882). In the 20th century introduction of *Mycobacterium bovis* as BCG vaccine and development of the antibiotic streptomycin played a crucial role in combating the disease. BCG vaccine was developed in 1921, by Albert Calmette and Camille Guerin which is still the only available vaccine. The BCG vaccine was first tested in 1921 on humans in France. Getting successful immunization BCG vaccination achieved widespread success in the US, Britain, and Germany after World War II. BCG vaccine works only for preventing childhood TB but does not provide protection against adult TB. In 1946, streptomycin, the first antibiotic against TB, was developed (MRC, 1948). Streptomycin antibiotic proved to be very effective against TB treatment and cure. Before the emergence of antibiotics, the only way to cure TB was surgical intervention like "pneumothorax technique" in which infected lung was collapsed to rest and the lesion was allowed to heal (Luca and Mihaescu, 2013).

Major landmark discovery occurred when effective medications started showing significant control over TB virulence. Long after the discovery of antibiotics sulfonamide and penicillin, Selman Waksman's and his group in 1944 identified the new antibiotic Streptomycin and in parallel, Jorgen Lehman synthesized para-aminosalicylic acid. Interestingly both these medication showed effective therapy against TB. Due to the less availability of streptomycin, the British Medical Research Council (BMRC) did the first

clinical trial using both antibiotics either alone or in combinations. The results showed that the combined effect is far better than the effect of single antibiotics. This further attracted researchers to work on a different combination. In 1952 the introduction of triple therapy which includes isoniazid in combination with streptomycin and para-aminosalicylic acid resulted in a cure of 90-95% patients but it required up to 24 months for complete treatment. The next major landmark in the history of TB cure was the introduction of rifampicin. Combination of rifampicin, isoniazid, streptomycin, and ethambutol resulted in a predicted cure of more than 95% patients in 8-9 months. Inclusion of pyrazinamide with rifampicin and isoniazid reduced the treatment time to 6 months (Smith et al., 2013; Nguyen, 2016)

2.2.1 DOTS treatment

DOTS treatment was first started in 1980 by Karel Styblo of the International Union against TB & Lung Disease. This treatment focused on giving intermittent regimens, twice or thrice weekly for over a period of 6 months. DOTS resulted in a steady decline of TB with an average of 7.8% per year in the USA. Globally DOTS has been recommended by the "World Health Organization" with some modifications and known as Directly Observed Treatment Short-course. In spite of its efficacy, DOTS has some limitations like the method used for diagnosis is based on microscopy of sputum samples using Ziehl-Neelsen staining and emergence of drug-resistant *M.tb* (Mitchison, 1986; Pablos-Me´ndez et al., 1998; Espinal et al., 2001).

2.3 The Pathogen: *Mycobacterium tuberculosis*

Mycobacterium tuberculosis belongs to the family Mycobacteriaceae. It was earlier known as tubercles bacilli and was first described by Robert Koch. *M.tb* is one of the members of complex that has nine members *M. tuberculosis, M. africanum, M. canetti, M. bovis, M. caprae, M. microti, M. pinnipedii, M. mungi,* and *M. orygis,* also known as "*Mycobacterium tuberculosis* complex" (MTC). But among all members, *M.tb* is the most pathogenic to humans as it cannot survive outside the host and has no environmental reservoirs (van Ingen et al., 2012).

2.3.1 Morphology of *M.tb*

M.tb is non-motile, slender and different from other bacteria in terms of its structure and growth rate. It appears as thin rods either alone or in groups in the form of threads **(Figure 2)**. N-glycolyl muramic acid is present instead of N- acetyl muramic acid in the cell wall of the bacteria, which renders it incompetent for taking gram staining making it difficult for it to be characterized as gram positive or gram negative. For its identification, other modified strain such as acid-fast Ziehl-Nielsen or auramine can be used. When seen under the microscope they appear as rod-shaped, curved in appearance and wrapped onto each other owing to the occurrence of fatty acid in their cell wall (Cudahy et al., 2016). The bacilli are 1-10 µm long and 0.2-0.6 µm wide. It may appear singly as well as in the form of threads.

2.3.2 Culture and growth conditions of *M.tb*

M.tb is a strictly aerobic organism and can grow on solid media as well as liquid media. It can be cultured in the laboratory in liquid media (7H9), egg-based, Lowenstein-Jensen or agar-based media, 7H11. Its growth rate is very slow in comparison to other bacteria. Their doubling time is 15-20 hours which is much higher compared to the bacteria like *E.coli* due to which colonies take a long time (approximately 21 days) to grow on the 7H11 plate. Its characteristic ability to generate catalase enzyme and niacin makes it stand different from the other bacteria.

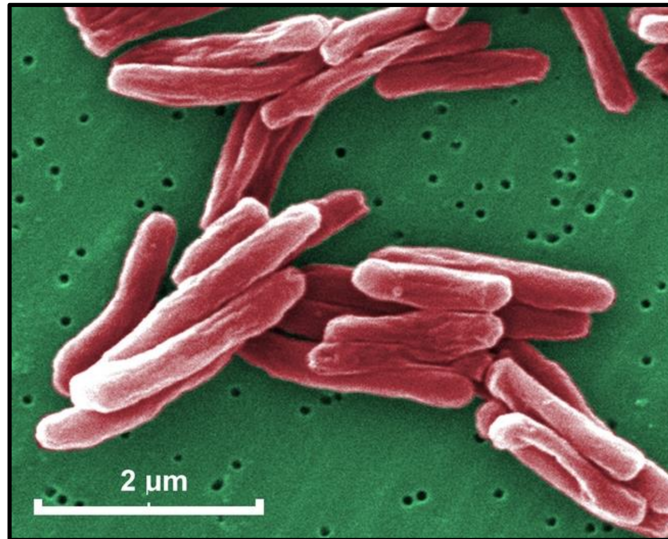

Figure 2. "Scanning Electron Micrograph (SEM)" of *Mycobacterium tuberculosis* (Image adapted from- CDC/Janice Haney Carr)

2.3.3 *M.tb* Genome

The genome of *M.tb,* H37Rv is 4.4 X 10^6 base pair long which codes for approximately 4000 genes (Cole et al., 1998). These genes are annotated to be involved in different process and pathways which include enzymes for lipid metabolism, cell wall synthesis, detoxification and many regulatory proteins (Bentley et al., 2002). Apart from that, it

harbors infectivity genes which are known as effector proteins. *M.tb* uses dedicated secretion systems known as type VII secretion system or ESX family secretion systems which code for different effectors protein based on which, the fate of *M.tb* inside host is decided; whether it will evade the host immune response or resist and persist inside the host cells **(Figure 3)** (Pym et al., 2003; Hsu et al., 2003). ESX is a special system that helps in the export of selectable proteins through the thick cell wall of *M.tb*. There are total five ESX systems explored in *M.tb* namely, ESX-1, ESX-2, ESX-3, ESX-4 and ESX-5 and out of the minimum three are necessary for virulence (Stanley et al., 2003). ESX-1 plays an indispensable part in providing resistance to *M.tb* from the host immune system and in "immune-evasion" mechanisms. It also has an important role in rupture of phagosomal compartments which eventually releases bacteria and its product into cytoplasm initiating a cascade of events related to immune response and host-pathogen interactions (Sasseti et al., 2013). Apart from ESX-1, ESX3 is known for iron acquisition and ESX5 is known to home of two conserve protein family, namely, PE (proline glutamate) and PPE (Proline-proline-glutamate) (Bottai et al., 2009; Abdullah et al., 2011; Houben et al., 2012). The term ESX and T7SS can be used interchangeably. Other types I- VI secretion system have already been studied in details in other bacterial systems but little knowledge is known about type VII secretion system (T7SS) of Mycobacterium which needs further exploration. Based on these studies it has been hypothesized that T7SS plays a role in maintaining cell envelope integrity, physiology, in the process of conjugation and in host-pathogen interactions. (Shah et al., 2016).

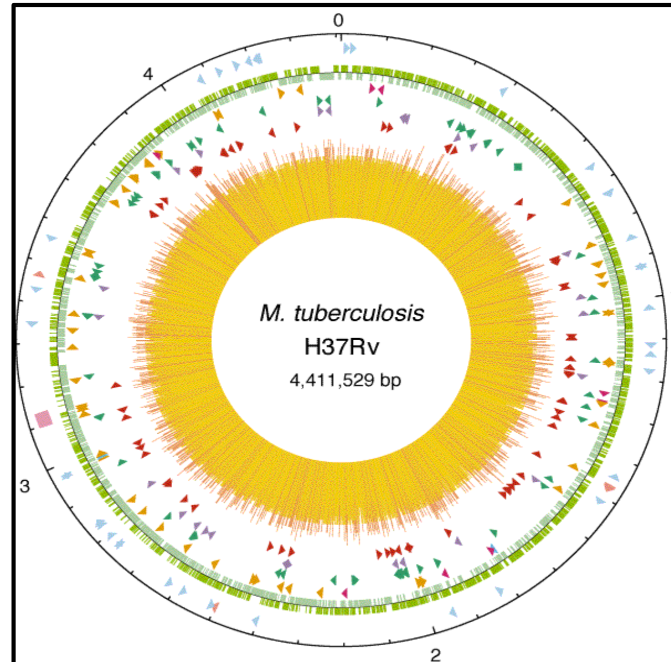

Figure 3. The diagrammatic representation of the *M.tb* genome (Adapted from Cole ST, Nature, 1998)

2.3.4 Pathogenesis of *M.tb*

M.tb has evolved to become a fully active human pathogen. Like other members of the group, mycobacterium is not able to survive outside a human host. It's only known reservoir is the human body. To survive inside human cells it has developed strategies to subvert and co-adapt with the human defense mechanisms. Being an intracellular pathogen, it persists within phagosome by modifying and seizing the phagolysosomal pathway. The prime target of *M.tb* is the human respiratory system where it infects the lungs and resides in the alveolar passages. The bacteria are inhaled into the lungs via the respiratory tract in the form of airborne droplets (Gupta and Chatterjee, 2005). Extrapulmonary TB affects between 10 to 25% of TB affected individuals' worldwide (Bloom and Small, 1998). TB is transmitted through aerosol droplets containing TB

bacteria which are expelled by patients with active TB. Alveolar macrophages engulf these bacilli containing droplets and recruit other cells of the immune system to form a granulomatous lesion of the lung to contain the bacteria within (Shoenfeld and Rose, 2005). Alveolar macrophages support the immediate killing of the bacilli to an extent; by utilizing different bactericidal mechanisms, like the generation of reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) (Kaufmann, 2001). The remaining bacteria reside in the lungs and replicate and initiate an inflammatory response by the action of various cytokines and chemokines (Algood et al., 2003; Flynn and Chan, 2005). The site of infection is invaded by macrophages and lymphocytes leading to granuloma formation, the hallmark of TB (Sandor et al., 2003). The "granuloma" is the form of host defense to fight the *M.tb* infection and eradicate the bacilli by the attack of immune cells. However, the bacterium finds shelter in the granuloma and persists in a latent state, often for decades (Silva-Miranda, 2012).

After entering into the lungs *M.tb* is identified by various arms of innate immune response like TLRs (Toll-like receptors), NOD-like receptors (NLRs), and C-type lectins. TLR2 and TLR4 initially recognize the *M.tb* cell surface ligands that include lipoprotein, glycoprotein, lipomannan (LM) and mannose-capped lipoarabinomannan (ManLAM) and starts the pro-inflammatory response by activating NF-kB pathway as a host defense. Activation of NF-kB results in the production of various pro-inflammatory cytokines like TNF-α, IL-1, IL-12, chemokines and nitric oxide. But as sustained activation of this pro-inflammatory pathway is atrocious for the host cell, it is negatively regulated by a family of receptor tyrosine kinases Tyro3/Axl/Mer (TAM); and this property of host is being exploited by *M.tb*. Its lipoproteins constitutively activate TLR2 signaling which

ultimately results in much reduced MHC-II expression and antigen processing by antigen presenting cells (APCs) at the site of infection. *M.tb* adapts itself inside the host by adopting one of these methods which are namely, (i) preventing the fusion of phagosome with lysosome by blocking the Early endosomal autoantigen 1 (EEA1). (ii) fusion of nutrient vesicles, (iii) presence of UreC gene for maintaining the favorable pH inside phagosome (iv) Preventing phagosome maturation by the production of diterpene isotuberculosinl (v) By its ability to neutralize free radicles and nitrogen intermediates. Besides, a bacterial entry in alveolar macrophages initiates a cascade of immune signaling pathways which result in the secretion of chemokines and cytokines like IFN-γ, TNF-α, IL-10, etc. These mediator cytokines assist in the assembly of different immune cells like macrophages, dendritic cells (DCs) and T lymphocytes. *M.tb* can survive for decades in the granuloma which on getting favorable environmental conditions or immune suppression gets reactivated, inducing the impairment of nearby bronchi and inducing the spread of the *M.tb* to other parts of the lung **(Figure 4)** (van Crevel et al., 2002).

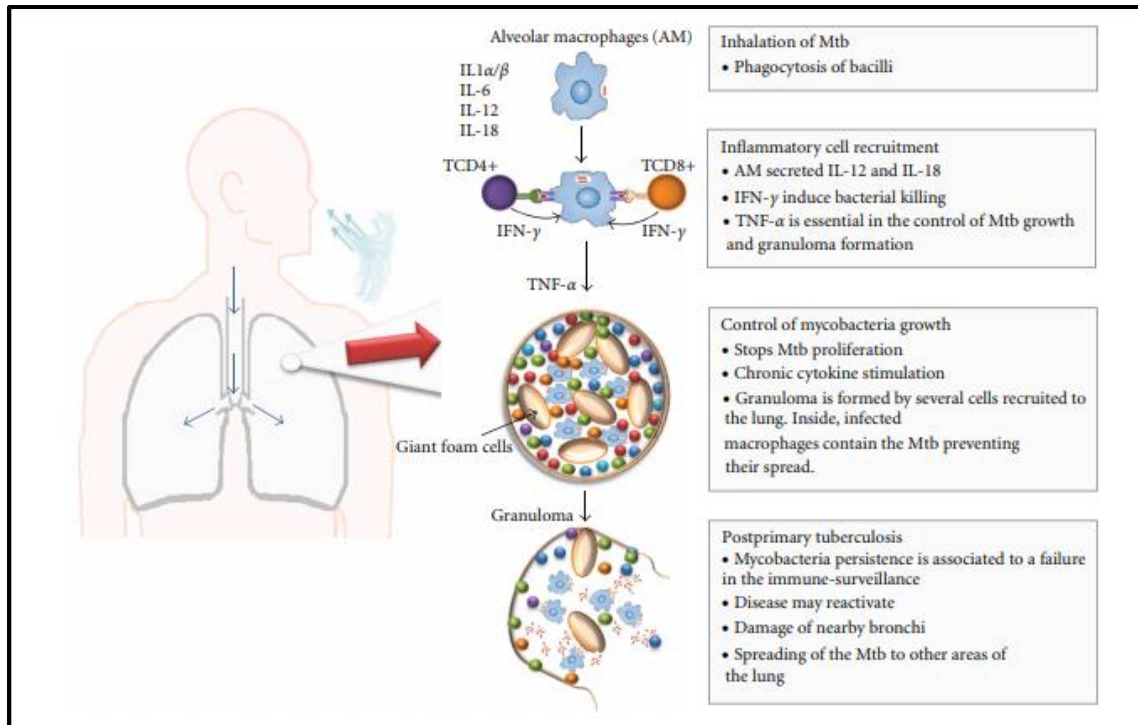

Figure 4. Pathogenesis of tuberculosis. (Adapted from Zuniga Joaquin et al, Clinical and Developmental Immunology, 2012).

2.3.5 Formation of the "Granuloma"

Granuloma formation is the "hallmark" of *M.tb* infection. Although it is formed as a part of a defense mechanism by the host, to prevent the dissemination of bacteria, it is used by *M.tb* as its niche to gain survival advantage for a long period (Sandor et al., 2003). It is composed of an accumulation of different cell types in different differentiation stages such as activated macrophages occupying the central position with a rim of activated lymphocytes particularly T- cells (Silva-Miranda, et al., 2012). Cells involved in the formation of granuloma include macrophages, epithelioid cells, multinucleated cells and different type of T cells (Flynn et al., 2011, Gonzalez-Juarrero, 2001). The central region of the granuloma is characterized by multinucleated giant cells also called Langerhans cells, formed by the fusion of activated macrophages. The Langerhans cells are further

enveloped by large macrophages which resemble epithelial cells known as epithelioid cells (Goldsby et al., 2003).

The granuloma is formed by the action of a highly activated immune system of the host against the invading bacteria (Ulrichs and Kaufmann, 2006). Secretion of TNF-α and other inflammatory cytokines or chemokines having an affinity for the CCR2 receptor are required for the initial entry and localization of macrophages. The recruitment and inter-adhesion of the cells during the formation of the granuloma is assisted mainly by the action of the cytokine, osteopontin which is released by macrophages and lymphocytes (Co et al., 2004). CCL19 along with CCL21 is probably involved in the recruitment and activation of IFN-γ-producing T cells. B-cell recruitment is assisted by CXCL13 (Khader, 2009). The role of TNF-α in granuloma formation is proved by the fact that in TNF-KO mice there is no recruitment of macrophages and CD4+ T around the granuloma (Roach et al., 2002). These cytokines and chemokines drive the recruitment of different immune cell types (neutrophils, natural killer (NK) cells, CD4+ T-cells and CD 8+ T-cells) secreting a pool of different cytokines thus amplifying the cellular migration process around the infection (Chackerian et al., 2003). This cascade of inflammation and cellular recruitment is regulated by the synthesis and secretion of IFN-γ. After the successful establishment of granuloma, the structure is further compacted and stabilized (Kaplan et al., 2003). A well-established granuloma is required for containment of infection during both latent and replicating *M.tb* infection. As the granuloma become mature, they form a marginal boundary encapsulating macrophages, granulocytes, giant cells and foamy macrophages (Ulrichs and Kaufmann, 2006). Recent research has established that the mesenchymal stem cells (MSCs) get recruited at the site of *M.tb*

infection around the granuloma (Raghuvanshi et al., 2010, Das et al., 2015). If the immunity is strong enough, it leads to latent infection without symptomatic manifestations and a non-transmissible state. In such a condition, the granuloma may get restricted to insignificantly small fibrous and calcified lesions (Ehlers et al., 2012). In case of weak immunity due to age, nutritional deficiencies or HIV coinfection, the TB granuloma undergoes caseation or cavitation- which is decaying into cellular debris with cheese like a resemblance and liquefies releasing thousands of live bacteria into the airways. This is responsible for the spread of the infection. Contradictory to the previous belief that granuloma is a static structure protecting the host by containment of the infection; recent researches have established that granuloma is a highly dynamic structure that may increase TB pathogenesis in certain circumstances (David and Ramakrishnan, 2009). Granuloma facilitates the development of latent infection but necrosis of the granuloma is exploited by the *M.tb* bacteria for its transmission (Ramakrishnan, 2012).

2.4 Tuberculosis Epidemiology

Tuberculosis is one of the frightening diseases in the modern world with alarming death rates. Complete eradication of the disease still remains a distant dream. Although the number of active TB cases declined in the 21st century but we are still far from what can be called complete eradication of the disease. TB largely affects the poorest population because its treatment requires better access to diagnostics and costly and lengthy treatment. Routine surveillance of the disease in hotspot population i.e. more prone to the disease is the best practice for monitoring the incidence of TB and the assessment drug resistance.

2.4.1 Epidemiology: Global perspective

TB is still among the top ten leading causes of death worldwide; being the leading cause of death due to any infectious agent. Till 2017, 1.3 million death in non- HIV infected individuals and 3 lakhs HIV positive (immune-compromised) individuals. According to the World Health Organization (WHO), 10 million people developed TB infection in 2017, of which men, women, and children were 5.8 million, 3.2 million and 1 million respectively. WHOs list of high TB burden countries, includes around 30 countries out of which one-third of the people are living in Africa alone and the remaining two-thirds in other developing countries of Indian continent which comprise 27% of the TB cases in India, 9% cases in China while Indonesia, Philippines Pakistan Nigeria, Bangladesh have 4-8% of the cases each **(Figure 5 and 6)**.

More threatful and dangerous is the rise in the incidence of drug-resistant bacteria. According to WHO, it was best estimated that around 558000 people develop TB which was resistant to the most effective first-line drug i.e. rifampicin known as rifampin-resistant TB (RR-TB) and isoniazid. Among them, 82% had multiple drug-resistant tuberculosis (MDR-TB). Of the total MDR cases, more than half were reported in India, China and Russia alone which percentage occurrence of 24, 13 and 10 respectively. Among new cases, 3.5% and among previously reported case around 18% developed MDR-TB and RR-TB. Among cases reported in 2017, around 8.5% were found to have extensively drug-resistant TB (XDR-TB). Apart from that, the most important concern in current TB management is the presence of latent TB in the majority of the global population. Around 23% (1.7 billion) people worldwide have a latent infection and can develop active TB anytime during their lifetime. It has been estimated that from 2020 the

rate of new TB cases per 1, 00,000 individuals will be slow down by 4-5% per year and rate of mortality (the case fatality ratio) will be controlled by 10%. From 2000, when the percentage of individuals who died of TB was 23%, it has been reduced to 16% in 2017. The best statistics of decline in TB death cases were reported in the WHO European region and African region (5% and 4% per year respectively) from 2013 to 2017. Worldwide, HIV negative people show a decline in death from 1.8 million in 2000 to 1.3 million in 2017 which is around 29% decline whereas better results have been obtained for HIV positive patients where death due to TB has been declined by 44% from 2000 to 2017 and by 20% since 2015. In 2017, 64% of the total estimated 10 million cases were only reported to WHO. The main reason for this gap is the under-reported and undiagnosed cases mainly in three countries which include India (26%), Nigeria (9%) and Indonesia (11%). To fill this gap and overcome under-reporting, WHO has collaborated with "Stop TB partnership" and "Global Fund" to fight AIDS, Tuberculosis, and Malaria in 2018 by the launch of an initiative program called "Find. Treat All". The aim of the joint program is to detect and treat around 40 million TB patients in the period 2018-2022.

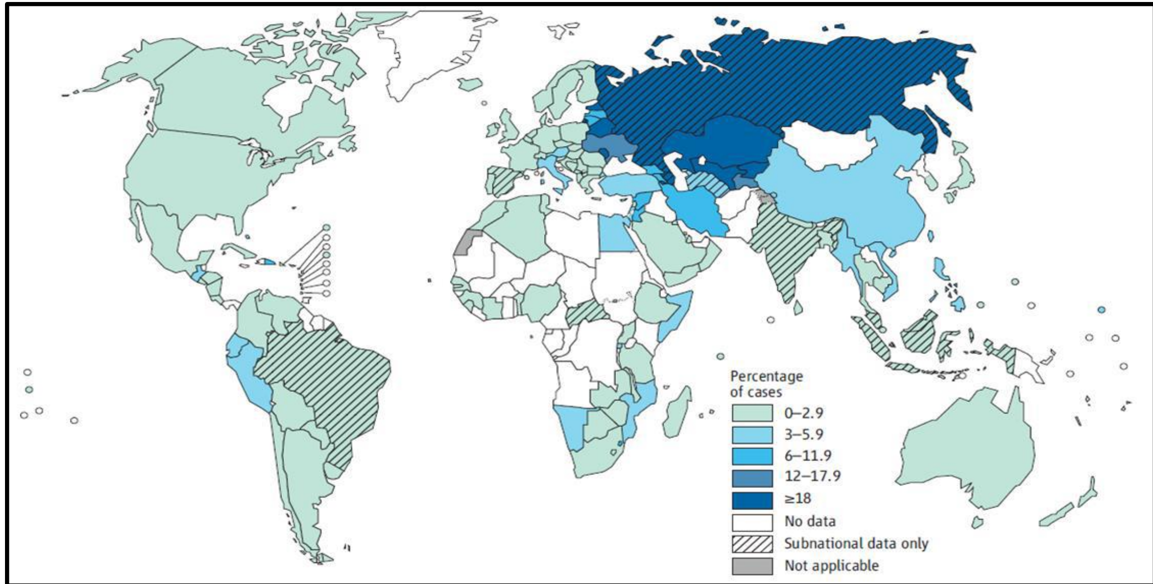

Figure 5. Representation of the percentage of new TB cases with MDR-TB in 2012 (WHO Report, 2013)

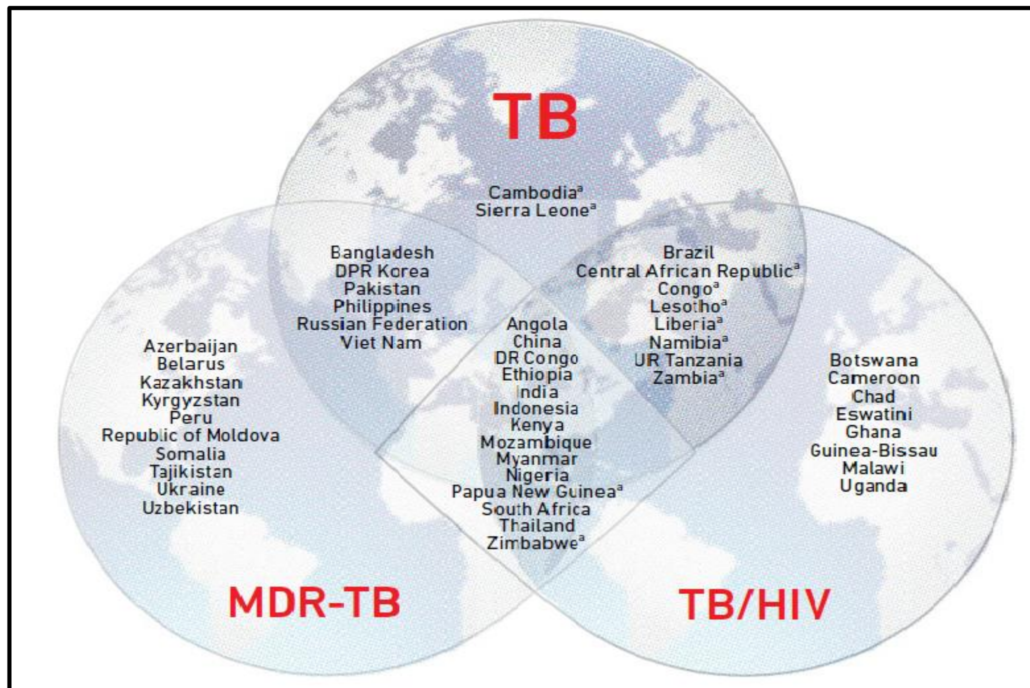

Figure 6 The Venn-diagram indicating 30 countries with huge TB burden (WHO Report, 2013)

2.4.2 Epidemiology: Indian Scenario

Total cases of TB reported in India were approximately 2.8 million as per global TB report, 2017. It is about one-quarter of total TB cases in the world. Out of that MDR-TB/RR-TB cases account for around 1, 47,000 individuals and HIV positive TB were reported to be around 85000 individuals. A more dangerous thing to ponder upon is 40% of the Indian population is a home for *M.tb* which is in a latent state. A total number of mortality recorded because of TB is approximately 4, 23,000 which exclude HIV positive patients. Thus India is at high risk for TB infection and major programs are required for the control and spread of TB among the population.

2.5 Diagnosis of tuberculosis and treatment

In ancient times people have a different perception for the TB because of less awareness and less information about the causative agent and disease. But with the passage of time and improvement in research and development, time changes and comes the era of facts and proof. Although TB is still the leading cause of death and is among the disease of high incidence rate, diagnosis and treatment played the commendable role in curbing the disease. On time diagnosis and treatment helped in saving the lives of many people. Diagnosis and treatment are based on different parameter like the appearance of sign and symptoms which is the traditional way to predict the TB and other ways include various laboratory test.

2.5.1 Diagnosis

Basic symptoms which appear on the onset of active Pulmonary TB include cough, sputum, fever, weight loss, hemoptysis, breathing problems, etc. But these problems are not uniquely present during TB, so it not affirmation to the presence of disease. So if the sign and symptoms like coughing appear for more than 2-3 week than for better diagnosis and confirmation more laboratory test is available. Early diagnosis help in effective treatment and patient management for active TB whereas people who are at risk because of latent TB infection can undergo test specific for latent TB like tuberculin skin test or IFN-γ based assay. However, many tests are available but the current era is still lacking more precise lack of time-consuming and very specific test for TB. Below are discussed some test for active as well as latent TB.

2.5.1.1 Radiologic studies

To diagnose pulmonary TB, the first method chosen is a chest X-ray. It is used for the evaluation of suspected or confirmed cases. Though, quite variable but this method is very useful in monitoring the complications and keeping a follow up of the patients. A major drawback of this test is that it can be normal for TB positive patients. S Therefore, to get a conclusive diagnosis it is accompanied by other more specific tests like sputum test for the detection of acid-fast bacilli. In chest X-ray, TB infection is identified as a cavitary disease in both the “superior segments of lower lobe” as well as the “apical/posterior segments of the upper lobes” (Woodring et al., 1986). Cavitation is the peculiar feature of post-primary TB which can be clearly seen in X-Ray films of 50% of

TB patients. To assure that the diseases are in an inactive state or absent; radiographic stability and negative sputum test should be conducted for at least 6 months. But this test has its limitation as it can only reveal whether the lesion is stable but does not confirm its dormant or active state (Karysl et al., 1994).

To overcome this limitation, a more advanced form of radiography technique i.e. chest computed tomography (CT) is practiced. CT scan provides more clear and valuable information of chest of bronchogenic dissemination, branching opacities and cavitation. Lobar and segmental distribution are the characteristics sign of branching opacities. When all these features are analyzed in proper clinical settings, it can establish a specific diagnosis of TB. Despite the added advantage of CT over chest X-ray, it does not have a significant impact on patient management, hence is followed by another test like the culture test of *M.tb* (Im et al., 1993).

2.5.1.2 AFB smear microscopy and culture

Sputum testing is a widely used test for pulmonary TB. It is a simple method in which sputum specimen is used to prepare smear which is followed by microscopy. Earlier Ziehl Neelsen staining was done to prepare smear slide which is visualized by using a conventional light microscope. This Ziehl-Neelsen microscopy is specific but its sensitivity is variable from 20% to 80%. So to make the diagnosis test more sensitive and less time consuming, the light microscope was replaced by a conventional fluorescence microscope. But, that too had a disadvantage as it's light source was mercury vapors, which required high maintenance. This problem was further resolved by technological advancement by replacing the light source with LED fluorescence. This new LED

fluorescence microscope was assessed by WHO for proper standards and accuracy to meet the international reference standard and it is proved to be more sensitive than conventional Zeihl-Neelson microscopy (Steingart et al., 2006).

Although microscopic examination of sputum helps indirectly diagnosing TB it has his limitation in terms of sensitivity and in identifying drug-resistant strains. So to overcome this, the culture of specimen sputum is advised. Sputum is collected and is either cultured in solid media or liquid broth media. Ideally, liquid media is assumed to be the "gold standard" for culturing *M.tb* as it is fast in comparison to solid media and yield obtained is high for further processing. Liquid media culture thus helps in proper patient management and identification of drug-susceptible mycobacterium faster. But as there are 5-10% chances of contamination in liquid media, it is followed in combination with solid media so as to strengthen the diagnosis and purity of the results (Kanchana et al., 2000).

2.5.1.3. Molecular methods

Nucleic acid amplification testing (NAA test) is a modern laboratory test for the diagnosis of TB. Although AFB smear test is important and cannot be ruled out NAA has added advantages over AFB smear test like (i) it is very specific (ii) it can rapidly confirm the *M.tb* infection in 50-80% smear negative and culture positive test (iii) it has more positive predictive value (iv) in 80-90% suspected patients, NAA helps in detection of *M.tb* many weeks before culture test. As this test can be used on specimen like sputum they are also called direct amplification test. There are different categories for this test which include in-house assays in which PCR method is used and others which are based

on commercial kits like amplified "*M.tb* Direct Test" (MTD) manufactured by Gen-Probe, Inc., San Diego and "Amplicor *M.tb* tests" by Roche Diagnostic Systems, Branchburg, NJ. Though these kits are highly specific and have high positive predicted value they have their limitations too. As their sensitivity is low, so, they cannot be used to give conclusive results in patients with low clinical indices and negative sputum smear test. Also, they cannot be used in a patient undergoing treatment as they cannot differentiate between viable and no-viable bacteria (Greco et al., 2006; Ling et al., 2008).

2.5.1.4 Drug-resistant *M.tb* identification based assay

Molecular beacon: There are specialized oligonucleotides which emit photons upon finding suitable conditions. Using these modified oligonucleotides, drug-resistant *M.tb* is identified. Molecular beacon assay is PCR based assay in which fluorescently labeled, hairpin-shaped DNA probes are used against amplified *M.tb* gene sequence. If there is wild type PCR product then fluorescence signals are detected but if there is some mutant of drug-resistant bacteria than there won't be any signal. This test has high sensitivity and high specificity especially for rifampicin resistant gene but low sensitivity for isoniazid-resistant bacteria. This test is not FDA approved and only used in either research laboratory or reference laboratories (Piatek et al., 1998).

Line probe assay: As conventional microscopy and culture methods based assays are more time consuming and cumbersome, more molecular-based assays were developed for rapid identification of drug-resistant TB. Line probe assay (LPA) is one such molecular test which uses specific gene markers associated with MDR-TB like rifampicin resistance or isoniazid-resistance or combination of both. *M.tb* becomes resistant to these drugs

because of mutation in some genes. Isoniazid resistance occurs due to a sequential mutation in katG, the active site of InhA and in the promotor of ahpC whereas, rifampicin mutation occur mainly due to a mutation in rpoB gene (Ramaswami et al., 1998 and Zhang et al., 2009).

LPA technology is performed is stepwise manner which includes: DNA extraction from the specimen, PCR amplification of resistance determining region, hybridization of oligonucleotides probes with PCR amplified product and the analysis of colorimetric development where probes hybridize with PCR products. This test holds high specificity for rifampicin resistant alone or rifampicin resistant in combination with isoniazid. In spite of all these major advantages over conventional culture and smear test, LPA cannot replace them as after identification, cultures test are needed for second-line anti-TB drugs (WHO, 2008).

2.5.1.5 Monteux test

Latent TB infection can be detected either by skin test or blood test and Monteux test belongs to the skin test category. It is the most widely used test since the 19th century. In this test, tuberculin which is basically the bacilli extract mix with glycerol is injected intradermally in the forearms of an individual. It is expected that administration of tuberculin will start an immune response if the person has been exposed to bacteria or bacterial proteins. After 24-48 hours duration, the formation of palpably raised and hardened area is measured and interpreted. If the diameter is zero millimeters then there is no infection. If it is above 5 mm then the person may be HIV positive or may be in recent contact be TB patient. If it is above 10mm then person categorized as a high-risk

individual. This test is non-specific as it can give false positive results also which may be due to earlier vaccination with BCG, infection with non-tuberculosis mycobacteria, or incorrect bottle of antigen used (Nayak et al., 2012).

2.5.1.6 IFN-γ release assay

With advances in immunology and genomics, T cells based assays are gaining importance. An in-vitro test is performed in which IFN-γ is measured after stimulation with *M.tb*. FDA has approved IFN-γ assay based kits QuantiFERON-TBGOLD, made by Cellestis, Inc for latent *M.tb* detection. It measures IFN-γ secretion in the plasma which is challenged with antigens specific to *M.tb* like ESAT-6 and CFP-10. These antigens which are *M.tb* specific are also sensitive for the identification of *M.tb* as they show less cross-reactivity with BCG vaccine, hence, this assay is good for both, in vivo as well as *in vitro* diagnosis. Despite the advantages of using this assay, there are several issues which still need to be resolved. It is not well documented whether these assays will be useful in patient with TB subgroups like immune-compromised patients, extrapulmonary TB patients, children, etc. Other limitations include fewer reports of association of ESAT-6 and IFN-γ, the effect of anti TB treatment, their high cost, etc. To assure the reliability, more research is needed in terms of latent and active TB infection and work is needed for cost-effectiveness studies (Pai, et al., 2014).

2.5.2 Treatment of tuberculosis

The treatment of TB involves the use of Directly Observed Treatment Short-course (DOTS) therapy. This treatment involves the use of multiple antibiotics constituting several first-line drugs such as isoniazid (INH) and rapamycin and various second-line drugs. World Health Organisation (WHO) has recommended 6 months of TB drug treatment of new patients. This includes "intensive" treatment phase of 2 months followed by "continuation phase" for an extended time (4 months).

During the initial two months, the patients are treated with the first line of antibiotics, isoniazid, and rifampicin with pyrazinamide and ethambutol. In the next 4 months of treatment isoniazid along with rifampicin is given to the patients (Elzinga et al., 2004).

Combination of antibiotics is given at a time instead of a single drug so as to minimize the danger of drug resistance development. It is also mandatory to complete the 6 months treatment duration. The drug dose given to the patient depends mainly on his body weight. Treatment of drug-resistant form of TB also involves the second line of drugs including fluoroquinolones, aminoglycosides, polypeptides and thioamides which comprise DOTS-Plus therapy (Iseman, 1998).

2.5 Challenges of tuberculosis control and cure

As per the WHO report of India, in the present time, TB takes the life of 2 people in every three minutes. Major challenges which result in failure to combat with TB include irrational and widespread use of anti TB drugs by unregulated private hospitals, compromised primary health care systems in underdeveloped cities and villages,

prevailing immunosuppressive disease like HIV, political and administrative incompetence (Murray et al., 2006). In a country like India consumption of raw milk from infected cattle can be a potent source of TB.

Another major problem is every year around 3.6 million cases go unnoticed (Herbert et al., 2014). They are either not diagnosed or treated or informed to the public health system. Those cases become the problem of either individual care or become responsible for continuous transmission. Those who get affected may develop multiple drug-resistant tuberculosis (MDR-TB). This MRD-TB is really a matter of concern and fights these there is an immediate need for expansion of rapid molecular diagnostic to every possible health care level. New medicine and drugs need to be included in the DOTS therapy to minimize the danger of emerging drug-resistant population (Falzon et al., 2015). Also, there is a need to improve and increase financial support as worldwide about 8 billion US dollar is required to cover the basic cost of TB case detection and treatment but in the current scenario around 6.4 billion US dollar is available which is not sufficient (WHO report, 2015). Above all, we need more accelerated research to develop new drugs and better diagnostics together with improved vaccine candidates. To meet this necessity, government and corporate sectors are needed to invest in research institution to fill the gap of the amount required to fulfill it.

2.6 Emergence of drug-resistant tuberculosis

Poorly organized and underfunded tuberculosis management programs in the developing countries result in unauthorized and inconsistent treatment, which ultimately leads to

treatment failures and the development of drug-resistant forms of *M.tb* (Caminero et al., 2010). Drug resistance poses an impending challenge to the global TB control program. There are different forms of drug-resistant *M.tb* namely multi-drug resistant (MDR) and extremely drug resistant (XDR). Recently a total drug-resistant (TDR) variant of *M.tb* is also discovered. MDR-TB is resistant to first-line treatment drugs, rifampicin and isoniazid and so cannot be treated using isoniazid and rifampicin and thus requires other drugs which form the second line of treatment for TB. Extensively drug-resistant TB (XDR-TB) includes MDR-TB conditions along with additional resistance to one or more drugs among fluoroquinolones and amikacin, capreomycin and kanamycin and account for 9% of the total MDR cases. TDR has been found resistant to all antibiotics available (Velayati, 2006). As per WHO report 2017, drug resistance poses a threatening situation in TB cure with reported 558 000 people developing rifampicin-resistant TB (RR-TB) which encompasses 82% of multidrug-resistant TB (MDR-TB) cases (WHO Report, 2017).

Drug resistance TB emerged soon after streptomycin was used as single drug therapy for the TB cure (Council, 1948). This led to the development of combination therapy using two or more drugs (Council, 1962). With the advent of HIV-TB coinfection, there has been a tremendous rise in the incidence of drug resistance. Early detection and proper time management is the only way to reduce the spread of these resistant strains.

2.7 Latency and Persistence in *M.tb*

Latent *M.tb* pose one of the major hindrances in the treatment of TB worldwide. Latent TB may be defined as the clinical syndrome after an individual is infected with *M.tb*

bacilli and the infection gets established and the immune system is triggered to control the spread of bacteria to a non-replicating, quiescent bacterium which displays an asymptomatic infection. Of the total individuals infected with *M.tb* only 10% develop active disease during their lifetime. The remaining 90% remain in a dormant state until the individual is affected by immune suppression due to malnutrition, cancer, diabetes, or HIV co-infection. Latent TB patients have a non-transmissible infection and therefore pose no threat to society. Latent TB is diagnosed mainly by two methods- first is tuberculin test using PPD (Purified Protein Derivative) and the second is the detection of lesions in the X-ray of the chest (Parrish et al., 1998). IFN-γ produced by T-cells along with TNF-α produced by macrophages and dendritic cells (DCs) activates more macrophages and T-cells. The activated CD8+ T-cells lyse and kill the bacteria while CD4+ T-cells mediate bacterial elimination indirectly. During immunosuppression, T-cells are depleted and the bacteria begin to multiply leading to reactivation of the disease. Defect in activation of macrophages and T-cells leads to disorganization of the granuloma which cannot contain the infection. For studying latent TB two mouse models are being used by researchers. The first was developed by the researchers at Cornell University, the USA in the 1950s. The Cornell model is the oldest and the most established model of latent TB in mice, where *M.tb* infection in mice is followed by treatment using antimycobacterial drugs such as isoniazid and pyrazinamide, which reduces the bacterial number to undetectable values, which may reactivate upon immune-suppression, similar to the human situation **(Figure 7)**. There are several variations of the Cornell model which are being used. The second model is in which mice are exposed to *M.tb* via aerosol route and the infection is controlled by the immune system in 3-4 weeks

on its own. The immune system controls the infection but does not simply eradicate it leaving a persisting population of bacteria in the different organs such as lung, spleen, bone marrow and liver of the mice (Tufariello et al., 2003). During this stage, the bacteria remain in a metabolically quiescent state resulting in a significant down-regulation of RNA and protein synthesis machinery. The genes which are characteristic of this nonreplicating state of *M.tb* constitute the DosR/devR regulon, a regulon of 48 closely related genes (Voskuil and Schlesinger, 2015). DosS and DosT (members of regulon) are the two sensor kinases containing the heme group at the active site. These activate the DosR, a transcriptional factor downstream which is responsible for the regulatory expression of 48 genes that constitute the regulon by phosphorylation at specific sites. This phosphorylation event is the direct outcome of hypoxia, nitric oxide and carbon monoxide sensing by the bacteria. Despite the fact that *M.tb* requires oxygen for its growth and division, it survives in the compromised anaerobic conditions for a long period of time, emphasizing on the importance of the role of DosR regulon in dormancy and maintenance of the granulomatous structure (Roberts et al., 2004; Kaur et al., 2016).

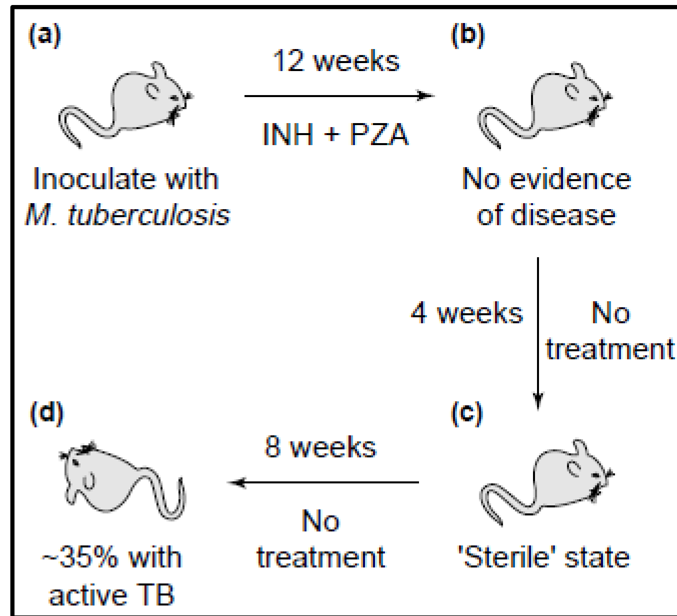

Figure 7. Diagrammatic representation of the Cornell model of latent tuberculosis (Adapted from Parrish NM et al, Trends in Microbiology, 1998).

2.8 Mesenchymal Stem Cells: Discovery and characteristics

“Mesenchymal stem cells” are nonhematopoietic stromal-cells having the ability of self-renewal and differentiation into multilineage cells under appropriate condition. They can be differentiated into various cell types like osteoblast, chondrocytes, adipocytes, and neurocytes (Miura et al., 2003). Due to their availability in different tissue types and lack of ethical problems for their use in the laboratory, its discovery is a boon for exploring tissue engineering and studies on stem cell therapy (Javazon et al., 2004; Krampera et al. 2006; Krampera et al., 2007).

2.8.1 Discovery of MSCs

Presence of MSCs in the bone marrow was first suggested around 130 years ago by a German pathologist Cohnheim. While studying the phenomenon of wound repair he

suggested the possibility of the presence of some fibroblast source that deposit collagen fiber during wound healing (Prockop, 1997). In 1924, Alexander Maximow identified multipotent precursor cells that can be differentiated into a variety of blood cells types. 40 years later, a team of McCulloch and James Till identified that stromal cells isolated from the spleen and bone marrow possess the ability of colony forming units and have clonal properties (Becker et al., 1963; Siminovitch et al., 1963). Almost after another decade in 1974, Friedensein and co-workers developed the ex-vivo assay and proved experimentally that bone marrow contains cells that possess the ability to differentiate into MSCs. They cultured BM cells in total, in culture dishes from which they removed non-adherent hematopoietic cells after the stem cells attached to the surface of the dish. They observed that cultured cells constituted a heterogeneous population; of which the cells with the highest adherence capacity had a characteristics spindle shape and formed a clump of 2-4 cells resembling a "foci". These cells remained as a highly inactivated and unresponsive population for an initial 2-4 days after which they started to divide actively. When these cells are passaged several times, they started resembling like fibroblasts. More interestingly, they discovered in these cells a potential to differentiate into colonies resembling either bone or cartilages (Friedenstein et al., 1974). This study was further carried out by other researchers who were able to differentiate Friedenstein's isolated cells into other cells such as bone (osteoblasts), cartilage (chondrocytes), adipose tissue (adipocytes) and muscles (myoblasts). These cells due to their characteristic of showing multi-lineage differentiation or multipotency were then referred to as mesenchymal stem cells or MSCs. Later these cells proved to be crucial for stem cell therapy and a first clinical trial was then conducted in 1995 followed by a series of trials for the treatment of

different diseases like cardiovascular disease, cancer, autoimmune disorders, etc (Wang et al., 2012). Recently, MSCs have been proved to be involved in different infectious diseases also like TB but more research is needed to explore various physiological and pathological roles of MSC in a different disease context.

2.8.2 Characteristics of MSCs

MSCs are an adherent population of cells isolated from both embryo and adult tissue types like the bone marrow, adipose tissue, amniotic fluid, fetal tissue, etc (Campagnoli, et al., 2001). Their enrichment can be achieved by passaging and expansion in selective media, resulting in the elimination of other cell types (In't Anker et al., 2003; Zuk al., 2002). The remaining cell population comprises of cells of different shape and sizes including spindle-shaped, polygonal or cuboidal cells (Nakahara, et al., 1991; Javazon et al., 2004).

Although the phenotypic markers of MSCs are not explicitly present on them but for identification they co-express certain sets of unique surface markers such as CD105, CD73, CD90, CD44, CD 106 and STRO-1 (Haynesworth, et al., 1992; Galmiche et al., 1993). These markers can be used to characterize and sort the MSCs from other population of cells using FACS. MSCs lack certain markers like CD45, CD34 characteristic of Hematopoietic Stem Cells (HSCs), and macrophage markers like CD14 and CD11 (Conget et al., 1999). MSCs are also devoid of the CD40, CD80, and CD86, which are the molecules responsible for the co-stimulatory function. There are reports of isolating and enriching MSCs using both the criteria of positive selection and negative selection (Pittenger et al., 1999; Sordi et al., 2005).

In spite of the presence of atypical markers in MSCs, the source and method of isolation also determine the variability or presence of some of the markers. MSCs isolated from adipose tissue have CD49b expression on them but not CD106 whereas MSCs isolated from bone marrow have CD106 on them but not CD49b (Zuk et al., 2002). MSCs isolated either from fetal blood, liver, spleen and bone-marrow or from the bone marrow of adult individual may seem phenotypically similar but exhibit heterogeneity in their potential to give rise to a certain population of cells. These differences in either type of population or different cell surface markers are governed by the effect of factors produced by accessory cells during initial culture. (In ’t Anker et al., 2003).

Another way to characterize MSCs is by their ability to get differentiated into the following different cells types’ like-

Osteoblast: To identify their differentiation to osteoblast MSCs are exposed to different concentrations of chemicals (ascorbic acid, glycerophosphate, and dexamethasone) for two to three weeks. Differentiation can be seen by the increased alkaline phosphatase expression, calcium accumulation and appearance of nodules or aggregates which can be stained by alizarin red and von Kossa techniques (Pittenger et al., 1999).

Adipocytes: To differentiate them into adipocytes, MSCs are treated with dexamethasone, insulin, isobutylmethylxanthine, and indomethacin which result in the formation of lipid-rich vacuoles and increased expression of PPARγ2 receptor (peroxisome proliferation-activated receptor). Oil red O staining can be used to observe lipids present inside the vacuoles. (Pittenger et al., 1999).

Chondrocytes: To differentiate them into chondrocytes, MSCs are centrifuged and pelleted micromass then stimulated with TGFβ (transforming growth factor).

Chondrocytes differentiation can be then visualized for the presence of glycosaminoglycan within the extracellular matrix by histological staining with toluidine blue (Mackay et al., 1998).

Myotubes: When MSCs. are treated with 5-azacytidine and amphotericin B, they get differentiated to myotubes. Which further get fused and form myotubes identified by their rhythmic beating (Wakitani et al., 1995)

2.9 Immunological phenotype and function of MSCs

2.9.1 Immunological phenotype

MSCs are considered to be non-immunogenic. This immunological phenotypic of MSCs is due to the absence of certain immunological markers. These markers are confirmed by flow cytometry which shows positive expression of MHC I but minimal MHC II expression and no expression of costimulatory markers CD40, CD80 and CD86. Presence of MHC I may stimulate T cell but due to the absence of these costimulatory markers, the secondary immune response is not generated hence making them best suitable for allogeneic transplantation in the host without using immunosuppression (Le Blanc et al., 2003). It has been well studied that MSCs possess immunomodulatory properties which inhibit dendritic cell and B cell activation, differentiation and proliferation. It has been shown that MSCs transplant in immunocompetent baboon from healthy one results in significantly prolonged survival of the MHC-mismatched skin graft. It has been tried in human also where hMSCs were administered and hematopoietic engraftment has been improved during allogeneic transplantation and graft versus host disease was hampered (Le Blanc et al., 2003; Bartholomew et al., 2002). These studies along with other reports

on different animal model paved way for their therapeutic use as immunosuppressor by targeting MSCs o the inflamed tissues.

2.9.2 Immunomodulatory function of mesenchymal stem cells

Host body usually rejects the allogeneic cell by their immune system. However, this rule is not applicable to MSCs. In addition to not being recognized by the immune system they successfully suppress the activation of proliferating of host immune cells which could be dendritic cells, B-cells, T cells or Natural Killer cells. They inhibited DCs by interfering in their pathway of endocytosis and inhibition of TNF-α and Interleukin-12 secretion (IL-12) thus inhibiting activation of alloreactive T cells. Allogenic MSCs have a strong impact on T cells proliferation as pro-inflammatory cytokines like TNFα or IFN-γ are unable to stimulate T cells activation in their presence. These MSCs also interfere with the process of naïve CD4+ T cells differentiation to either type of T helper cells (Th1 or Th2), By doing so they act as an anti-inflammatory in nature. Also, when MSCs are premixed with CD8+ T lymphocytes or NK cells it reduces their cytotoxicity. MSCs also exert their effect on B-cell by reducing their differentiation into plasmocytes hence reducing the secretion of immunoglobin like IgA, IgM or IgG. (Tse et al., 2003; Corcione et al., 2006; Spaggiari et al., 2008; Spaggiari et al., 2009; Asari S et al., 2009)

2.10 Role of stem cells in tuberculosis and the emergence of drug resistance

MSCs have evolved as a new and fifth arm of immune responses initiated as a defense mechanism of the host during TB infection along with the well-known antigen presenting cells such as macrophages and dendritic cells, classical T cells, and non-classical antigen-

presenting immune cells. MSCs of non-hematopoietic lineage is of multipotent nature that isis characterized by self-renewal capacity and multilineage differentiation. MSCs are reported to express cell surface receptors such as Toll-like receptors (TLRs) and also known to secrete different cytokines, chemokine, and certain growth factors. MSCs have been reported for their use in therapeutics for various autoimmune diseases and cancer because they possess the immunomodulatory properties and differentiation capacity. Their importance of utilization was also seen during organ transplantation as they are proved to have the potential to avoid graft rejection. MSCs produces cytokines and chemokines which act as potent immuno-modulators factors and dampen T cell responses while shifting macrophage differentiation from M1 to M2 phenotype. MSCs imply autophagy and synthesis of NO (nitric oxide) to curb the growth and replication of *M.tb* inside the host cells. MSCs supposedly play a dual role in TB pathogenesis. MSCs also provide a nutrient-rich favorable environment for the intracellular existence of *M.tb*. It was first reported by Raghuvanshi et al., that $CD3^{-}$ $B220^{-}Sca\text{-}1^{+}$ MSCs are present in the granulomatous structure of H37Rv (*M.tb* strain) infected mice. (Raghuvanshi S et al., 2010). MSCs suppress T cell responses by the action of secreted nitric oxide, aiding the survival of *M.tb*. It was proved that TGF-βRIIDN transgenic mice become more susceptible to *M.tb* infection when naïve MSCs are infused in it. Additionally, in lymph nodes section of TB patient, MSCs were found to be established with acid-fast bacteria (Raghuvanshi S et al., 2010). These reports suggested that *M.tb* infected MSCs may circulate from the lungs to other extra-pulmonary organs and may regulate the persistence and dissemination of *M.tb* from its primary target organ, lung. This study of the role of MSCs during TB was followed by two exiting researches which demonstrated that human

CD271$^+$ and CD133$^+$ MSCs harbored viable *M.tb* (Das et al., 2013; Garhyan et al., 2015). Moreover, it was found that in H37Rv infected BALB/c mice; *M.tb* was distributed into the bone marrow mononuclear cells from the lungs which are primarily infected after aerosol challenge. Viable *M.tb* was found in MSCs purified from TB patients after DOTS therapy. These studies in MSCs confirmed the presence and survival of *M.tb* in human MSCs which may provide a niche for persistent bacilli even after antibiotic therapy. In yet another study of latency, *M.tb* bacilli were detected in bone-marrow derived MSCs in drug-treated mice establishing the fact that MSCs may assist in the survival of bacteria even in the presence of antibiotics thus giving rise to persisting populations of *M.tb.* It has further been confirmed that MSCs provide an antibiotic protective niche to *M.tb* due to the presence of efflux pumps present on the MSCs (Beamer G et al, 2014).

2.11 Role of autophagy in tuberculosis

Autophagy is a name given to collective processes required for maintaining homeostasis; namely, macroautophagy, chaperone mediate autophagy and microautophagy that deliver the cytosolic contents to the lysosomes for degradation and removal in case of cellular stress and starvation (Mizushima et al., 2011). During autophagy, the host cell upon getting sensitized by oxidative stress and other environmental challenges eliminates dysfunctional and excessive cellular components in a sequence of events which may or may not result in cell death (Aggarwal et al., 2016; Jurkuvenaite et al., 2015). Autophagy increases cellular life by removing unwanted cellular constituents and maintaining cellular homeostasis (Jurkuvenaite et al., 2015). In the process of autophagy, the cellular components for degradation, such as proteins, lipids, and cell organelles, are sequestered

into double-membrane-bound vesicles called autophagosomes and are transported to endosomes or lysosomes which fuse to become autophagolysosomes. Hydrolase enzymes, present in the autophagolysosomes, digests the autophagic constituents into their basic forms– fatty acid and amino acids which may be reused by the cell for energy generation and cell maintenance.

Autophagy plays a role in the intracellular killing of *M.tb* mainly through the lytic and antibacterial properties of autolysosomes- formed by the fusion of autophagosomes and lysosomes. Factors associated with autophagy such as SLR, Sequestosome 1/p62 and NDP52 have proven that autophagy plays an indispensable role in the defense against *M.tb* (Ponpuak et al., 2010; Watson et al., 2012). Induction of autophagy upon *M.tb* infection accelerates the bacterial killing by phagosomal maturation and acidification leading to the development of structures called autophagosomes. (Gutierrez et al., 2004; Fabri et al., 2011). Thus autophagy checks the *M.tb* strategy of inhibiting phago-lysosomal fusion (Harris et al., 2007; Vergne et al., 2004) and helps in the elimination of bacteria by the action of trafficking pathways which deliver proteins with anti-microbial properties like cathelicidin (present in the lysosomes) and other partially digested cytosolic proteins with antimicrobial properties (Yuk et al., 2009; Fabri et al., 2011, Ponpuak et al., 2010). Even though autophagosome formation kills the majority of the intracellular bacteria, a small number of bacteria still escape the killing and move to the cytoplasm. They then undergo killing in the cytoplasm by selective autophagy (van der Wel et al., 2007; Watson et al., 2012).

Autophagy in combination with frontline anti-tuberculosis drugs like isoniazid and pyrazinamide helps in better clearance of *M.tb* from the host in comparison to drugs

alone (Kim et al., 2012). Treatment with anti-mycobacterial drugs leads to the dissemination of bacterial constituents which activate autophagy and generate ROS from the mitochondria in macrophages (Kim et al., 2012). Several compounds work in combination with conventional antibiotic drugs and activate autophagy inhibiting the cellular survival of *M.tb* in the host (Floto et al. 2007; Sundaramurthy et al., 2013). Thus, autophagy may promote the development of host-based therapeutics helping in attaining sterile clearance of the bacterium (Lam et al., 2012).

2.12 Quiescence in stem cells

Quiescence is the innate property of tissue resident stem cells when the stem cells exist in a non-proliferating, cell cycle arrested stage known as G0 phase of the cell cycle. Induction of quiescence is responsible for maintaining tissue homeostasis and regeneration. Quiescence is characterized by slower metabolism, small cell size, increased nuclear to cytoplasmic ratio and autophagy (Schultz et al, 1978). The cellular changes accompanying quiescence are transient and may be reverted back unlike the permanent change in cellular function in the process of senescence which is characterized by terminal differentiation of the cell. When required as in the case of tissue injury the quiescent cells become active, replicate and differentiate into cells required for tissue regeneration (Schultz et al, 1978). There has been a shift in the concept of cellular quiescence. Earlier, it was believed that quiescence was a default phenomenon undergone by cells upon facing challenges to proliferation. Recently, it has been found that cells, predominantly stem cells, embrace the quiescent state to conserve key functional characteristics (Cheung and Rando, 2013). Genetic markers characteristics of quiescent

stage are PCNA (proliferating cell nuclear antigen), MCM2 (minichromosome maintenance-2) and Ki67 (Bravo and Macdonald-Bravo, 1984). Molecular mechanisms regulating the induction of quiescence include the notch and forkhead signaling pathways which are upregulated in case the cells attain the quiescent state. FOXO3 promotes the quiescent state by activating Notch signaling in adult stem cells (Chapouton et al., 2010; Gopinath et al., 2014).

2.13 Role of lipids in tuberculosis infection

Bloch H et al, 1956 and others have recognized that *M.tb* uses host lipids as their major energy source. The crucial role that fatty acids play as an energy source for *M.tb* survival has been demonstrated by the importance of the key enzyme of glyoxylate pathway, isocitrate lyase in *M.tb* pathogenesis (Munoz-Elias and McKinney, 2005). Triacylglycerol (TAG) have been proposed as the storage house of energy for latent *M.tb* because of the studies which state that fatty acids are stored in the form of TAG in adipose tissue of mammals and as inclusion bodies composed of lipids in prokaryotes during stressful conditions (Daniel et al., 2004). The presence of intracellular lipid inclusion bodies in *M.tb* was observed approximately six decades ago and recently lipid bodies have been studied in *M.tb* detected in the sputum of TB infected individuals (Burdon, 1946; Garton et al., 2002). These findings have strongly established that the accumulation of TAG in *M.tb* infected cells is crucial and necessary for latency. Deletion of triacylglycerol synthase 1 (tgs1), which plays a vital role in triacylglycerol synthesis reduces the accumulation of TAG in *in vitro* generated latency conditions (Sirakova et al., 2006; Deb et al., 2009). The initial sources of lipids that accumulate as lipid droplets in the *M.tb*

infected cells remain largely unknown. In patients with untreated TB (pulmonary as well as extra-pulmonary), caseous granuloma within the lungs has been reported to carry lipid-loaded macrophages typically known as foamy macrophages (Hunter et al., 2007). These foamy macrophages are believed to have formed in the hypoxic environment of the lungs (1% O_2) and other extra-pulmonary organs infected by *M.tb* (Russell, 2007; Peyron et al., 2008). Foamy macrophages consist of huge accumulation of triacylglycerol which may provide a lipid-rich niche for *M.tb* growth and survival (Reed et al., 2007). *M.tb* in the foamy macrophages have been reported to utilize host TAG by depositing it inside the bacterial cells to meet their nutrient requirement (Garton et al., 2008).

2.14 Role of cytokines and chemokines in *M.tb* infection

Infection by *M.tb* presents antigens which stimulate the onset of inflammatory responses, which are required to be regulated in order to maintain a balance between checking the infection and avoiding tissue damage. Therefore it is important to have a deep understanding of the functions and communications between cytokines and chemokines to design an effective strategy to combat TB. In the context of TB, the major cytokines which are necessary for a proinflammatory response are IFN-γ and TNF-α while anti-inflammatory cytokines involve IL-10, IL-4, and IL-13.

Cytokines of the Th17 pathway namely IL-17, IL-23 also play a protective role while Tregs regulate the immune system to avoid self-destruction. The main cytokines involved are the cytokines of the interferon family (IFN) and Tumor Necrosis Factor (TNF) family.

IFN family: Interferons (IFNs) class of cytokines are categorized into two types depending on the specificity of the receptor and sequence-homology (Schroder et al., 2004). IFN-γ is the only type II interferon whereas IFNα and IFNβ constitute the type I interferons (Schroder et al., 2004). IFN- γ is mandatory for survival after infection with *M.tb* because of its protective role for the host whereas types I IFNs are mainly utilized by the bacteria for its own survival thus being harmful to the host protection. Type I IFNs and type II IFNs binds to different receptors. The binding of cytokine with its receptor induces signaling primarily by activating the JAK-STAT pathway. The activated signaling pathways within the cells activate the others cells to perform their functional role including NK cells, T cells and tissue macrophages (Greenlund et al., 1994; Kovarik et al., 1998; Frucht e al., 2001).

The major function of IFN-γ is the activation of phagocytes by stimulating phago-lysosomal fusion therefore in case of defect for deficiency in the IFN-γ producing pathway the patient succumbs to early death due to reduced phago-lysosomal fusion, low reactive oxygen and nitrogen intermediates (ROI and RNI) production and de-acidification of lysosomes (Filipe-Santos et al., 2006). Individuals having a genetic defect in IFN-γ production suffer from a condition called MSMD (Mendelian susceptibility to mycobacterial disease) (Zhang et al., 2008).

TNF family: The primary source of secretion of TNF-α are the macrophages but other cell types also produce and secrete it (Wajant et al., 2003; Jiang et al., 1999). TNF-α has two binding receptors which area is known as TNF-Receptor 1 (TNF-R1) and TNF-Receptor 2 (TNF-R2) both of which have different cellular localizations. Whereas TNF-R1 is present on all cells, TNF-R2 is confined in expression mainly on certain cell types

like neuronal, endothelial, a few subsets of T cells, MSCs and certain other cells types (Carswell et al., 1975). TNF-α plays a vital role in inflammatory and migratory potential of cells it acts on (Faustman et al., 2013). It also plays a role in inducing apoptosis as a means of immune protection in cells infected with *M.tb* (Keane et al., 1997; Keane et al., 2000). TNF-α produced by the virulence strain of *M.tb* has less bio-potential and activity compared to that produced by avirulent bacteria. This is an example of host immune evasion strategy employed by the virulent *M.tb* (Balcewicz-Sablinska et al., 1998). Later, in the infection cycle TNF-α support the development and maintenance of the granuloma as well (Serbina et al., 1999).

CHAPTER 3
Rationale of the Study and Objectives

Tuberculosis (TB) is a deadly disease causing the highest number of deaths due to a single infectious agent (WHO report, 2017). The pathogen, *Mycobacterium tuberculosis* (*M.tb)*, infects the alveolar passages of the individuals and inhabits the lungs (McDonough et al., 1993). Out of the total number of individuals infected with *M.tb*, only approximately 10% develop the active disease while the other 90% is mostly eliminated by the strong host immune response against the foreign pathogen (Blumberg and Ernst, 2016). A small number of these bacteria survive the host immune response and find protection in the host cells where they live for decades, as non-replicating asymptomatic bacteria. This condition is known as latency. Dormant bacteria are characterized by low metabolism and unresponsiveness to anti-tuberculosis drugs (Ignatov et al., 2015) and are responsible for the induction of latency. Directly Observed Treatment Short-course (DOTS) therapy treats active TB but dormant bacteria are tolerant to the drugs so are not eliminated. Treatment of dormant *M.tb* is a challenge for TB control strategy. These dormant populations of bacteria revert to active form on confronting immune-suppressive conditions. The emergence of HIV co-infection in the twentieth century has made these dormant bacteria very prone to reactivation posing a major threat to the health and well-being of mankind (Blumberg and Ernst, 2016). Noncompliance to the DOTS treatment regime has also led to the development of drug-resistant variants of the TB bacteria which are very difficult to cure owing to the lack of new antibiotics which have become approved for use to treat TB (Almeida Da Silva et al., 2011). Therefore it is the need of the present time to discover new treatment and host-mediated therapies that may reduce the length of the treatment and kill dormant bacteria so as to combat both latency and drug resistance in the TB prone population.

In 2010, it was first time reported by Raghuvanshi et al that granulomatous structure in the lungs consisting of macrophages, epitheloid cells, dendritic cells, B-cells and T-cells also recruit mesenchymal stem cells and help the bacteria in evading host immunity by huge NO secretion (Raghuvanshi et al., 2010). This study was followed by two more comprehensive studies stating that *M.tb* infects MSCs and those $CD271^+$ MSCs harbor dormant and persisting bacteria (Das et al., 2013). Viable *M.tb* was identified in $CD271^+/CD45^-$ Bone marrow (BM) MSCs in patients who had already completed 6 months long DOTS treatment which hinted that MSCs may harbor dormant and drug-tolerant population of *M.tb*. The researchers suggested that CD271+ MSCs may provide a protective intracellular niche in the host for the long-term persistence of dormant *M.tb* in the host because of the hypoxic environment in which the MSCs reside. There is an aggravated incidence of drug resistance in TB around the globe. In this regard, Beamer et al suggested that BM-MSC provide suitable factors for the emergence of drug-resistant TB because of the presence of drug efflux pumps, mainly ABCG2 efflux pump (Beamer et al., 2014).

These studies make MSCs an interesting area of research in the pathogenesis of TB. They seem to be involved both in latency and drug resistance, which are a prominent threat in TB treatment at present and may further aggravate the burden of TB. Although MSCs play an important role in shielding the bacteria from the host immunity the exact mechanism employed by MSCs to induce latency and drug resistance is uncovered. Moreover, the killing mechanisms used by MSCs to reduce the bacterial number also needs more elaborate and intensive research.

Therefore, to decipher and study the molecular mechanism of adaptation of *M.tb* in the host MSCs we designed the present study with the following objectives:

1. To culture and characterize Mesenchymal Stem Cells (MSCs).
2. To compare the mechanism of *Mycobacterium tuberculosis (M.tb)* infection in MSCs to professional phagocytes.
3. To study the cellular response of MSCs post infection with *M.tb.*
4. To study the effect of soluble factors and cytokines released from MSCs infected with *M.tb* on tuberculosis pathogenesis

CHAPTER 4
Materials and Methods

4.1 Ethics Statement

This study was ethically approved by the Institutional Committee for Stem Cell Research, All India Institute of Medical Sciences (AIIMS), New Delhi, India; (Reference number: IC-SCR/47/16(R)). Animal experiments were performed according to the guidelines approved by the Institutional Animal Ethics Committee of the International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India and the Department of Biotechnology guidelines (Government of India).

4.2 Reagents

Rapamycin (Cat. No. R8781), isoniazid (Cat. No. I3377), phorbol 12-myristate 13 acetates (PMA) (Cat. No. P1585) and paraformaldehyde (Cat. No. P6148) were obtained from Sigma Aldrich Co (St Louis, USA). The immunosuppressant dexamethasone was also obtained from Sigma, Cat. No. D1756. Triacsin C, used to inhibit lipid synthesis was purchased from A.G Scientific, Inc., (Cat. No. T1242). Primers were purchased from Integrated DNA Technologies (IDT), USA. Primary antibodies used were Rab 5 (SantaCruz, Cat.sc-46692) and Phalloidin (Invitrogen, Cat. A22287). Alexa Fluor-conjugated secondary antibodies and lipidTox (Invitrogen, Cat. H34477) were bought from Invitrogen molecular probes, USA. All antibodies used for flow cytometry were bought from BD Bioscience. Antibodies for western were procured from Cell Signaling Technologies (CST, 9946T).

4.3 Animals

C57BL/6 strain of mice (6-8 week old) was used throughout the study, which was provided by International Centre for Genetic Engineering and Biotechnology (ICGEB),

New Delhi, India. All mice used for the study were sacrificed at the particular time point by asphyxiation in the carbon dioxide chamber according to institutional guidelines and Department of Biotechnology, Government of India guidelines and regulations.

4.4 Mesenchymal Stem Cell (MSC): Culture and Characterization

MSCs were isolated from the bone marrow (BM) of healthy donors according to the institutional ethical guidelines. Cryopreserved human BM-MSC (n=3) were revived and cultured in low glucose containing Dulbecco's Modified Eagle's Medium (LG-DMEM), (Gibco, Gaithersburg, MD, USA) supplemented with 10 % Fetal Bovine Serum (Gibco, Gaithersburg, MD, USA) and incubated at 37˚ Celsius and 5% CO_2 in a humified CO_2 incubator. For the experiments, MSCs were used at passage number 3 throughout the study. For all experiments, cells were seeded at the required cell density, 24 hours before the experiment.

Cells were stained with MSC surface markers CD105, CD29, CD73, CD90, HLA-I and HLA-II, and Hematopoietic Stem Cell Marker CD34/45 and acquired on BD FACS LSRII and data was analyzed with Flow Jo (Tree star, USA). MSCs were subjected to tri-lineage differentiation for the characterization of human MSCs.

4.5 Bacterial Culture

H37Rv strain of *M.tb* used for infection was a kind gift from Colorado State University. *M.tb* was grown up to mid-log phase in 7H9 media (Middlebrooks, DifcoTM) supplemented with 10% OADC (Ovalbumin, Dextrose and Catalase, Difco^{TM}, USA), 0.05% Tween-80 and 0.2% glycerol. Cultures were cryopreserved in 20 % glycerol and preserved at -80° C until used for infection.

4.6 *M.tb* infection and estimation of Colony Forming Units (CFU)

Human BM-MSCs were infected at MOI of 1:50 for 6 hours and THP-1 (Human monocytic cell lines) (ATCC®TIB-202TM) and PBMC derived macrophages at 1:10 for 4 hours to obtain equal internalization of bacteria at 0 hours. After infection, cells were treated with 100µg/ml of Gentamicin (Gibco, USA) for 2 hours followed by washing and incubation at 37° C and 5% CO_2 in complete LG-DMEM and complete RPMI-1640 (Invitrogen) medium respectively. For in vivo experiments mice were infected with low-dose aerosol infection, with approximately 200 CFU per mouse. 15 ml of M. tuberculosis, H37Rv ($20x10^6$ bacteria/ml) in single cell suspension was used in nebulizer chamber to deliver the desired CFU of bacteria to the lungs of mice kept in the aerosol chamber for a 15-minute cycle. In-vitro bacterial colony numbers were counted by lysing 10,000 cells per well by using 250 µl of 0.05% SDS buffer and incubating for 5 minutes for both MSCs and macrophages. Dilutions were made and plated on 7H11 plates supplemented with 10% OADC (Difco, BD). The colonies were counted on day 21 and plotted.

4.7 Flow Cytometry

For flow cytometry, the cells were harvested, pelleted and washed twice with FACs buffer. Cells were stained with the antibodies for surface markers and incubated for half an hour. After incubation cells were washed twice with PBS and suspended in PBS. The acquisition was done on BD FACS LSRII and data were analyzed with Flow Jo (Tree star, USA). MSCs ($CD45^{-}Sca1^{+}$) were sorted from the bone marrow of *M.tb* infected

mice and macrophages ($CD45^+CD11b^+$) were sorted from the lungs of *M.tb* infected mice.

4.8 Peripheral Blood Mononuclear Cell (PBMC) isolation from blood and differentiation to macrophages

Blood was collected from the healthy donor in heparin-coated tubes and diluted in DPBS (Gibco, 14190250) in ratio 1:2. This blood DPBS diluent was layered onto Ficoll-Paque TM Plus (Cat. No. GE17-1440-02) and centrifuged at 800g for half an hour. The interphase cells containing the lymphocytes and monocytes were transferred to a new Falcon and subjected to RBC lysis. The PBMCs were then plated on Poly-D- lysine coated flasks and allowed to stay at 37° Celsius and 5 % CO_2 for 3-4 hours in DMEM without FBS. The non-adherent cells were washed with DPBS and the adherent population was treated with 50 ng/ml Macrophage Colony Stimulating Factor (MCSF) from R&D Systems. The media was changed every 2 days for 7-8 days. The attached macrophages were trypsinized and seeded for CFU experiment at 10,000 cells per well.

4.9 Transcriptomic and data analysis

Total RNA was isolated from MSCs infected with H37Rv using the Trizol method (Invitrogen). The RNA quality was analyzed by Agilent Bioanalyser and by running on the gel. Sequencing libraries were prepared using the Illumina Platform to generate~6 GB data per sample. The fastq files were analyzed using the Tuxedo protocol. The files were aligned against (GRCh37/hg19) version of the human genome. ClustVis, an online tool was used for generating heat maps (30).

4.10 Real-Time PCR

RNA was isolated using the Trizol method. cDNA was prepared using iScript cDNA synthesis kit (Cat. No. 1708890, Bio-Rad, USA), for MSCs and for bacterial cDNA synthesis, RevertAid First Strand cDNA synthesis kit (Cat. No. K1622, Thermo Fischer Scientific, USA) was used. Real Time PCR (qPCR) was performed in 10 µl reaction volume according to the manufacturer's instructions using the CFX96 Real-Time PCR Detection System (Bio-Rad, USA). All reactions were normalized to GAPDH for human MSCs and 16S rRNA for *M.tb*. See the supplemental material for primer sequences.

4.11 Confocal Microscopy

BM-MSCs and THP-1cells were seeded on glass coverslips (Cat. No. 2845-22, Corning) and infected with *M.tb*-GFP at the MOI mentioned above. After 6 hours and 4 hours of infection respectively, cells were washed twice with media supplemented with 10 % FBS and treated with 100 µg/ml gentamicin for 1 hour. After 2 hours, cells were washed twice, supplemented with fresh media and kept at 37° C and 5% CO_2. At the desired time point, the cells were washed twice with PBS and fixed in 2% Paraformaldehyde in PBS, pH 7.4 for 15-20 minutes. After fixation cells were washed three times with PBS at pH 7.4 and kept in 4˚C until used for staining. Staining was done using Rab5 (Cat. No. sc-46692), Phalloidin (Invitrogen, Cat. No. A22287) and LipidTox (Invitrogen, Cat. No. H34477) according to the manufacturer's protocol. Images were taken on Leica confocal SP5 microscope using 40X and 60X objectives as per the requirement. Analysis of images was done using the Leica Application Suite software.

4.12 Transmission Electron Microscopy (TEM)

MSCs were cultured in a 100 mm culture dish in LG-DMEM medium with 10% FBS. Cells at 70% confluency were infected with *M.tb* at MOI of 1:50. Cells were incubated at 37° C in 5% CO_2. At 96 hours' time point, infected cells were washed three times with PBS. Following this, cells were harvested (using a scraper) and fixed using 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and 4% PFA for 24 hrs at $4^{O}C$ (24, 25) with shaking at intervals and processed for sectioning at Advanced Instrumentation Research Facility (AIRF), JNU, New Delhi. Ultra-thin sections were cut (50-60nm) and collected on the nickel grid. Electron microscopic images were taken using a transmission electron microscope facility at AIIMS, New Delhi.

4.13 Western Blot

The whole cell lysate was prepared from uninfected and *M.tb* infected MSCs by using RIPA lysis buffer using Protease and Phosphatase inhibitor cocktail (Thermo Scientific, Cat. No. 78441). Western Blot analysis was done for Forkhead Signaling Kit (CST, 9946T). 40µg of protein was loaded into each well and electrophoresed on 12% SDS-PAGE and transferred to Nitrocellulose Membrane. Blots were probed with antibodies against NOTCH-1,p-FOXO3a(s318/321), p-FOXO3a(s253), FOXO1,p-FOXO1, FOXO4 and GAPDH.

4.14 Statistical Analysis

Statistical Analysis was performed using GraphPad 5.0 software. Student t-test and ANOVA followed by Bonferroni post-test was used to determine statistical significance.

Error bar represent S.E.M. *** represents P<0.001, ** P<0.01 and *P<0.05. P>0.05 is taken non-significant (NS).

Table 1: List of primers used in the study

Gene Name	Primer Sequence (5'→3')
Rv0001 F	GCGACGTAGACGTGCTGTTG
Rv0001 R	GGCATTGTGCAAGG TGTTGA
dnaA F	CCAGACACCACAACCGACAA
dnaA R	TGGCCAACTGTGCTGGTTATC
ftsZ F	GCAGCGACTTGGGCTT GTTC
ftsZ R	GGGTGAGCGGCGTCTTGTAC
parB F	GCGTAAGCCGATTCAGATGCC
parB R	CC GACGCGAACTCCACCAC
smc F	TGAACTGGATCAAGGCGAGGTC
smc R	CAACGCGGCCACAGTACG
Rv0058 F	GAGAGGCGACGCGTACTGGG
Rv0058 R	ATGACAACGTCGGCATCTTG
Rv0169 F	CCGGAATCGTTGGCGGCGCG
Rv0169 R	TGCATAAGGCGAGCCAACCT
dnaN F	CGGTGAGACGGTGGTTTTG
dnaN R	CGACGCCGACCACTTCAG
parA F	TCACCACCGTGATCCTTACCA
parA R	CTTGATCGGCGAGCT TTGTC
ftsQ F	TGACGTTGGCCGATGGCCGCG
ftsQ R	GCCTGGCTGGGTCAACAGCGC
rodA F	TGACCTTCCTCAAGGACCAC
rodA R	GCCGAAAAGAAGATCAGCAG
pbpA F	GTGAGGCATTCGTCAAATCA
pbpA R	TTTTGGCCGATACTGGTCAT
Rv0571c F	ACG GTG CGG ACA AGG TGG TGC TG
Rv0571c R	CGC CAA ACA CAC CAC CTC ATC GG
narK2 F	CTG GTA CCA GCC GGC GCG
narK2 R	AAC CGC GGG GTG AAG AAC GC
Rv1738 F	CGA CGA ACA CGA AGG ATT GA
Rv1738 R	ACA CCC ACC AAT TCC TTT TCC
ctpF F	CAG CAC CAC GGT CAT CTG
ctpF R	ATC TCA CCG TGG GGT GTC
otsB1 F	GAT CTA CCC TGA GCG CTG TC
otsB1 R	GAT ATC GGA GCG CAG GAC

fdxA F	TGT CCG GTC GAC TGT ATC TAT GA
fdxA R	GGC AGG CCG GTT TGC
hspX F	CGC ACC GAG CAG AAG GA
hspX R	ACC GTG CGA ACG AAG GAA
acg F	GCT TTT GAG ACT TCT GAG GGC ATA
acg R	GGT GAC CCG GTC ACT TTC G
tgs1 F	TGG CTG CCG GGC CTT TCC C
tgs1 R	GCA GGG CCA AAG GTC CTC C
Rv3131 F	CGA TCA GGC CGA TGT CGC CTT
Rv3131 R	TCA CCT CCT GGC ACC GGC C
devR F	CCG ATC TGC GCT GTC TGA TC
devR R	GTC CAG CGC CCA CAT CTT T
Rv3134c F	CTG GCT GGG TCG GCC TTA
Rv3134c R	GCT GAC CTG GGA GGT TGT CG
Human PCNA F	GGCTCTAGCCTGACAAATGC
Human PCNA R	CTAGCTGGTTTCGGCTTCAG
Human CCNA1 F	GCCACCTGCAGTTCTTCTTC
Human CCNA1 R	CAACGTGCAGAAGCCTATGA
Human FoxO3 F	GGCGGACTTTGTGTTTGTTT
Human FoxO3 R	AAGCCACCTGAAATCACACC
Human SKP2 F	GTAGAGACGGGGTTTCACCA
Human SKP2 R	GCAGTTGCTCATGCCTGTAA
Human gapdh F	GGCCTCCAAGGAGTAAGACC
Human gapdh R	AGGGGTCTACATGGCAACTG
Human notch1 F	GGAGGCATCCTACCCTTTTC
Human notch1 R	TGTGTTGCTGGAGCATCTTC

Table 2: List of materials used in the study

Material	Source	Identifier
RPMI-1640 Medium	Sigma	Cat# R8755
L-Glutamine	Cell Clone	Cat# CC4023
Antibiotic Solution 100X Liquid	Himedia	Cat# A001A
Lipofectamine 2000 Reagent	Invitrogen	Cat# 11668-091
Hygromycin B Solution	Himedia	Cat# A015
Trypsin- EDTA Solution 1X	Himedia	Cat# TCL007
OptiMEM	Gibco	Cat# 31985-070
HEPES buffer	Sigma	Cat# H3375-500G
Penicillin	Sigma	Cat# 13752
Ampicillin	Sigma	Cat# 10835242001
Streptomycin	Sigma	Cat# S9137
Sodium bicarbonate	Himedia	Cat# TC230
Potassium phosphate monobasic	Himedia	Cat# GRM3943
Sodium chloride	SRL	Cat# 41721(1949134)
DPBS/MODIFIED	Gibco	Cat# 14190250
Sodium pyruvate	Sigma	Cat# S8636
DMEM	Sigma	Cat# D5796

Eosin	Sigma	Cat# HT110332-1L
DPX mountant	Sigma	Cat# 06522-100ML
10% SDS	Bio-Rad	Cat#1610416
Beta-marcaptoethanol	Bio-Rad	Cat# 1610710
TEMED	Bio-Rad	Cat# 161-0801
30% Bis-acrylamide	Bio-Rad	Cat# 1610156
Protein ladder	Bio-Rad	Cat# 161-0380
Potassium chloride	Sigma	Cat# P9541
Magnesium chloride	Sigma	Cat# M8266
Skimmed milk	MERK	Cat# 1.15363.0500
Methanol	SDFCL	Cat# 39192-S25
FBS	Gibco	Cat# 10270
Molecular grade ethanol	MERK	Cat# 1.00983.0511
Nitrocellulose membrane	Bio-Rad	Cat# 1620112
Rapamycin	Sigma	Cat# R8781
Isoniazid	Sigma	Cat# I3377
Phorbol 12-myristate 13 acetates (PMA)	Sigma	Cat# P1585
Paraformaldehyde	Sigma	Cat# P6148
Dexamethasone	Sigma	Cat# D1756

Triacsin C	A.G Scientific, Inc	Cat# T1242
Rab 5	SantaCruz,	Cat# sc-46692
Phalloidin	Invitrogen	Cat# A22287
LipidTox	Invitrogen	Cat# H34477
Forkhead Signalling antibody Kit	CST	Cat# CST# 9946T
7H9	BD DifcoTM	Cat# DF0713-17-9
7H11	BD DifcoTM	Cat# 212203
OADC	BD DifcoTM	Cat# 211887
Gentamicin	Gibco	Cat# 15750045
Ficoll-Paque TM Plus	GE healthcare	Cat# GE17-1440-02
MCSF	RnD Biosystem	Cat# 216-MC-010
Trizol	Ambion	Cat# 15596018
iScript cDNA synthesis kit	Bio-Rad	Cat# 1708890
RevertAid First Strand cDNA synthesis kit	Thermo	Cat# K1622
Coverslips	Corning	Cat#2845-22
Phalloidin	Invitrogen	Cat# A22287

CHAPTER 5
Results

5.1 Culture and characterization of Mesenchymal Stem Cells (MSCs)

Bone marrow (BM) is the main reservoir of adult hematopoietic stem cells (HSCs) that refurbish circulating blood elements. BM also contains mesenchymal stem cells (MSCs), which contribute to the regeneration of mesenchymal tissues such as bone, muscle, cartilage, tendon, ligament, stroma and adipose tissues (Prockop 1997; Friedenstein et al., 1976; Ashton et al., 1980). The process of density gradient centrifugation is used for isolation of MSCs from the other cell types present in the BM.

A very small proportion of the isolated cells (approximately 0.001 to 0.01%) grew and formed fibroblast-like colonies after 5-7 days of initial *ex vivo* culture. Other non-adherent cells such as RBCs and HSCs were removed by washing with fresh complete media. In our study we have isolated Human BM-derived mesenchymal stem cells (BM-MSCs) from healthy donors (n=5) after their consent from AIIMS, New Delhi. BM cells were seeded in a 60mm culture dish. After adherence, culture media was changed every third day until the cell confluency reached to 80%. Adherent cells were passaged with 0.05% trypsin-EDTA and reseeded at the required cell density for all experiments.

International Society for Cellular Therapy (ISCT) has recommended three minimal criteria's to define human MSCs.

1. MSCs must be plastic adherent expressing CD105, CD73 and CD90 and should not express hematopoietic markers, CD45, CD34, and CD14 OR CD19 and HLA-DR surface molecules.

2. They should be capable of differentiation to osteogenic, adipogenic and chondrogenic lineages (Dominici et al., 2006).

3. They should have the ability to form colony forming units (CFU) which is the characteristic of MSC's self-renewal property. (La Rocca et al., 2009)

MSCs upon culture has a spindle-shaped /fibroblastoid morphology. Throughout the study, human bone marrow stem cells (BM-MSC) at passage 3 were used for experiments **(Figure 1)**. MSCs were initially known as fibroblastoid colony-forming-cells because of their feature of adhering to tissue culture dish and formation of stem cell colonies. The efficiency with which they form colonies still remains an important assay for assessing the quality of the cell preparations.

The BM-MSCs were estimated for their colony forming ability and the self-renewal capacity by Colony Forming Unit-Fibroblast (CFU-F) assay. The MSCs were seeded at a density of 1×10^5 in DMEM supplemented with 10% FBS. The cells were incubated at 37 °C in 5 % CO_2 incubator. The medium was changed after 7 days initially and afterward, every 3rd day. After 2 weeks of culture, the stem cell colonies were stained with 0.05 % crystal violet and counted. Clusters with more than 50 cells together, were considered as colonies **(Figure 2).**

Flow cytometry analysis

MSCs express surface markers characteristic of stem cells. These markers include CD 105, CD 73, CD 90, CD 29 and sca1. MSCs are distinguished from HSCs by the absence of CD 45 and HLA II (Prockop, 1997). For the characterization, the cells were scrapped and washed twice with PBS containing 2 % FBS. FITC conjugated anti-human CD29, anti CD34/45 and anti-HLA-II, PE-conjugated anti-human CD73, APC conjugated anti-CD105 and anti-HLA-II and PerCP-Cy 5.5 conjugated anti-human CD90 antibodies were

used for staining the cells. All antibodies were purchased from BD Biosciences. BM-MSCs cultured and characterized by us showed the expression of MSC markers and lacked HSC markers **(Figure 3).**

In-vitro differentiation of BM-MSCs to osteogenic, adipogenic and chondrogenic lineages

The differentiation potential of MSCs was identified by their osteogenic, adipogenic and chondrogenic differentiation and demonstrated by positive staining with Alizarin red, Oil O red and Alcian blue staining, respectively (Kulterer et al., 2007; Schilling et al., 2007).

Osteogenic differentiation

BM-MSC at 3rd passage were treated with osteogenic differentiation medium comprising of complete DMEM-LG, 50 µM ascorbic acid-2-phosphate, 0.1 µM dexamethasone and 10 mM ß-glycerophosphate for 4 weeks with a change in media every 2 days. The differentiation into osteoblasts was confirmed by Alizarin Red S staining **(Figure 4)**.

Chondrogenic differentiation

For chondrogenic differentiation, commercially available kit from GIBCO was used. Single cell suspension of BM-MSC at 3rd passage was prepared with a concentration of 1.6×10^7 viable cells/ml. The micro-mass culture was created by seeding of 5 µl droplets of cell solution in 35 mm tissue culture plate and incubated for 2 hours at 37 °C and 5% CO2. The media was replaced every 3rd day for 14 days duration. Untreated MSCs were taken as controls. The differentiated cells were stained with Alcian Blue staining to confirm the differentiation state **(Figure 5).**

Adipogenic differentiation

BM-MSC at 3rd passage were treated with adipogenic differentiation medium containing complete DMEM-LG, 100 μM indomethacin, 1 μmol/l dexamethasone, 500 μM 3-isobutyl-1- methylxanthine, 1 μg/ml insulin for 21 days (5). Untreated MSCs were considered as an experimental control. The medium was changed every third day. The differentiated cells were stained by Oil Red O **(Figure 6).**

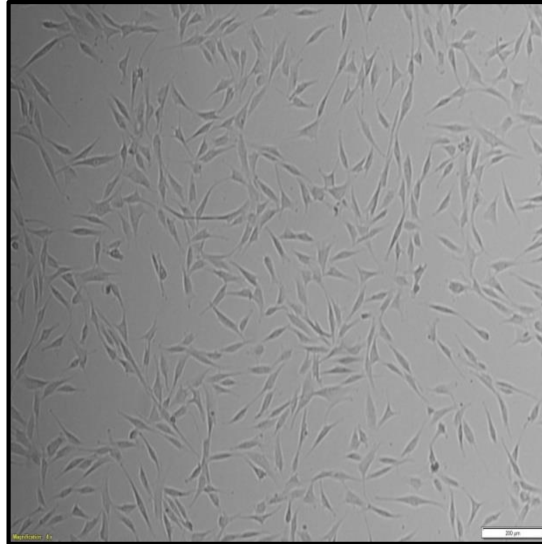

Figure 1. Image of Bone Marrow derived Mesenchymal Stem Cells (BM-MSCs) at passage 3 adhered to the surface of 100mm culture dish (Scale bar 200 μM).

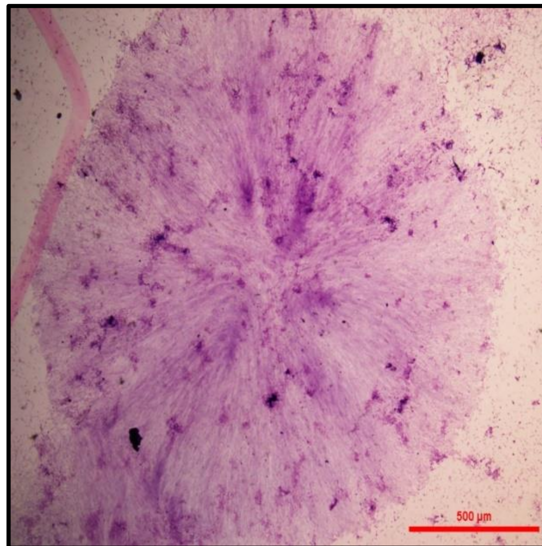

Figure 2. CFU-F assay for the estimation of self-renewal capacity of BM-MSCs. After 2 weeks of culture, the stem cell colonies were stained with 0.05 % crystal violet and counted. Clusters with more than 50 cells together, were considered as colonies (Scale bar 500 μM).

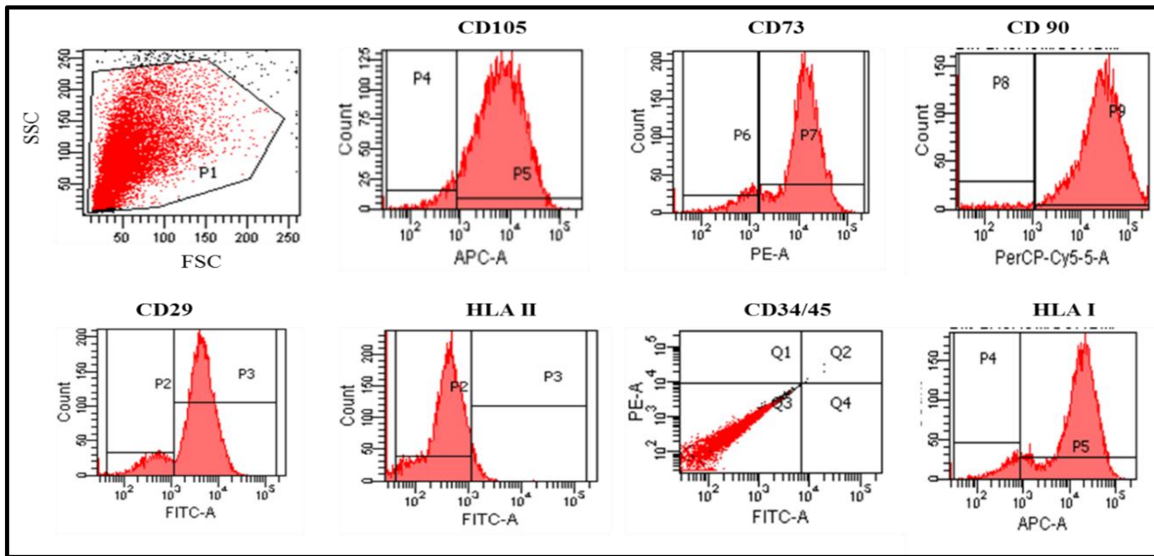

Figure 3. Positive expression of mesenchymal stem cell markers (CD105, CD 73, CD90 and CD 29) and negative expression of hematopoietic markers (CD34/45, HLA-I and HLA-II) in BM-MSC at passage 3.

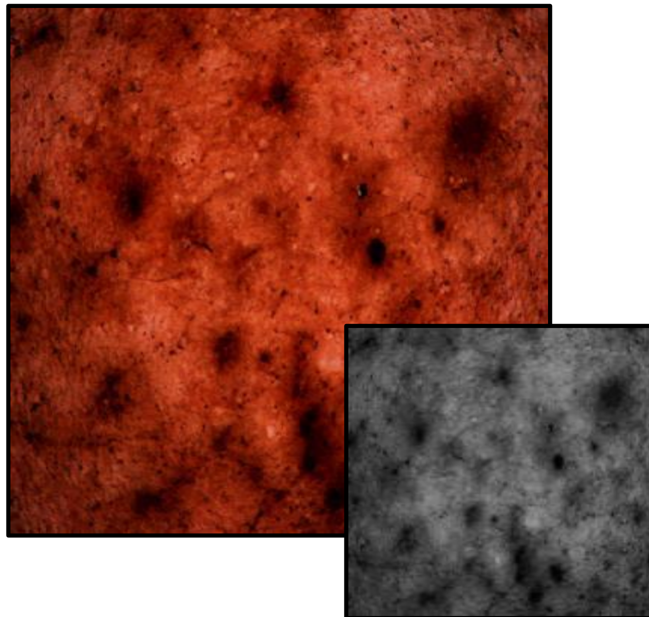

Figure 4. Image showing the differentiation of hMSCs into osteocytes (Alizarin Red Staining).

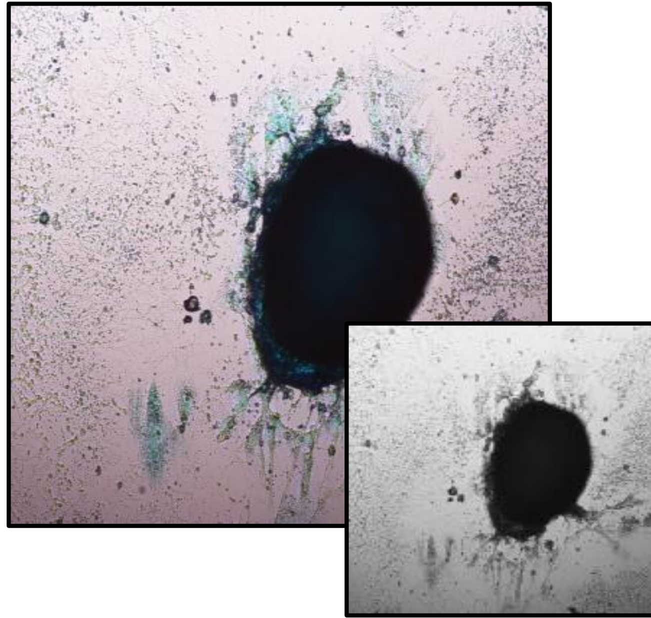

Figure 5. Image showing the differentiation of hMSCs into chondrocytes (Alcian Blue Staining).

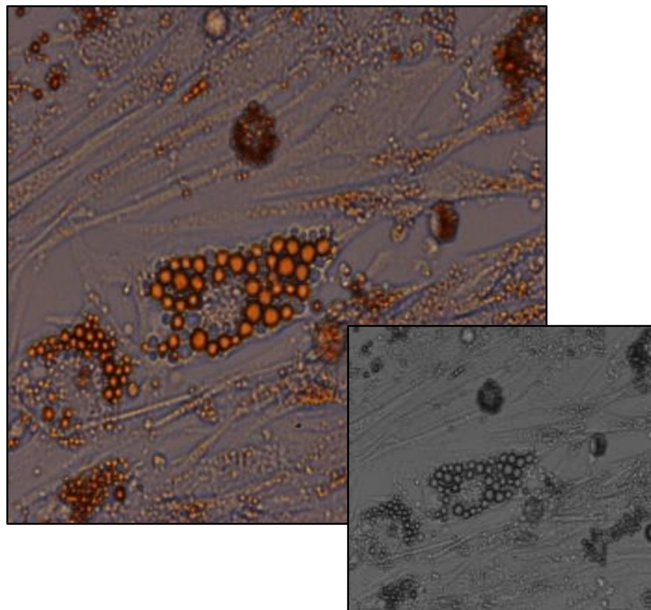

Figure 6. Image showing the differentiation of hMSCs into adipocytes (Oil Red O Staining).

5.2 *M.tb* (H37Rv) infects MSCs and macrophages differentially

Tuberculosis is difficult to treat because of the ability of *M.tb* to attain a dormant, non-replicating and asymptomatic form and its ability to evade the host immunity. Professional antigen presenting cells such as macrophages and dendritic cells (DCs) are already known host for *M.tb* infection and growth. Recently, we and others have shown that *M.tb* supports recruitment of Mesenchymal Stem Cells (MSCs) at the site of infection and by doing so suppresses the host immune response (Raghuvanshi et al., 2010; Das et al., 2015, Khan et al., 2017). MSCs are associated with non-replicating bacteria (Das et al., 2015). Therefore to investigate whether MSCs are the natural reservoir of *M.tb* infection, we infected MSCs and PBMC derived macrophages with *M.tb* and found that interestingly, MSCs uptake a very low number of bacteria as compared to macrophages. Upon increasing multiplicity of infection (MOI) in MSCs at MOI 50 for 6 hours infection and MOI 10 for 4 hours infection in macrophages, equal number of bacteria internalize in both cell types. Interestingly, with the progression of time, bacteria continued to multiply within macrophages till the cells underwent cell death after 72 hours. Conversely, in MSCs, *M.tb* numbers continued to decrease, reached a plateau by 72 hours and remained there in viable form till an extended period of time **(Figure 7).** These results support the fact that *M.tb* infects MSC albeit at a lower rate and establishes infection within MSCs which comprises mainly of viable non-replicating bacteria. MSCs support the survival of very few bacteria at 72 hours as compared to macrophages as indicated by the above figure. Thus, our confocal microscopy data reveals the number of bacteria per cell in MSCs and macrophages. The bacteria in MSCs are very few compared to the macrophages (THP-1) which are the natural reservoir of

M.tb infection supporting our CFU data of Figure 7, and thus indicating that MSCs support a minimal number of *M.tb* organisms which remains unchanged till 120 hours **(Figure 8).**

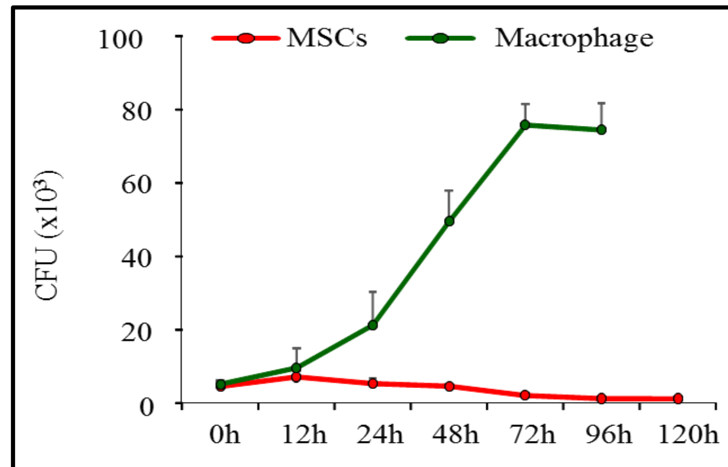

Figure 7. Growth Kinetics of *M.tb* in BM-MSCs and PBMC derived macrophages at different time points in a time-course experiment.

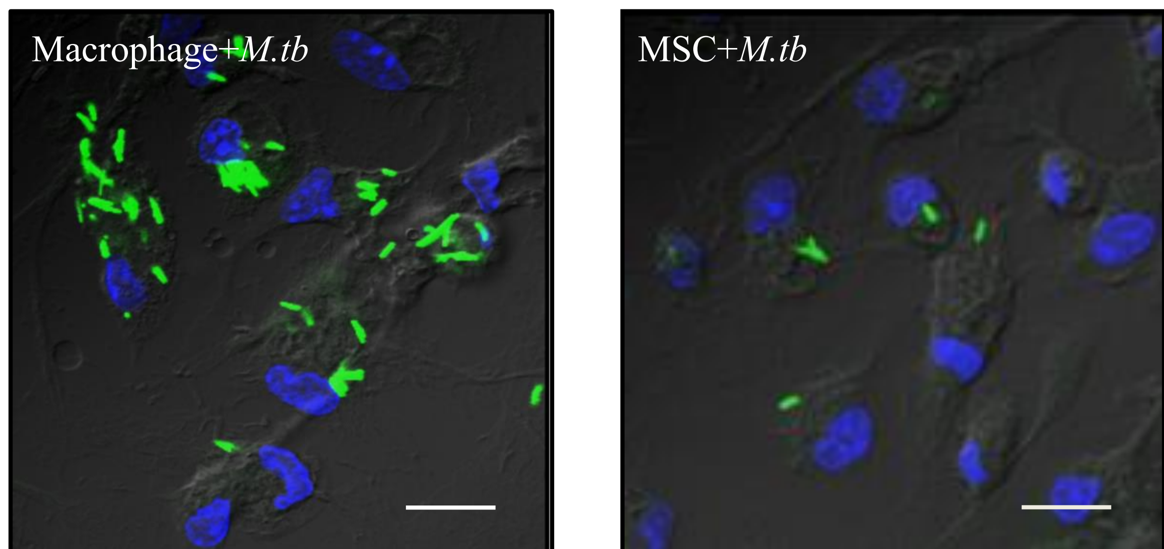

Figure 8. Confocal microscopy images of macrophage and MSCs infected with *M.tb*-GFP at 72 hours post infection (Taken at 40X, Scale bar 200 μM).

5.3 *M.tb* resides in different cell compartments after internalization in MSCs and macrophages

To understand the differential behavior of *M.tb* survival in macrophages and MSCs, we examined the localization of *M.tb* in MSCs and macrophages immediately after infection (0 hours) and after reaching the plateau in MSCs (72 hours) and at 96 hours. Interestingly, we found that immediately after infection, macrophages have maximum bacteria localized in the early endosomes whereas in MSCs maximum number of bacteria localized in the cytosol (**Figure 9, 10)**. We used Rab5 as an endosomal marker, which stains early endosomes and phalloidin as a marker for cytosolic localization. Phalloidin binds to F-actin which is abundant in the cytosol of the cells (Frimmer M, 1987). When we analyzed the percentage co-localization of *M.tb* with Rab5 and phalloidin in both MSCs and macrophages, we found a significant difference in localization pattern in the two cell types. In macrophage, more than 90 percent of bacteria colocalized with early endosomes, whereas in MSCs, this reduced to approximately 15 percent. In contrast, in MSCs approximately more than 85 percent bacteria resided in the cytosol whereas for macrophages this number reduced to just 20 percent **(Figure. 11).**

]

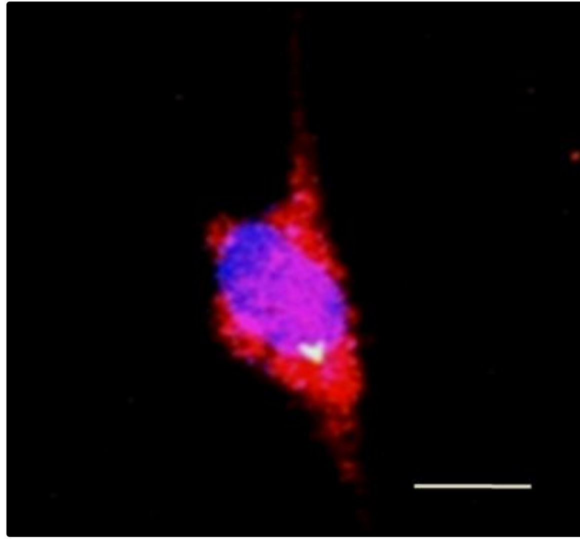

Figure 9. Confocal microscopy images of *M.tb*-GFP infected macrophages with *M.tb* localizing in the early endosome immediately after infection (0 hour). Scale 200 μM.

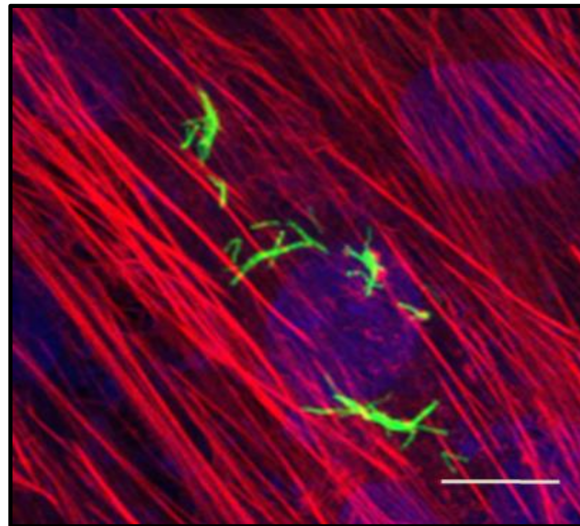

Figure 10. Confocal microscopy images of *M.tb*-GFP infected MSCs with *M.tb* localizing in the cytosol immediately after infection (0 hour). Scale 200 μM

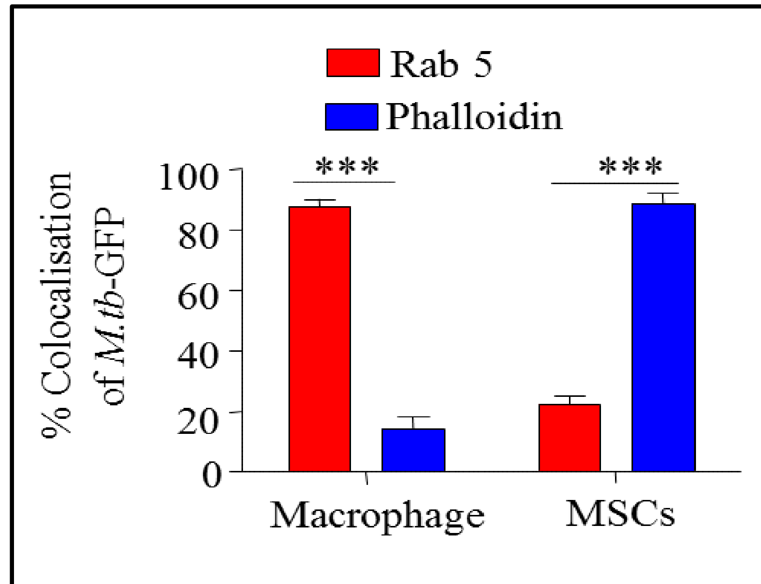

Figure11. Graphical representation of percentage colocalization of *M.tb* with endosomes (Rab5) and cytosol (Phalloidin) in both MSCs and macrophages (THP-1). Red bar (Rab5) and Blue bar (Phalloidin).This data was plotted after calculating percentage co-localisation of *M.tb* in 30 fields each of macrophage and MSCs immediately after infection. Error bar represent S.E.M. *** represents $P<0.001$, ** $P<0.01$ and *$P<0.05$. $P>0.05$ is taken non-significant (NS).

5.4 MSCs shield the harbored bacteria in lipid droplets by the activation of lipid synthesis pathway

Many studies have reported the role of lipids in affecting the growth of *M.tb* in macrophages (Gengenbacher and Kaufmann 2012; Singh et al., 2009). Thus, many studies have established that host lipids integrate into mycobacterial triacylglycerols, which has been associated with dormancy like phenotype in *M.tb* (Sirakova et al., 2006; Daniel et al., 2011). Other studies have also shown that foamy macrophages containing lipid bodies facilitate the persistence of *M.tb* (Peyron et al., 2008; Russell et al., 2009). Role of lipids in *M.tb* infection in MSCs has not been elucidated so far. We performed RNA sequencing in MSCs at 48 hours and 96 hours after infection to get an insight into the pathways which were getting differentially expressed in MSCs. We found that after infection out of the several pathways, sphingolipid pathway was getting significantly altered in MSCs indicating that lipid synthesis is an important pathway in *M.tb* infected MSCs and the expression of the genes involved was higher compared to macrophages. **Figure 12** shows the heatmap of genes involved in sphingolipid synthesis pathway which were getting differentially expressed.

Moreover, we checked for the accumulation of lipid droplets after *M.tb* infection in both macrophages and MSCs using confocal microscopy; using lipid binding strain LipidTox. The LipidTOX™ neutral lipid stain binds to neutral lipids with an extremely high affinity and can be detected by fluorescence microscopy. Interestingly we found abnormal lipid accumulation in MSCs **(Figure 13)** which was much higher than in macrophages **(Figure 14).** Then we sought to determine the percentage colocalization of *M.tb* with the lipid droplets in both MSCs and macrophages. Surprisingly, the percentage colocalization of

M.tb with lipid droplets was much higher in MSCs than macrophages **(Figure 15).** Moreover, the intensity of lipid droplets was found to be significantly higher in MSCs compared to macrophages and it continued to increase in a time-dependent manner **(Figure 16).** These findings establish that MSCs have higher lipid content than macrophages which is utilized by the *M.tb* to establish successful infection and use host lipid for its survival in the host.

Electron microscopy data also confirmed the presence of *M.tb* in neo-lipid droplets thwarting the antimicrobial defense mechanism **(Figure 17)**. Lipid droplets observed as round structures in microscopy studies (Fujimoto et al., 2011). They have diameters ranging from 0.1–5 μm in non-adipocytes, to 100 μm in the white adipocyte. The lipid droplets are not bound by a unit membrane but enclosed by a phospholipid monolayer (Tauchi-Sato et al., 2002). Phospholipids making up the lipid droplet boundary include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol, lysoPC, and lysoPE (Leber et al., 1994; Tauchi-Sato et al., 2002; Bartz et al., 2007). They also incorporate free cholesterol mostly at the boundary surface (Prattes et al., 2000).

Taken together, *M.tb* utilizes host lipid to survive and maintain a non-replicative state in the MSCs which is essential for its survival and for bypassing the host immune response.

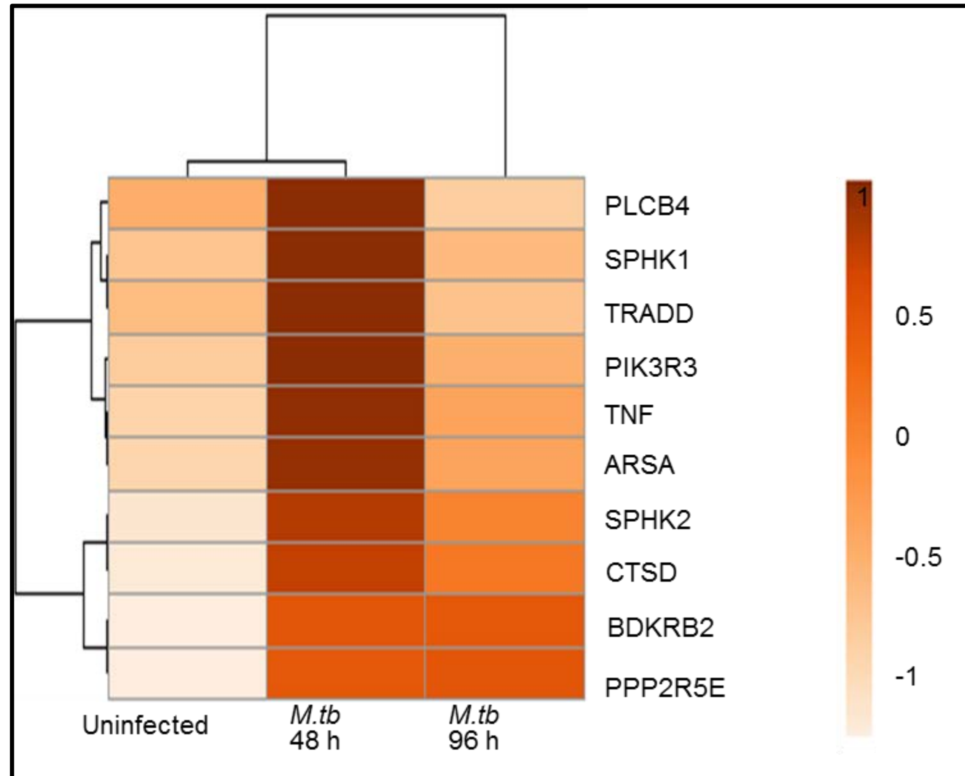

Figure 12. Heat map showing differential regulation of genes involved in sphingolipid synthesis pathway.

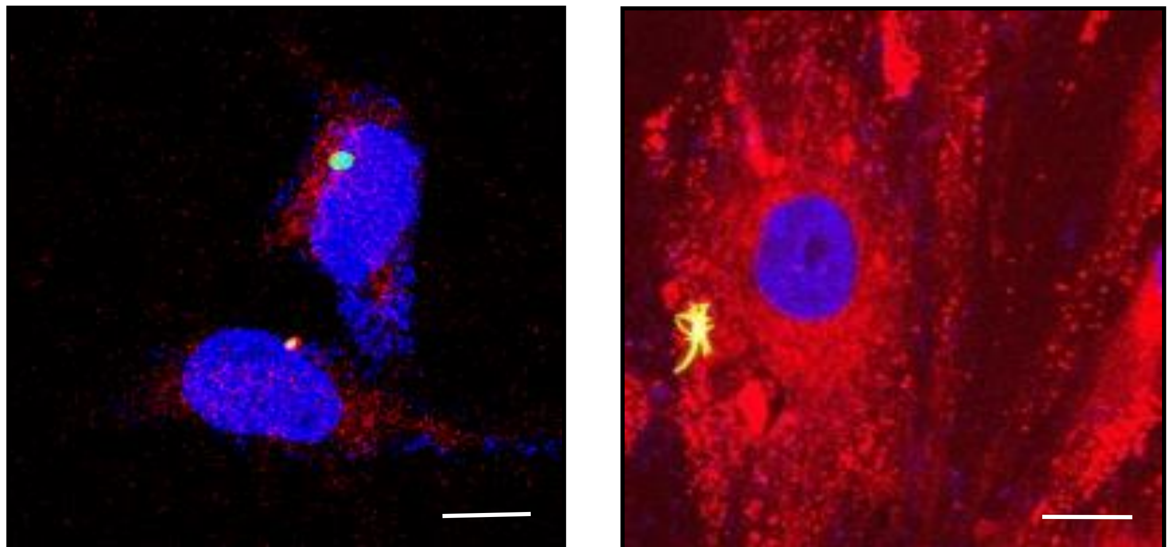

Figure 13. Confocal microscopy images showing colocalization of *M.tb*-GFP with lipid bodies stained with LipidTox in macrophages (THP-1) and MSCs at 72 hrs post infection. Scale bar represents 200 µM

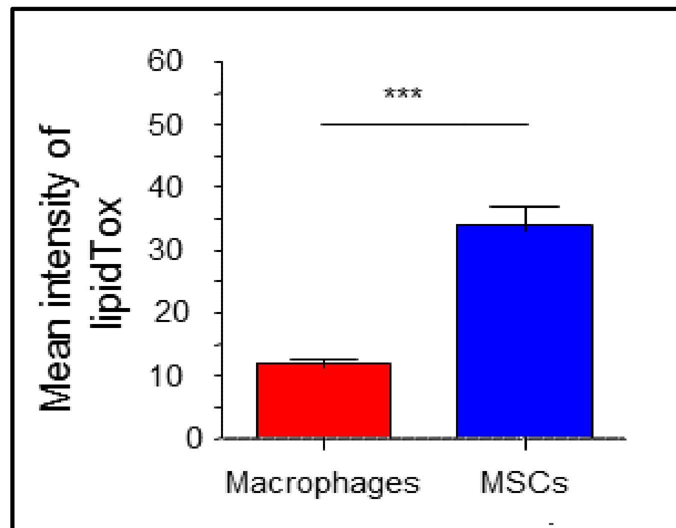

Figure 14 Graphical representations of the mean intensity of lipid bodies stained with LipidTox in macrophages (THP-1) and MSCs post infection with *M.tb*-GFP at 72 hours. Error bar represent S.E.M. *** represents $P<0.001$, ** $P<0.01$ and *$P<0.05$. $P>0.05$ is taken non-significant (NS).

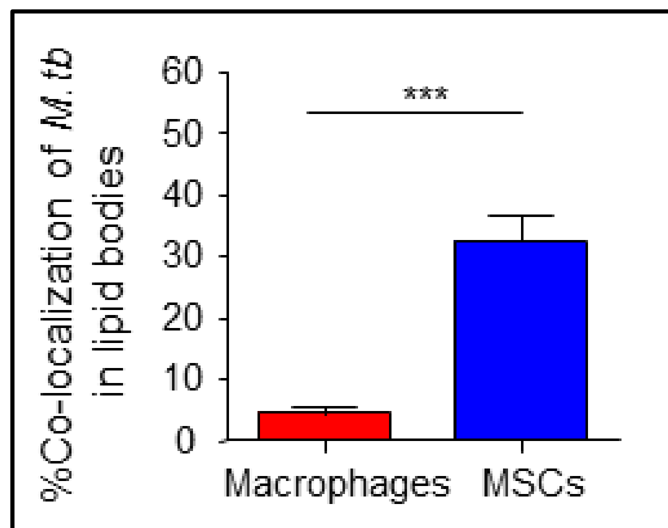

Figure 15 Graphical representation of the percentage co-localization of *M.tb*-GFP with lipid bodies in both macrophages (THP-1) and MSCs. Error bar represent S.E.M .*** represents $P<0.001$, ** $P<0.01$ and *$P<0.05$. $P>0.05$ is taken non-significant (NS).

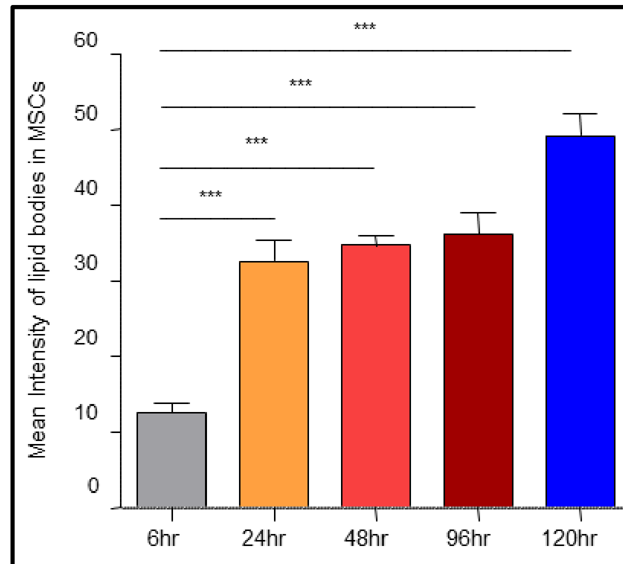

Figure 16 Graphical representation depicting the increasing mean intensity of lipid bodies stained with LipidTox in MSC from 0 hours upto 120 hours. Error bar represent S.E.M. *** represents $P<0.001$, ** $P<0.01$ and *$P<0.05$. $P>0.05$ is taken non-significant (NS).

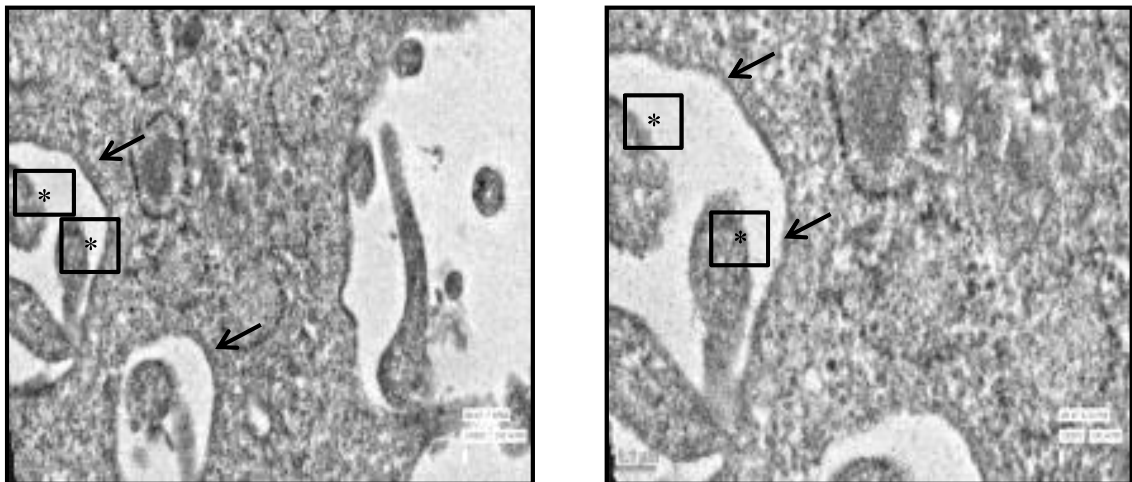

Figure 17 Transmission Electron Microscopy (TEM) images of MSCs infected with *M.tb*, 72 hrs post-infection. Lipid droplets (arrowheads) and *M.tb* (*asterisk) are indicated. Images were taken at 9900X (left) and 19500X (right).

5.5 MSCs and macrophages are differentially programmed to support dormant and active form of *M.tb* respectively

Our previous results establish that macrophage and MSCs are programmed differently to respond to *M.tb* infection. MSCs yield non-replicating bacteria whereas macrophages bear actively replicating bacteria. MSCs have high lipid synthesis owing to which they shield *M.tb* from host immune system. Bacteria use the niche provided by the lipid droplets to acquire nutrition and for their unhindered survival. To decipher this difference of survival conditions of *M.tb*, we checked for the expression of dormancy regulon of *M.tb* and replication genes at 72 hours, the time point at which *M.tb* in MSCs neither increased in number nor decreased, and reached a plateau with most bacteria in non-replicating form. Dormancy acquired by *M.tb* is the main reason behind the failure of anti-tuberculosis drugs. The genes responsible for this non-replicating state constitute the dormancy survival regulator (DosR) regulon which is composed of 48 co-regulated genes. These co-regulated genes are mainly responsible for *M.tb* persistence in the host. The DosR regulon is composed of a two-component regulatory system comprising of two sensor kinases-DosS (Rv3132c) and DosT (Rv2027c) kinase, and a response regulator DosR (Rv3133c). The regulatory mechanism of expression of DosR regulon is very complicated. Many factors are involved in the expression of the dormancy genes, oxygen tension being the most important. The DosR regulon enables the pathogen to persist during hypoxic conditions and stress.

We checked for the expression of dormancy and replication genes both in macrophages and MSCs. Interestingly; we found that there was sustained expression of dormancy associated devR/dosR regulon genes in *M.tb* isolated from MSCs but not macrophages

(Figure 18). However, the genes that are involved in various stages of replication such as replication initiation and cytokinesis were enriched in *M.tb* isolated from infected macrophages but not MSCs **(Figure 19)**. This difference in the expression of dormancy and replication genes in the two cell-types indicated that MSCs provide the niche to dormant bacteria while macrophages do not support the dormant population.

To explore in vivo relevance, and to confirm that BM-MSCs support dormant *M.tb*, we sorted $CD45^-Sca1^+$ MSCs from the BM of infected mice. To compare, we sorted $CD45^+CD11b^+$ macrophages from the lungs of the same infected mice. After sorting we got 99% pure population of both MSCs and macrophages. We analyzed the expression of dormancy and replication related genes in these cells. Consistent with the *in vitro* data, we found that *M.tb* in MSCs express dormancy-related genes whereas *M.tb* that are in macrophages express replication-related genes **(Figure 20 and 21)**. Thus our *in vitro* and *in vivo* data strongly confirm that BM-MSCs shield dormant bacteria which lead to the dormant phenotype of the bacilli in the host.

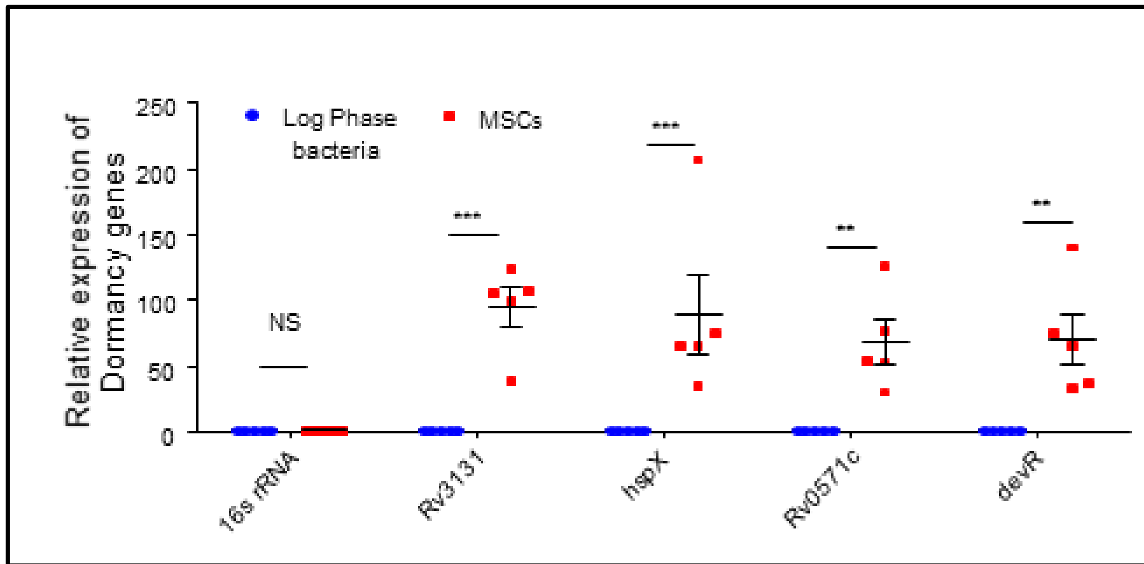

Figure 18 Graphical Representation depicting the relative expression of dormancy genes of *M.tb* within MSCs and macrophages (derived from human PBMCs, from 5 donors) at 72 hours post infection as compared to log phase bacteria. Error bar represent S.E.M. *** represents P<0.001, ** P<0.01 and *P<0.05. P>0.05 is taken non-significant (NS).

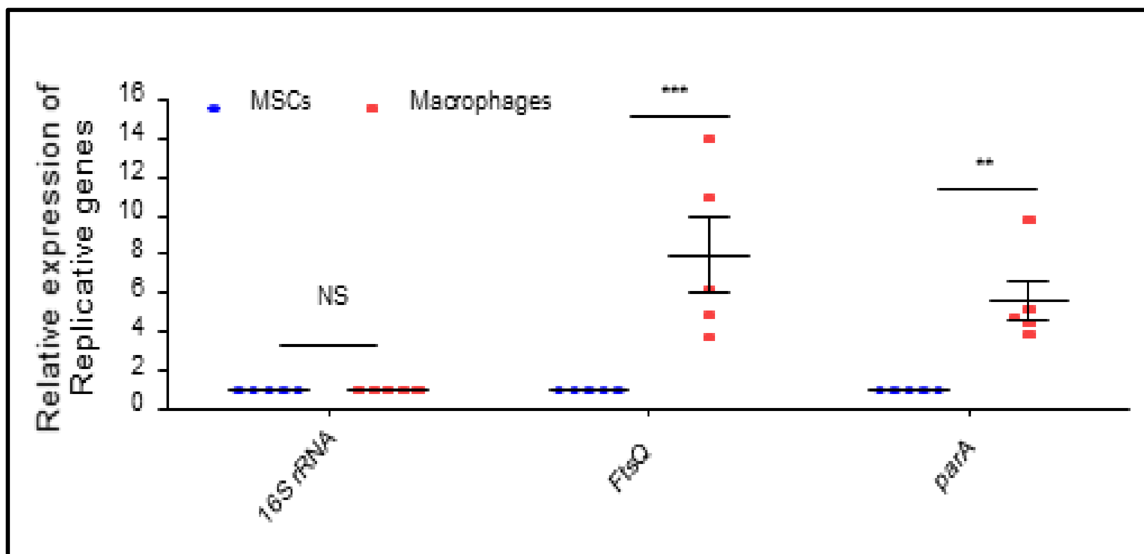

Figure 19. Graphical representation depicting the relative expression of replicative genes of *M.tb* in MSCs and macrophages (derived from human PBMCs, from 5 donors) at 72 hours post infection. Error bar represent S.E.M. *** represents P<0.001, ** P<0.01 and *P<0.05. P>0.05 is taken non-significant (NS).

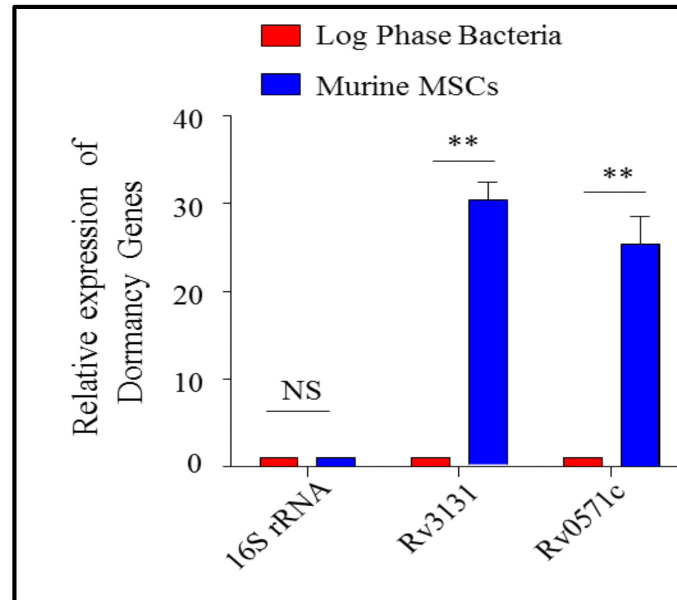

Figure 20 Graphical representation depicting the relative expression of dormancy genes of *M.tb* in CD45-Sca1+ MSCs sorted from the bone marrow of infected mice as compared to log phase bacteria. Error bar represent S.E.M. *** represents P<0.001, ** P<0.01 and *P<0.05. P>0.05 is taken non-significant (NS).

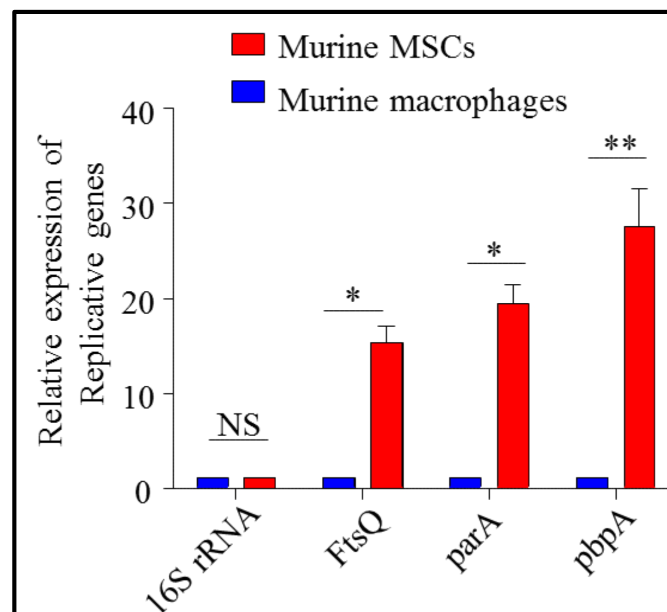

Figure 21 Graphical representation depicting the relative expression of replicative genes of *M.tb* in CD45+CD11b+ macrophages sorted from the lungs of infected mice as compared to MSCs from the bone marrow. Error bar represent S.E.M. *** represents P<0.001, ** P<0.01 and *P<0.05. P>0.05 is taken non-significant (NS).

5.6 MSCs induce a quiescent state upon infection with *M.tb* and remain viable for an extended period

Mesenchymal Stem Cells (MSCs) are self-renewing cells, prompting us to explore whether *M.tb* infection affects MSC replication. Surprisingly we found that *M.tb* infection inhibits MSC replication in a time-dependent manner. Therefore, we measured the expression of quiescence markers characteristic of stem cells. RNAseq analysis showed up-regulation of several quiescence markers and down-regulation of cell cycle progression markers in human MSCs infected with *M.tb* **(Figure 22)**. This was confirmed by qPCR of selected quiescence-markers such as FOXO-3, NOTCH-1 and SOX-9, which were upregulated in MSCs as compared to macrophages **(Figure 23)**. These quiescence markers were also are present in macrophages but at dramatically reduced levels than in MSCs, which agrees with a previous report (Holt DJ and Grainger DW, 2012). In contrast, cellular proliferation -markers, S-phase kinase 2 (SKP2) and CCNA encoding cyclin A2 were highly up-regulated in macrophages **(Figure 24)**. To further confirm the phenomenon of quiescence in MSCs we performed western blot analysis using antibodies for quiescence markers. Western blot analysis confirmed enhanced expression of NOTCH-1, FOXO-3 and p-FOXO-3 at Ser318/321 **(Figure 25)**. Phosphorylation of FOXO-3a at Ser318 and/or 321 causes its nuclear -exclusion and inhibits its transcriptional activity (Wang et al., 2017). Thus, the increased FOXO-3a phosphorylation might be essential in modifying the transcriptional activity to inhibit MSC proliferation. Although phosphorylation of FOXO-3a at Ser253 is known to exert an inhibitory response on its transcriptional activity (Wang et al., 2017), we did not observe any significant change in the phosphorylation status of FOXO-3a at this site.

Additionally, there was no significant difference in the protein levels of FOXO-1 and FOXO-4 or p-FOXO-1 **(Figure 26)**, suggesting that these quiescence markers might play a prominent role in attaining a quiescent state in MSCs. This observation implied that upon infection, *M.tb* acquires dormancy whereas MSCs enter into a quiescent state.

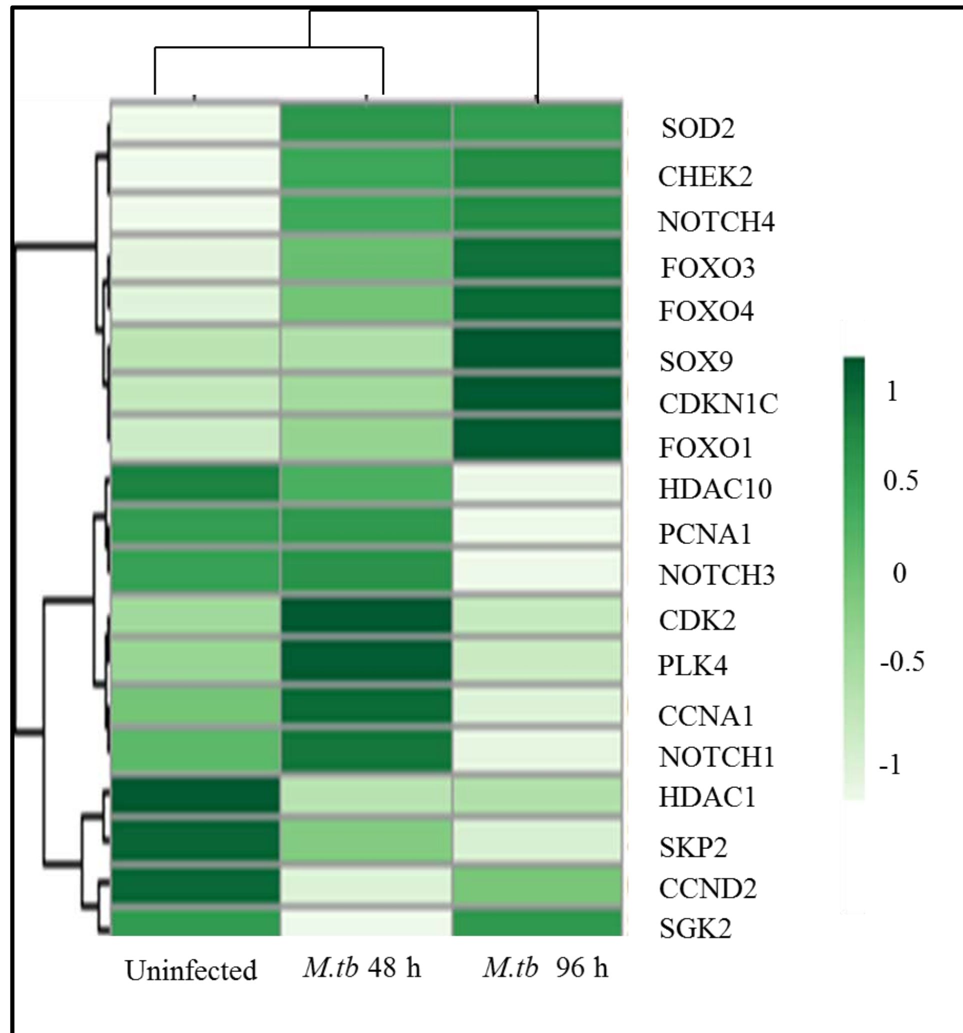

Figure 22 Heat map showing the relative expression fold of cell proliferation and quiescence genes in uninfected and *M.tb* infected MSCs at 48 and 96 hrs post infection.

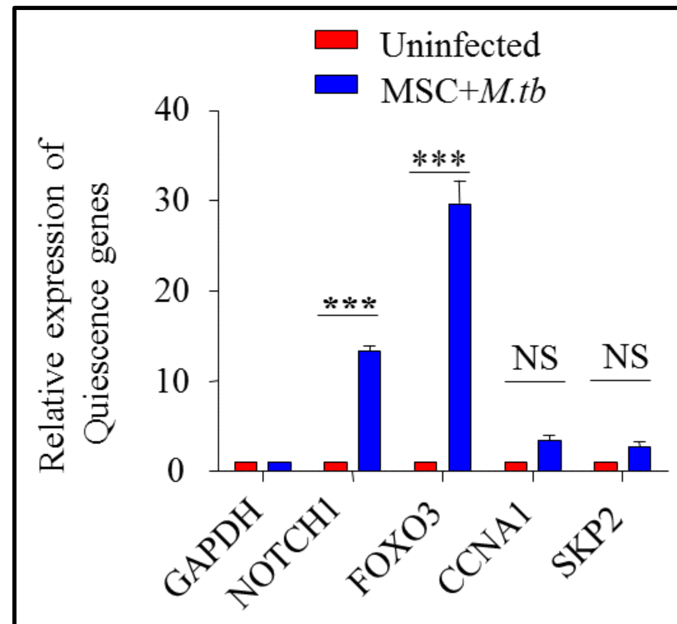

Figure 23 Graphical representation of validation of relative expression of cell proliferation and quiescence genes in MSCs as compared to uninfected control at 72 hours. These experiments are representative of three independent experiments with triplicates (n=3). Error bar represent S.E.M. *** represents P<0.001, ** P<0.01 and *P<0.05. P>0.05 is taken non-significant (NS).

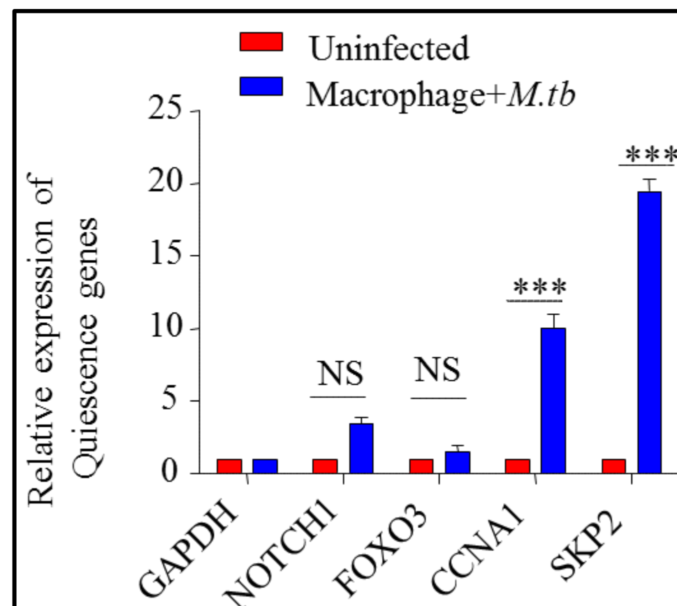

Figure 24 Validation of relative expression of cell proliferation and quiescence genes in macrophages as compared to uninfected control at 72 hours. These experiments are representative of three independent experiments with triplicates (n=3). Error bar represent S.E.M. *** represents P<0.001, ** P<0.01 and *P<0.05. P>0.05 is taken non-significant (NS).

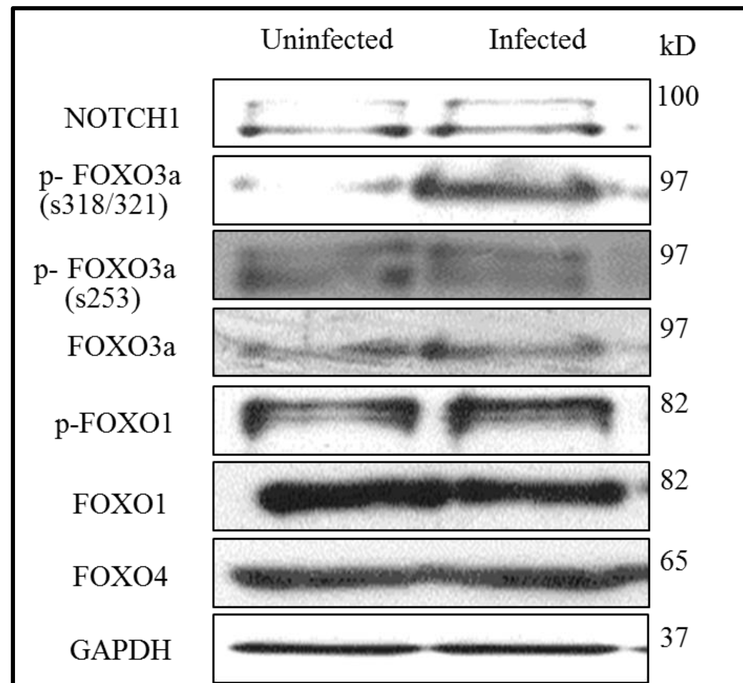

Figure 25 Western blots showing forkhead signaling pathway from uninfected and *M.tb* infected MSCs at 96 hours.

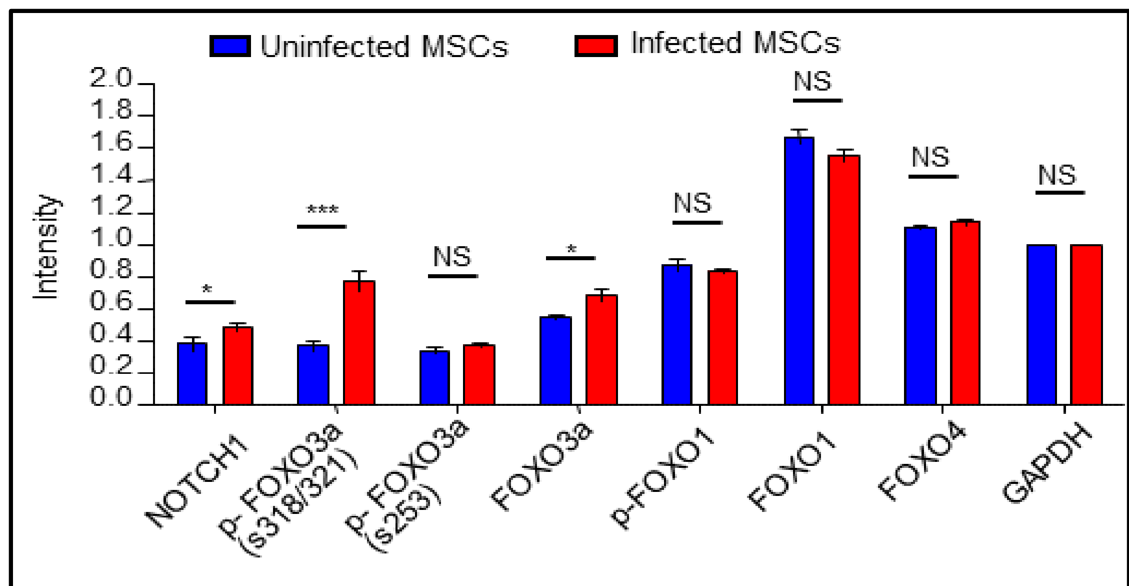

Figure 26 Graphical Representation of densitometry data of the western blot of the forkhead signaling pathway. Error bar represent S.E.M. *** represents P<0.001, ** P<0.01 and *P<0.05. P>0.05 is taken non-significant (NS).

5.7 *M.tb* hides in lipid droplets to attain dormancy in MSCs which is reversed upon inhibition of lipid synthesis

To examine the relation of lipid synthesis with dormancy, we employed the lipid synthesis inhibitor, Triacsin C. Triacsin C is a potent inhibitor of fatty acyl-CoA synthetase that strongly interferes with lipid metabolism by blocking the de-novo synthesis of diacylglycerols, triacylglycerols and cholesterol (Namatame et al., 1999). Inhibition of lipid synthesis resulted in profound downregulation of dormancy-related gene expression in *M.tb* **(Figure 27)** with significant alteration in the expression of replicative genes **(Figure 28)**. These results strongly imply that *M.tb* induces lipid-synthesis in MSCs and compartmentalize themselves within neo-lipid-droplets, hence thwarting antimicrobial host-defense mechanisms.

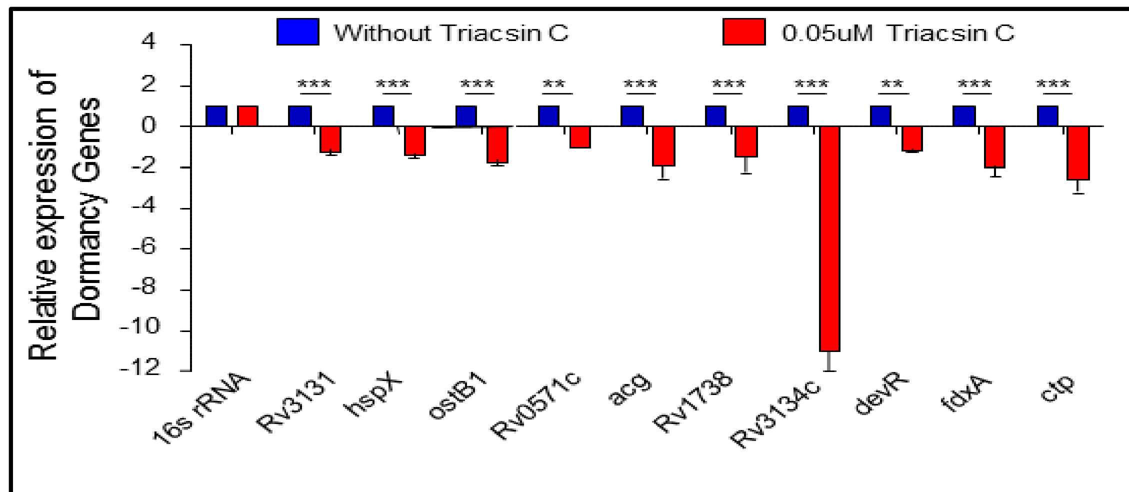

Figure 27 Graphical representation of the relative expression of dormancy genes of *M.tb* in infected MSCs treated with or without lipid inhibitor, Triacsin C (0.05 μM) at 72 hrs post infection. These experiments are representative of three independent experiments with triplicates (n=3). Error bar represent S.E.M. *** represents P<0.001, ** P<0.01 and *P<0.05. P>0.05 is taken non-significant (NS).

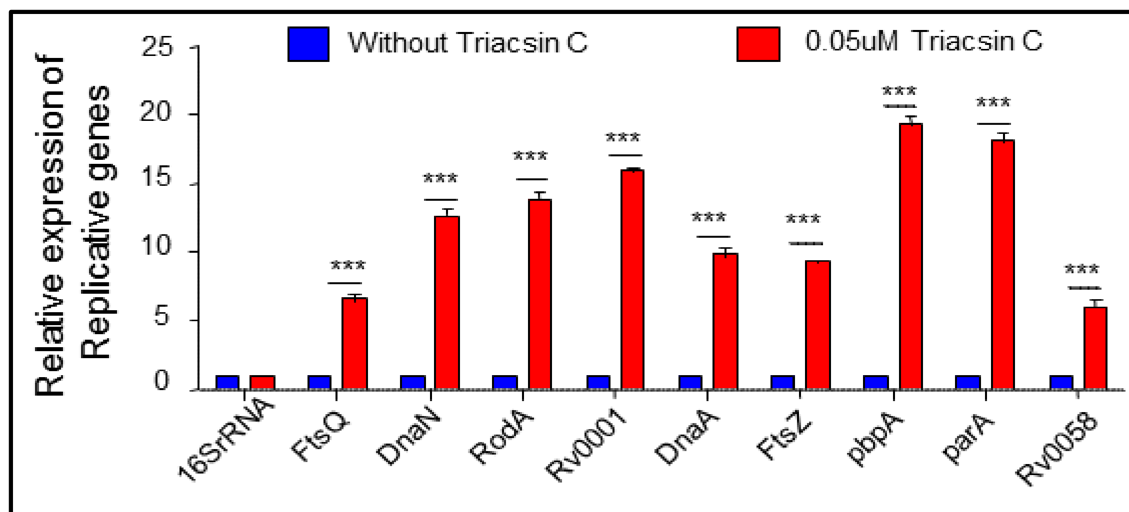

Figure 28 Graphical representation of the relative expression of replicative genes of *M.tb* inside MSCs treated with or without Triacsin C (0.05 μM) compared to macrophages at 72 hours post-infection. These experiments are representative of three independent experiments with triplicates (n=3). Error bar represent S.E.M. *** represents P<0.001, ** P<0.01 and *P<0.05. P>0.05 is taken non-significant (NS).

5.8 *M.tb* replication inside MSCs is regulated by autophagy and *M.tb* in MSCs become non-responsive to isoniazid in a time-dependent manner

Excitingly, mesenchymal stem cells are known to exhibit intrinsic autophagy which is assumed to be crucial for their self-renewal, maintenance of pluripotency, differentiation state and attainment of quiescent state (Phadwal et al., 2013). Therefore we were keen on knowing how intrinsic autophagy in MSCs restricts the growth of *M.tb*. It is known that MSCs do not possess well specialized phagosomal compartments as compared to macrophages. To obtain insight into the mechanism by which MSCs provide a niche for the dormancy of *M.tb*, we analyzed RNAseq data and found that MSCs strongly induce the expression of autophagy-related genes **(Figure 29)**. Inhibition of autophagy is one of the most widely adopted host-evasion mechanisms used by virulent strains of *M.tb* (Gutierrez et al., 2004; Khan et al., 2017). Therefore, we tested if the induction of autophagy by rapamycin can eliminate *M.tb* in MSCs. We treated infected human macrophages and MSCs in vitro with isoniazid/or rapamycin and assessed the viability of *M.tb* thereafter. Interestingly, we observed that addition of rapamycin reduced bacterial loads in both macrophages and MSCs in a time-dependent manner. However, effects on MSCs were more significant than macrophages **(Figure 30 and 31)**. As our ex-vivo data indicated that INH eliminates replicating bacteria in macrophages whereas induction of autophagy by rapamycin kills non-replicating *M.tb* in MSCs, we validated these observations in a mouse model of TB. As expected, the addition of rapamycin along with antibiotics was able to achieve sterile cure of TB, as compared to INH treatment alone **(Figure 32)**. Rapamycin is known to induce autophagy by inhibiting the mTOR pathway (Araki et al., 2009). To further test if the addition of rapamycin along with antibiotics

indeed attains sterile cure, we employed dexamethasone to suppress immunity in animals that were previously treated with INH or with the combination of INH and rapamycin. Suppression of the immune response with dexamethasone reactivated TB disease in INH-treated animals but not in animals treated with the combination of INH and rapamycin **(Figure 33 and 34)**. This observation indicated that autophagy can eliminate both active and dormant TB residing in macrophages and MSCs, respectively, thus preventing disease reactivation.

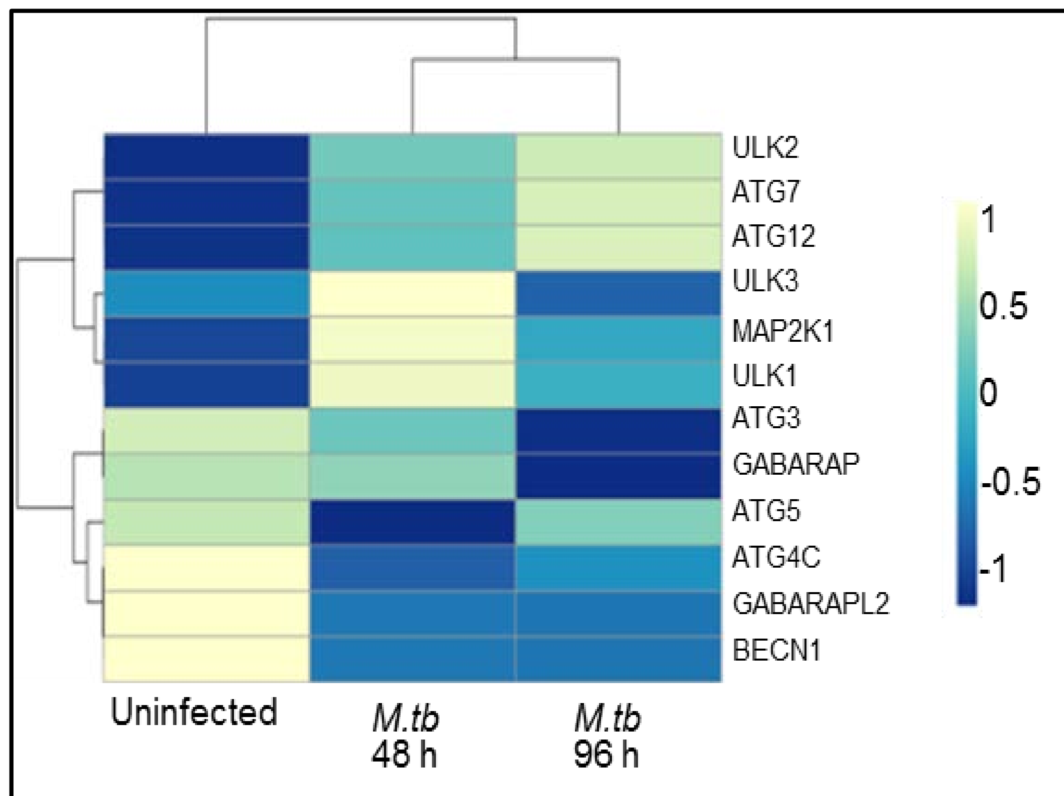

Figure 29 Heat map showing the relative expression of autophagy pathway genes in uninfected and *M.tb* infected MSCs at 48 hrs and 96 hrs post infection.

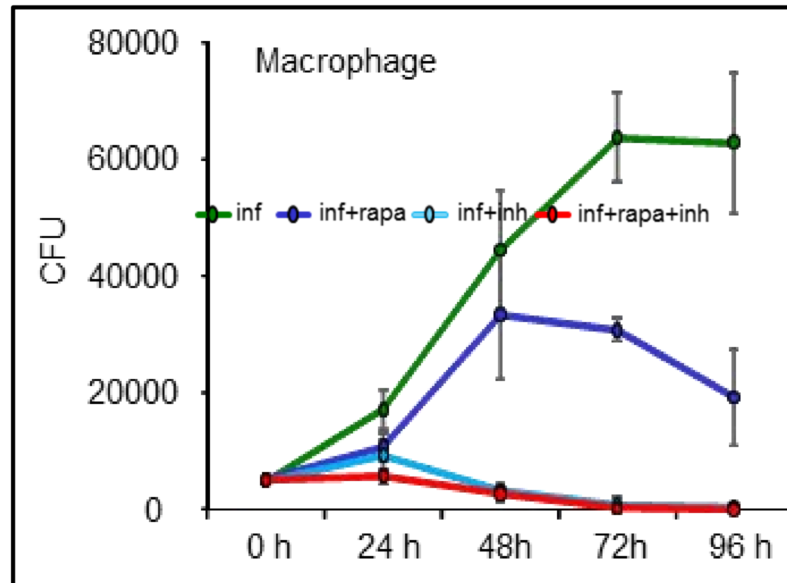

Figure 30 Growth kinetics of *M.tb* in macrophages (PBMC-derived) either infected alone with *M.tb* and/or treated with rapamycin or isoniazid (10 µg/ml) and isoniazid + rapamycin. Each experiment is representative of three independent experiments (n=5).

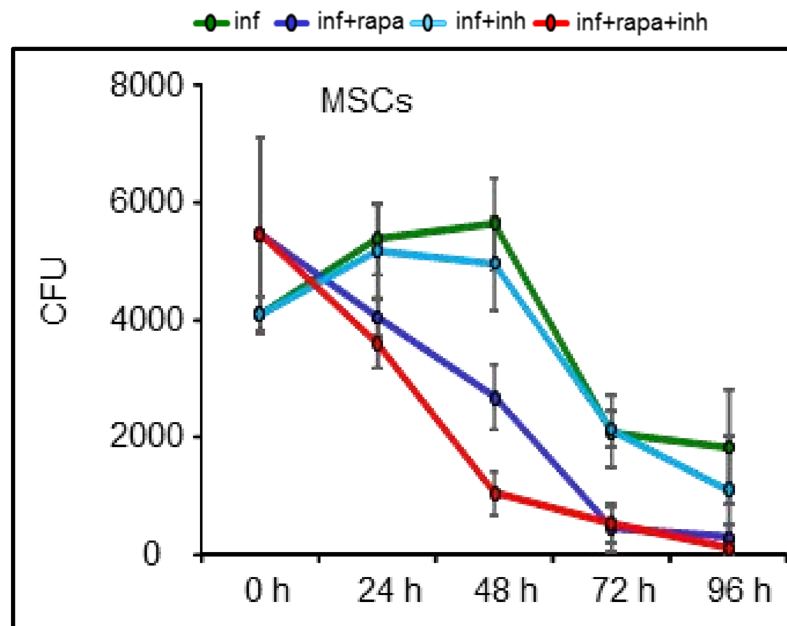

Figure 31 Growth kinetics of *M.tb* in MSCs either infected alone and/or treated with rapamycin (1 µM for 3 hrs before infection), isoniazid (10 µg/ml) and isoniazid + rapamycin. Each experiment is representative of three independent experiments (n=5).

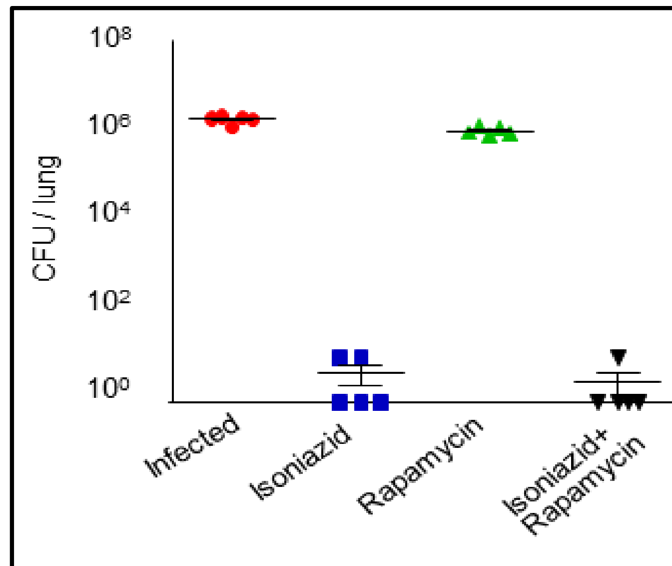

Figure 32 *M.tb* burden in lungs isolated from mice treated with or without isoniazid, rapamycin or isoniazid+rapamycin (n=5).

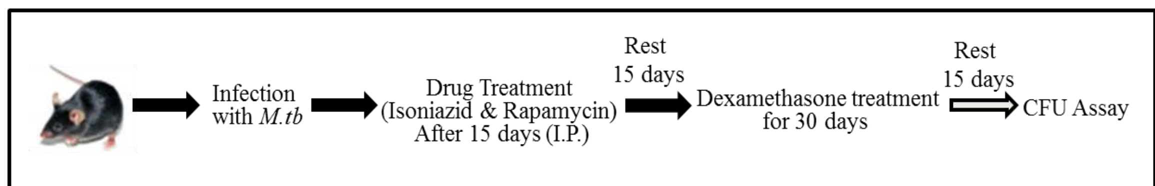

Figure 33: Schematic representation of the reactivation experiment.

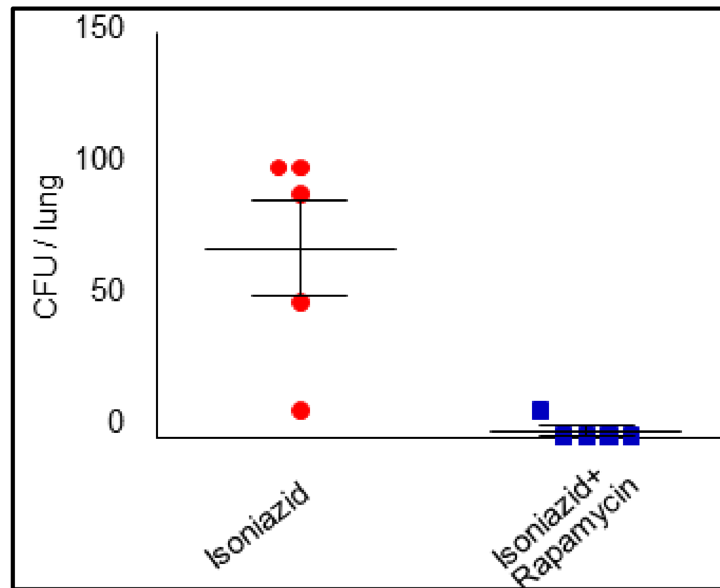

Figure 34 *M.tb* reactivation in lungs isolated from mice treated with isoniazid and isoniazid+rapamycin followed by dexamethasone treatment (n=5).

5.9 Dormant *M.tb* in MSCs is reactivated to active replicating bacteria upon immunosuppression

We investigated the status of dormancy and replicative gene expression in bacilli from bone-marrow and lungs of *M.tb* infected mice that were treated or untreated with INH. We found that the bacilli residing in bone-marrow of the INH treated mice were enriched with dormancy-related genes and expressed fewer replication-associated genes **(Figure 35 and 36)**. We also observed similar trends in lung of INH treated mice **(Figure 37 and 38)**. These data indicated that stem-cells not only from bone -marrow but also from the lungs harbor dormant *M.tb*.

These bacteria residing in the BM which showed dormancy phenotype converted to actively replicating state upon immune suppression with dexamethasone in reactivation

experiment. Dexamethasone is a corticosteroid which acts as an immune-suppressant impairing the T-cell response by increasing inhibitory receptors on the T-cells particularly PD1 and CTLA-4 (Xia et al., 1999). Dexamethasone treatment strikingly upregulated replicative -genes in the harbored *M.tb* in these animals **(Figure 39)** and dramatically reduced expression of dormancy-related genes **(Figure 40)**, indicating that immune-suppression converts dormant-bacteria into an active form in these animals. Taken together these observations strongly imply that our mouse model of dormancy triggers the induction of dormancy and these bacteria become highly unresponsive to conventional antibiotic treatment which may lead to active TB in case of immunosuppression as with HIV-TB co-infection.

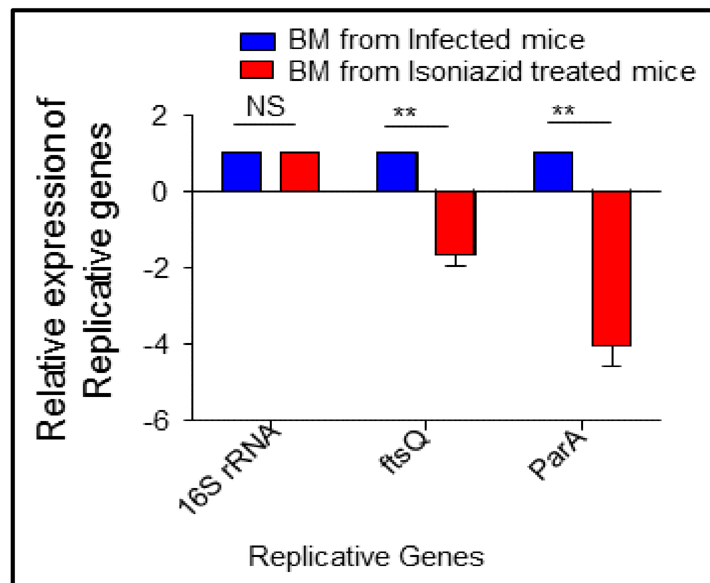

Figure 35 Graphical representation depicting the relative expression of replicative genes of *M.tb* from the bone marrow of isoniazid-treated mice compared to infected control (n=5). Experiments shown are representative of an independent experiment (n=5). Error bars represent S.E.M. *** represents P<0.001, ** P<0.01 and *P<0.05. P>0.05 is taken as non-significant (NS).

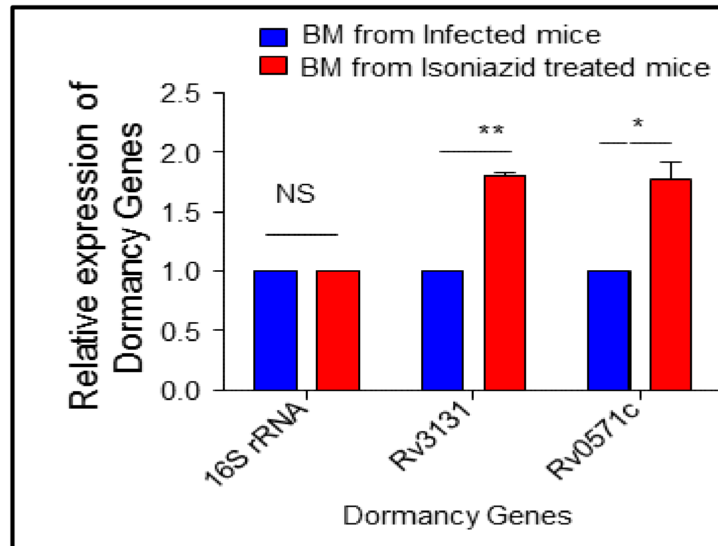

Figure 36 Graphical representation depicting relative expression fold of dormancy genes of *M.tb* from bone marrow of isoniazid-treated mice compared to infected control (n=5). Experiments shown are representative of an independent experiment (n=5). Error bars represent S.E.M. *** represents P<0.001, ** P<0.01 and *P<0.05. P>0.05 is taken as non-significant (NS).

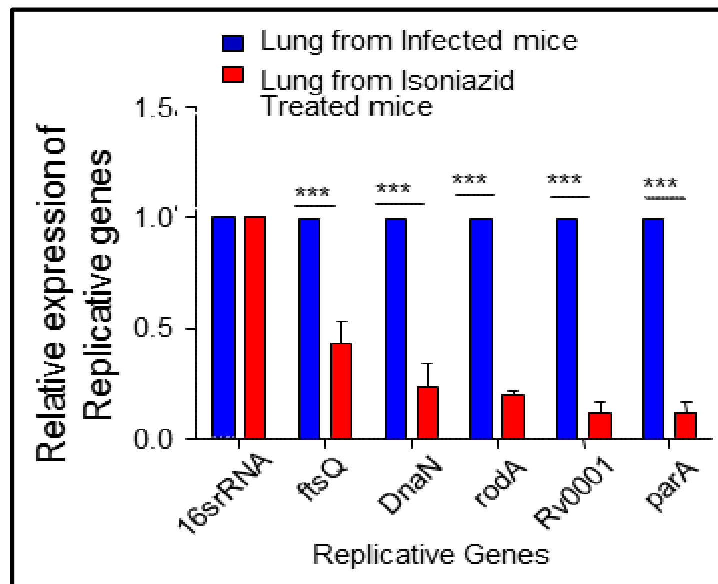

Figure 37 Graphical representation depicting relative expression of replicative genes of *M.tb* from lungs of isoniazid-treated mice compared to infected control (n=5). Experiments shown are representative of an independent experiment (n=5). Error bars represent S.E.M. *** represents P<0.001, ** P<0.01 and *P<0.05. P>0.05 is taken as non-significant (NS).

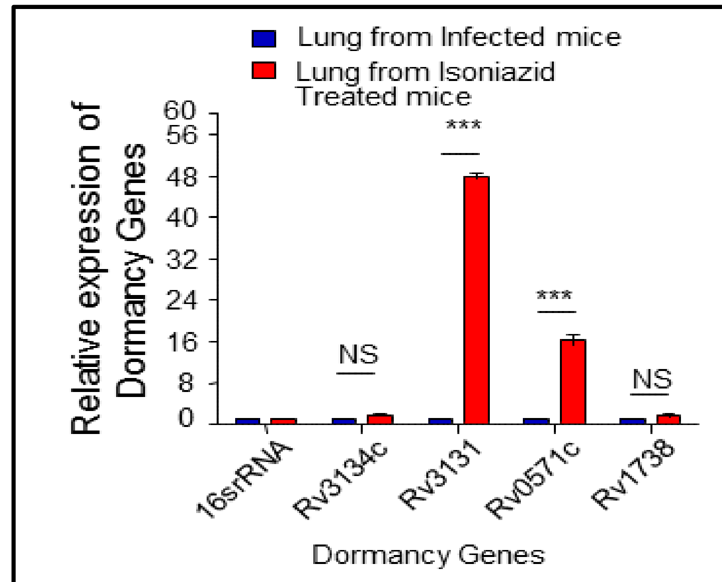

Figure 38 Graphical representation depicting the relative expression of dormancy genes of *M.tb* from the lung of isoniazid-treated mice compared to infected control (n=5). Experiments shown are representative of an independent experiment (n=5). Error bars represent S.E.M. *** represents P<0.001, ** P<0.01 and *P<0.05. P>0.05 is taken as non-significant (NS).

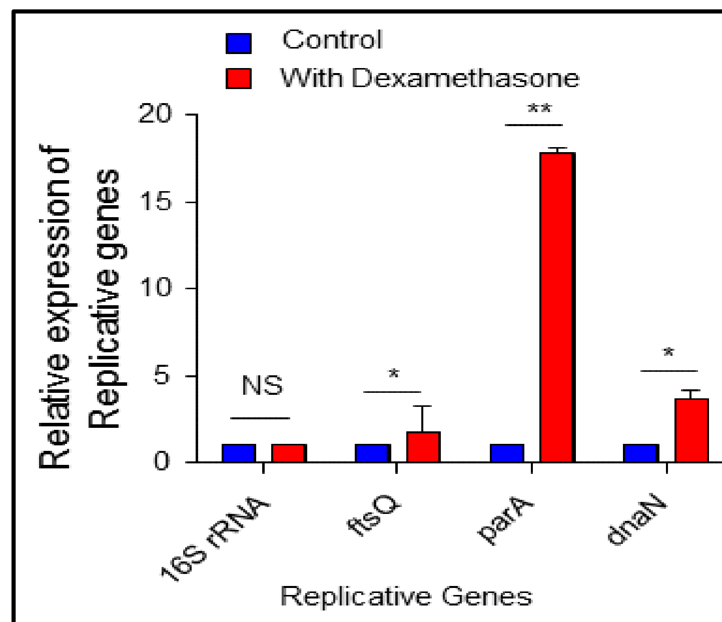

Figure 39 Graphical representation depicting the relative expression of replicative genes of *M.tb* from the bone marrow of dexamethasone-treated mice compared to INH control (n=5). Experiments shown are representative of an independent experiment (n=5). Error bars represent S.E.M. *** represents P<0.001, ** P<0.01 and *P<0.05. P>0.05 is taken as non-significant (NS).

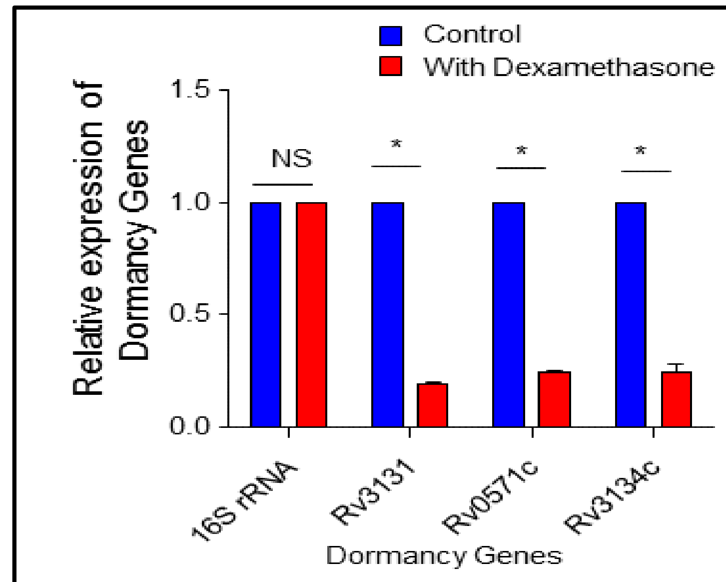

Figure 40. Graphical representation depicting the relative expression of dormancy genes of *M.tb* from the bone marrow of dexamethasone-treated mice compared to INH control (n=5). Experiments shown are representative of an independent experiment (n=5). Error bars represent S.E.M. *** represents $P<0.001$, ** $P<0.01$ and *$P<0.05$. $P>0.05$ is taken as non-significant (NS).

5.10 Secretory mediators or cytokines secreted by MSCs upon infection by *M.tb*

Mycobacterium tuberculosis induces a robust host immune response upon infection but even then the bacterium shields itself from the host response and establishes its long term survival in the host. This special behavior of *M.tb* immunity intrigued us to check for the cytokines and chemokines which are secreted by MSCs upon infection by *M.tb* which may assist in the establishment of the dormant population in MSCs. Chemokines are chemoattractant cytokines that help in the regulation of trafficking of monocytes, macrophages and lymphocytes to the sites of infection and inflammation (Domingo-Gonzalez et al., 2016). Interestingly, we found significant overexpression of IL-6, IP-10,

MCP-1 and VEGF in MSCs compared to macrophages while little or almost no secretory level of IFN-γ and TNF-α. These chemokines may help in the recruitment of T-cells and establishment of dormancy. IP-10 is known to selectively attract activated T lymphocytes. MCP-1 is known to attract monocytes and lymphocytes. These chemokines may help in the killing of *M.tb* in MSC leaving a small population which uses host lipids to survive and sustains itself as dormant bacteria for an infinite time **(Figure 41)**.

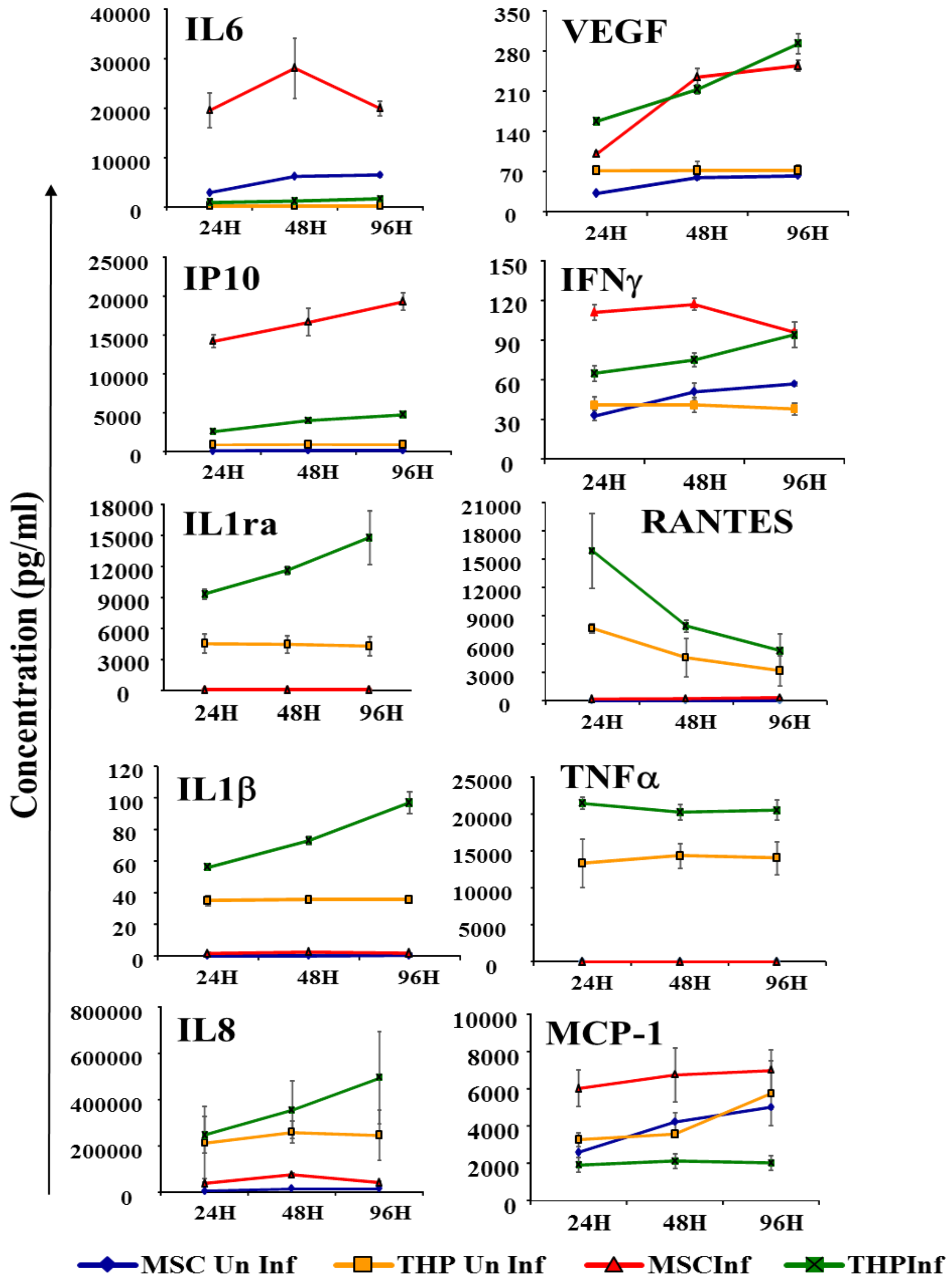

Figure 41. Cytokines secreted upon *M.tb* infection in MSCs compared to macrophages (THP-1) cells.

CHAPTER 6
Discussion

It has now become increasingly clear that upon infection with *M.tb,* the bacilli are engulfed by the macrophages and dendritic cells in the lungs. Following the taking up of bacilli by the phagocytic cells the host immunity mounts a killing response against the pathogen by recruiting T-cells, B-cells, neutrophils and mast cells and forming a granuloma. Raghuvanshi et al, for the first time, established that mesenchymal stem cells (MSCs) are also recruited in the vicinity of the granuloma and help the bacilli to evade the host immunity (Raghuvanshi et al, 2010). It has been established that MSCs immigrate to the periphery of the granuloma in mice, infected with tuberculosis through aerosol challenge and provide a stable niche for the growth and safe survival of *M.tb*. It was documented that MSCs secrete nitric oxide (NO) which restricts the growth of harboring *M.tb* and on the other hand, it implicates the suppression of T- cell responses that help the bacteria survive within granuloma-like structures, by avoiding the hostile immune responses. Therefore, a balance between bacterial growth and the host immune response leads to the establishment of latent TB. MSCs also secreted TGF-β cytokine which inhibits the protective immune response of TB by inducing Treg cells. Previously, it has been established that TGF-βReceptor-II Dominant negative (TGF-βRIIDN) animals, which are relatively more resistant to TB infection due to blockade of TGF-β dependent activation of Treg cells (Chen et al., 2003). Infusion of naïve MSCs, followed by infection with *M.tb,* enhanced the susceptibility to *M.tb* infection in this strain of transgenic animal (Raghuvanshi et al., 2010). With the progression of the disease, *M.tb* spreads in various parts of the body and causes extra-pulmonary TB. We hypothesized that MSCs aid in the distribution of the bacilli from the lung to the other extra-pulmonary organs; where the bacilli may exploit the local environment induced, and thus providing a

mechanism of distribution and long term survival of *M.tb* in the host both within the lung granuloma but also other organs outside the lung granuloma.

This study of the role of MSCs in immune evasion in mice model was further elaborated by two comprehensive studies analyzing human ($CD271^+$ and $CD133^+$) MSCs. The human $CD271^+$ and $CD133^+$ MSCs were reported to contain viable *M.tb* (Das et al, 2013). They infected MSCs with *M.tb in vitro* and observed an interesting growth pattern in serum-free and with serum media. *M.tb* grown in media without serum were fully viable and replicating whereas MSCs grown in media with serum-supplemented displayed a reduction in the bacterial load in a time-dependent manner (Das et al., 2014). Mice infected through aerosol challenge showed the presence of viable *M.tb* in the bone marrow (BM) cells. Viable *M.tb* and *M.tb* DNA was detected in MSCs sorted from TB patients who had undergone treatment with DOTS (Das et al., 2013). Together, these studies demonstrated that MSCs shield viable *M.tb* and provide a survival niche for *M.tb* to remain viable even after the drug treatment and acquire persistence in the host.

In view of the above findings, we sought to determine whether MSCs are a natural reservoir for *M.tb* infection and dormancy that renders these organisms non-responsive to antibiotic treatment. We infected human MSCs and peripheral blood mononuclear cell (PBMC) derived macrophages with H37Rv strain of *M.tb* and found that both were infected at equal efficiency at MOI 10 for macrophages and MOI 50 for MSCs. But, interestingly, as time progressed, *M.tb* continued to replicate in the macrophages with the macrophages becoming necrotic by 96 hours of infection. Contradictorily, *M.tb* numbers gradually decreased in MSCs, reached a plateau by 72 hours, and remained there in a viable form for an extended period of time. This result intrigued us to study the dormancy

status of the bacteria in MSCs after they stop multiplying and remain in the non-replicating state and compare it with macrophages. Upon studying the gene expression level in both the cell types, we found unrelenting expression of dormancy-related devR/dosR regulon genes in *M.tb* isolated from infected MSCs but not macrophages. DevR/dosR regulon is a well-studied gene expression marker for dormant bacteria (Kaur et al., 2016). Its expression in *M.tb* isolated from MSCs is being reported for the first time. However, genes that are involved in various steps of *M.tb* replication such as replication initiation and cytokinesis were enriched in *M.tb* isolated from infected macrophages which were minimally expressed in *M.tb* isolated from MSCs. Thus, our results established for the first time, through genetic analysis, the presence of dormant *M.tb* in MSCs by studying their mRNA expression pattern. Our data strongly claims that upon infection, *M.tb* in macrophages is in actively dividing state as compared to those in MSCs, where most of the bacteria attain a non-dividing metabolically inactive, dormant state. To further validate this finding we infected C57BL/6 mice with *M.tb* through aerosol challenge and precisely sorted $CD45^{-}Sca1^{+}$ MSCs from the BM and $CD45^{+}CD11b^{+}$ macrophages from the lungs of the mice. Expectedly, consistent with the *in vitro* data, we observed that *M.tb* isolated from the sorted MSCs express dormancy-related genes whereas *M.tb* isolated from the macrophages express replication-related genes that strengthen ours *in vitro* observations. Therefore, it is highly likely that MSCs and macrophages are programmed differently to counteract to the bacterial infection, which is demonstrated at the gene level by the expression level of DosR regulon genes in *M.tb*. Moreover, the non- replicating dormant *M.tb* inhibits MSC replication in a time-dependent fashion. This phenomenon, better known as quiescence was confirmed by the

differential expression of quiescence markers and cell cycle progression markers through RNA sequencing (RNAseq) analysis and qPCR of selected quiescence and cell proliferation markers such as FOXO-3, NOTCH-1 and SOX-9, S-phase kinase 2 (SKP2) and CCNA encoding cyclin A2. The quiescence markers were significantly up-regulated in MSCs compared to the macrophages. The reverse was true for cell progression markers. Quiescence in *M.tb* infected MSCs was strongly confirmed by western blot analysis of NOTCH-1, FOXO-3, and p-FOXO-3 at Ser318/321. ***These observations strongly implicated that upon infection, M.tb acquires dormancy in MSCs, inducing a state of quiescence in the stem cells. This dual strategy of taking the host cell in a metabolically silent stage with only a few expressing genes while itself attaining a dormant state may assist M.tb to better shield itself from the host immune system and establish long-lasting survival in the host.***

Previously, it has been reported that *M.tb* infects MSCs through scavenger receptors (Khan et al., 2017), unlike macrophages which uptake the bacilli through the mostly phago-lysosomal pathway. MSCs, not being the professional antigen presenting cells (APCs) lack a well-defined phagosomal pathway. To decipher the exact location of *M.tb* inside MSCs after internalization, we determined the intracellular localization of GFP-labeled *M.tb* bacteria in human macrophages and MSCs. We observed that immediately after infection, in macrophages, most of the *M.tb* bacteria localized to early endosomes, whereas the majority of bacilli in MSCs were found in the cytosol; which is in contrast to its localization in macrophages. After getting some interesting insight at the localization of *M.tb* in MSCs interestingly, we also observed abnormal lipid droplets accumulation in MSCs, whose intensity increased over time. *M.tb* co-localized with these lipid droplets to

a very higher extent. It has already been reported that *M.tb* utilizes host lipids in the macrophages for obtaining nutrition (Peyron et al., 2008). Here we found that the intensity of lipid droplets was significantly much higher in MSCs as compared to the macrophages. Through RNAseq analysis, we got an insight that lipid synthesis pathways, especially genes involved in sphingolipid synthesis, were highly up-regulated in infected MSCs, compared to infected macrophages. These results intrigued us to check if lipid synthesis was associated with the attainment of dormancy by the *M.tb* inside the MSCs. Therefore we used a lipid synthesis inhibitor which blocks all neutral lipids, triacylglycerol, and cholesterol, all major lipid sources. Upon inhibition of lipid synthesis, we found dramatic down-regulation in dormancy-related gene expression in *M.tb* residing in MSCs. In contrast, we did not register any noticeable changes in *M.tb* that were residing in the macrophages. Thus, host lipid synthesis may be one of the major reasons for driving the *M.tb* bacteria to a dormant state. ***These results strongly imply that M.tb organisms strongly induce huge lipid synthesis in MSCs compared to that of macrophages and compartmentalize themselves within neo-lipid droplets, increasing the expression of dormancy genes in the bacilli. Dormant M.tb utilizes the host components itself for its long-term survival in the host by shielding itself from the attacking immune system.***

Although the cells predominantly infected by *M.tb* are the macrophages and DCs, other cells have also been reported to get infected by *M.tb* (Russell, 2001). The *M.tb* antigen is presented to T-cells after being processed by both MHC-I and MHC-II pathway in macrophages as well as DCs, which lead to the activation of $CD8^+$and $CD4^+$ T cells

(Harding and Boom, 2010). Among the other cells infected by *M.tb*, epithelial cells are also reported to present antigens to T-cells (Scordo et al., 2016).

Interestingly, MSCs have a low expression level of both MHC-I and MHC-II receptors (Jacobs et al., 2013). Within the macrophages, *M.tb* is able to evade phagosome-lysosomal fusion to minimize antigen processing and presentation (Singh CR et al., 2006). In MSCs although the mechanism remains largely unknown the mycobacterial antigens are not processed efficiently through the phago-lysosomal pathway indicating that MSCs lack a well-documented phagosomal pathway. Therefore, to kill intracellular *M.tb,* MSCs may rely mainly on innate host defense pathways. MSCs also express drug efflux pumps such as ABCG2, thus making MSCs the perfect survival niche for *M.tb* by shielding the bacilli from the effect of drugs through the action of efflux pumps (Zhou et al., 2001).

M.tb is known to hide in the intracellular niche of MSCs in a non-replicating state but the mechanism that restricts the growth and mediates killing of *M.tb* inside MSCs is hardly known. Recent studies have indicated the role of innate immunity in the intracellular killing of *M.tb* in the MSCs (Khan et al., 2017; Naik et al., 2017). Khan et al, 2017 reported that MSCs regulate the intracellular growth of *M.tb* through intrinsic autophagy and NO synthesis. The killing of *M.tb* was further augmented upon inducing autophagy in cells by using rapamycin (Khan et al., 2017). Another study suggested that an antimicrobial peptide, cathelicidin helps in the killing of avirulent *mycobacteria* but had no effect on the virulent strains of *M.tb* (Naik et al., 2017). These studies confirmed that innate immunity is mainly responsible for the killing of *M.tb* in MSCs.

Here, through analysis of RNAseq data and mice experiments, we found that MSCs strongly induce the expression of autophagy-related genes and that addition of rapamycin reduced the bacterial loads further, in both macrophages and MSCs in a time-dependent manner. However, the effects on MSCs were more significant than on macrophages. This observation indicated that autophagy can eliminate both active and dormant TB residing in macrophages and MSCs, respectively. We also studied that after treating the *M.tb* infected mice with INH, the bacilli residing in the BM of the mice were enriched with dormancy-related genes and expressed fewer replication-associated genes. This dormant resident population of *M.tb* is reactivated to actively replicating bacteria upon dexamethasone treatment. Addition of rapamycin along with antibiotics to these dormant bacteria was able to achieve sterile cure of TB in a mouse model of TB infection and this combination therapy induced rapid clearance of *M.tb* organisms as compared with INH treatment alone. ***Taken together these observations strongly suggested that autophagy is mainly involved in the killing of M.tb in MSCs and when induced in combination with conventional drug treatment, a combination of INH and rapamycin can be used to eliminate actively replicating as well as latent bacteria to achieve sterilizing TB cure.***

Cytokines play an indispensable role in the tuberculosis pathogenesis. Cytokines are categorized into pro-inflammatory and anti-inflammatory groups based on the role they play in host protection. The two most important cytokines secreted by the host immune system in response to *M.tb* challenge are IFN-γ and TNF-α (Domingo-Gonzalez et al., 2016). Other cytokines and chemokines play a part in the recruitment of other cell types to the site of infection and inflammation. The major producers of cytokines are T-cells and macrophages. Other cells such as MSCs, fibroblast and epithelial cells also secrete

few cytokines and chemokines (Orme et al., 2015). MSCs recruited at the interface of the granuloma and T-cells helps in shielding dormant bacteria, it was intriguing for us to study the cytokines which help the MSCs to create an environment which aided in the shielding and attainment of the dormant phenotype of the *M.tb*. We analyzed cytokines from the supernatant of both infected macrophages and MSCs and found that in MSCs, there was a significant up-regulation in the level of IL-6, IL-8, IP-10, MCP-1, and VEGF. Further study is required to elucidate and characterize the role of these cytokines and chemokine, released by MSCs in dormancy and immune evasion.

Thus, our study strongly establishes that M.tb acquires dormancy in MSCs and upon infection simultaneously drives host MSCs to enter into a non-replicative quiescent state. This two-fold strategy employed by M.tb helps it to get enhanced shielding protection from the host immune system and simultaneously avoiding the effect of drugs used for treatment, through the presence of efflux pumps thus establishing dormancy and persistence. M.tb induces huge lipid synthesis in MSCs and protects themselves within host lipid droplets, obtaining nutrition for the dormant state from the host lipids. Host lipids play a major role in the attainment of the dormant phenotype by the M.tb organisms. Elimination of dormant M.tb hiding in MSCs is essential to achieve sterile cure of TB which is achieved through a combination therapy consisting of INH and rapamycin. Taken together, this therapy can be used to eliminate actively replicating as well as latent bacteria to achieve complete TB cure.

CHAPTER 7
Summary

The present research highlights the importance of the role of mesenchymal stem cells (MSCs) in tuberculosis pathogenesis. Here we report for the first time through gene expression analysis that MSCs are the sites where actively dividing *M.tb* organism attains an asymptomatic stage characterized by dormancy and drug tolerance. After uptake of the bacilli in the MSCs, the expression of dormancy associated DosR/DevR regulon genes are induced in the active bacteria in a time-dependent fashion which drives the pathogen towards a dormant non-replicating state. The state of dormancy of the bacteria is also widely known as latent tuberculosis. Once inside the MSCs the bacteria mainly inhabit the cytosolic compartment, unlike in macrophages where most of the bacteria cover the phago-lysosomal compartments. In the MSCs, *M.tb* organisms, while residing in the cytosol influence the host cells to enter within quiescence and persuade the cells to undergo rapid and prolonged lipid synthesis and accumulate these lipids in the form of lipid bodies. These lipid bodies shield the viable persisting population of the bacteria and drive the *M.tb* organism towards dormancy. Reduction in the synthesis of lipids also decreases the expression of dormancy regulon genes. The dormancy status of the bacteria residing in the MSCs was also confirmed by *in vivo* experiments. These dormant bacterial populations which surpass INH treatment revert to actively dividing state upon immune suppression, in mice model of tuberculosis. MSCs deploy the innate immune mechanism of autophagy to eliminate dormant bacteria from the host. While the active bacterial population in macrophage responds to INH, a major constituent of the DOTS therapy; the dormant bacteria of MSCs respond poorly to INH and thus autophagy is the major mechanism for the killing of the bacteria. A combination of induced autophagy and INH could prevent disease reactivation in a mouse model of infection. Thus, MSCs provide a

new ray of hope in clearing dormant *M.tb* and any new knowledge which helps in attainment of complete TB eradication would help in the achievement of the dream of "TB free world".

References

Abdallah, A. M., Bestebroer, J., Savage, N.D., de Punder, K., van Zon, M., Wilson, L., Korbee, C.J., van der, Sar, A.M., Ottenhoff, T.H., van der, Wel. N.N., Bitter, W. and Peters, P.J. Mycobacterial secretion systems ESX-1 and ESX-5 play distinct roles in host cell death and inflammasome activation. *J. Immunol.* 2011. 187, 4744–4753.

Aggarwal, S., Mannam, P. and Zhang, J. Differential regulation of autophagy and mitophagy in pulmonary diseases. *Am J Physiol Lung Cell Mol Physiol*. 2016. 311(2), L433-52.

Algood, H.M., Chan, J. and Flynn, J.L. Chemokines and tuberculosis. *Cytokine Growth Factor Rev*. 2003.14(6):467-77.

Almeida Da Silva, P.E. and Palomino, J.C. Molecular basis and mechanisms of drug resistance in Mycobacterium tuberculosis: classical and new drugs. *J Antimicrob Chemother*. 2011. 66(7), 1417-30.

Armstrong, J.A. and Hart, P.D. Response of cultured macrophages to Mycobacterium tuberculosis, with observations on fusion of lysosomes with phagosomes. *J.Exp.Med.* 1971. 134(3), 713-740.

Asari, S., Itakura, S., Ferreri, K., Liu, C., Kuroda, Y., Kandeel, F. and Mullen, Y. Mesenchymal stem cells suppress B-cell terminal differentiation. *Exp Hematol*. 2009. 37(5), 604-15.

Ashton, B.A., Allen, T.D., Howlett, C.R., Eaglesom, C.C., Hattori, A. and Owen. M. Formation of bone and cartilage by marrow stromal cells in diffusion chambers in vivo. *Clin Orthop Relat Res*. 1980. (151), 294 –307.

Balcewicz-Sablinska, M.K., Keane, J., Kornfeld, H. and Remold, H.G. Pathogenic Mycobacterium tuberculosis evades apoptosis of host macrophages by release of TNF-R2, resulting in inactivation of TNF-alpha. *J Immunol.* 1998, 161, 2636–2641.

Bartholomew, A., Sturgeon, C., Siatskas, M., Ferrer, K., McIntosh, K., Patil, S., Hardy, W., Devine, S., Ucker, D., Deans, R., Moseley, A. and Hoffman, R.

Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol* 2002. 30, 42– 48.

Bartz, R., Li, W.H., Venables, B., Zehmer, J.K., Roth, M.R., Welti, R., Anderson, R.G., Liu, P. and Chapman, K.D. Lipidomics reveals that adiposomes store ether lipids and mediate phospholipid traffic. *J Lipid Res*. 2007. 48(4), 837-47.

Beamer, G., Major, S., Das, B. and Campos-Neto, A. Bone marrow mesenchymal stem cells provide an antibiotic-protective niche for persistent viable Mycobacterium tuberculosis that survive antibiotic treatment. *Am J Pathol*. 2014. 184,3170e5.

Becker, A.J., McCulloch, E.A. and Till, J.E. Cytological Demonstration of the Clonal Nature of Spleen Colonies Derived from Transplanted Mouse Marrow Cells. *Nature*. 1963. 197(4866), 452–4.

Bloom, B. (ed). Tuberculosis: pathogenesis, protection and control. *American Society for Microbiology*, 1994.Washington, D.C.

Bloom, B.R., Atun, R., Cohen, T., Dye, C., Fraser, H., Gomez, G.B., Knight, G., Murray, M., Nardell, E., Rubin, E., Salomon, J., Vassall, A., Volchenkov, G., White, R., Wilson, D. and Yadav, P. Tuberculosis. In: Holmes KK, Bertozzi S, Bloom, B.R. and Jha, P. editors. *Major Infectious Diseases. 3rd edition. Washington (DC): The International Bank for Reconstruction and Development / The World Bank.* 2017. Chapter 11.

Bloom, B.R. and Small, P.M. The evolving relation between humans and Mycobacterium tuberculosis. *N Engl J Med.* 1998. 338(10), 677-8.

Blumberg, H.M. and Ernst, J.D. The Challenge of Latent TB Infection. *JAMA*. 2016. 316(9), 931–933.

Bottai, D. and Brosch, R. Mycobacterial PE, PPE and ESX clusters: novel insights into the secretion of these most unusual protein families. *Mol. Microbiol.* 2009. 73, 325–328

Bravo, R. and Macdonald-Bravo, H. Induction of the nuclear protein 'cyclin' in quiescent mouse 3T3 cells stimulated by serum and growth factors. Correlation with DNA synthesis. *EMBO J.* 1984. 3(13), 3177-81.

Burdon, K.L. Fatty material in bacteria and fungi revealed by staining dried, fixed slide preparations. *J Bateriol.* 1946. 52, 665–678.

Caminero, J.A. Multidrug-resistant tuberculosis: epidemiology, risk factors and case finding. *Int J Tuberc Lung Dis.* 2010. 14(4), 382-90.

Campagnoli, C., Roberts, I.A., Kumar, S., Bennett, P.R., Bellantuono, I. and Fisk, N.M. Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. *Blood.* 2001. 98(8), 2396-402.

Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N. and Williamson, B. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Nat Acad Sci USA*. 1975. 72, 3666–3670.

Chackerian, A., Alt, J., Perera, V. and Behar, S. M. Activation of NKT cells protects mice from tuberculosis. *Infection and Immunity,* 2002. 70(11), 6302–6309.

Chapouton, P., Skupien, P., Hesl, B., Coolen, M., Moore, J.C., Madelaine, R., Kremmer, E., Faus-Kessler, T., Blader, P., Lawson, N.D. and Bally-Cuif, L. Notch activity levels control the balance between quiescence and recruitment of adult neural stem cells. *J Neurosci.* 2010. 30(23), 7961-74.

Chen, W., Jin, W., Hardegen, N., Lei, K.J., Li, L., Marinos, N., McGrady, G. and Wahl, M. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med.* 2003. 198(12), 1875-86.

Cheung, T.H. and Rando, T.A. Molecular regulation of stem cell quiescence. *Nat Rev Mol Cell Biol*. 2013. 14(6), 329-40.

Corcione, A., Benvenuto, F., Ferretti, E., Giunti, D., Cappiello, V., Cazzanti, F., Risso, M., Gualandi, F., Mancardi, G.L., Pistoia, V. and Uccelli, **A.** Human mesenchymal stem cells modulate B-cell functions. *Blood.* 2006, 107(1), 367-72.

Co D. O., Hogan, L. H., Kim, S. I and Sandor, M. T cell contributions to the different phases of granuloma formation, *Immunology Letters*, 2004. 92(1-2), 135–142.

Conget, P.A. and Minguell, J.J. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. *J Cell Physiol.* 1999. 181:67–73.

Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E. 3rd, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M.A., Rajandream, M.A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J.E., Taylor, K., Whitehead, S. and Barrell, B.G. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature.* 1998. 393(6685), 537-44.

Cudahy, P. and Shenoi, S.V. Diagnostics for pulmonary tuberculosis. *Postgraduate Medical Journal.* 2016. 92 (1086), 187–93.

Das, B., Kashino, S.S., Pulu, I., Kalita, D., Swami, V., Yeger, H., Felsher, D.W. and Campos-Neto, A. CD271 (+) bone marrow mesenchymal stem cells may provide a niche for dormant Mycobacterium tuberculosis. *Sci Transl Med.* 2013. 5.170ra113.

Davis, J.M. and Ramakrishnan, L. The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell.* 2009. 136(1), 37-49.

Daniel, T.M. The history of tuberculosis. *Respir Med*. 2006. 100(11), 1862-70.

Daniel, J., Deb, C., Dubey, V.S., Sirakova, T., Abomoelak, B., Morbidoni, H.R. and Kolattukudy, P.E. Induction of a novel class of diacylglycerol acyltransferases and

triacylglycerol accumulation in Mycobacterium tuberculosis as it goes into a dormancy-like state in culture. *J Bacteriol*. 2004. 186, 5017–5030.

Deb, C., Lee, C.M., Dubey, V.S., Daniel, J., Abomoelak, B., Sirakova, T.D., Pawar, S., Rogers, L. and Kolattukudy, P.E. A novel in vitro multiple-stress dormancy model for Mycobacterium tuberculosis generates a lipid-loaded, drug-tolerant, dormant pathogen. *PLoS One.* 2009. 4, e6077.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D.J., Horwitz, E. Minimal criteria for defining multipotent mesenchymal stromal cells. *Cytotherapy.* 2006. 8(4), 315-7. The International Society for Cellular Therapy position statement.

Domingo-Gonzalez, R., Prince, O., Cooper, A. and Khader, S.A. Cytokines and Chemokines in Mycobacterium tuberculosis Infection. Microbiol Spectr. 2016. 4(5):10.1128/microbiolspec.TBTB2-0018-2016.

Ehlers, S. and Schaible, U.E. The granuloma in tuberculosis: dynamics of a host-pathogen collusion. *Front Immunol*. 2013. 3(411).

Elzinga, G., Raviglione, M.C. and Maher D. Scale up: meeting targets in global tuberculosis control. *Lancet.* 2004. 363(9411): 814–9.

Espinal, M.A., Laszlo, A., Simonsen, L., Boulahbal, F., Kim, S.J., Reniero, A., Hoffner, S., Rieder, H.L., Binkin, N., Dye, C., Williams, R. and Raviglione, M.C. Global trends in resistance to antituberculosis drugs. *N Engl J Med,* 2001. 344, 1294–1303.

Fabri, M., Realegeno, S.E., Jo, E.K. and Modlin, R.L. Role of autophagy in the host response to microbial infection and potential for therapy. *Curr Opin Immunol.* 2011. 23, 65–70

Falzon, D., Mirzayev, F., Wares, F., Baena, I.G., Zignol, M., Linh, N., Weyer, K., Jaramillo, E., Floyd, K. and Raviglione, M. Multidrug-resistant tuberculosis around the world: what progress has been made? *Eur Respir J.* 2015. 45, 150-60.

Floto, R.A., Sarkar, S., Perlstein, E.O., Kampmann, B., Schreiber, S.L. and Rubinsztein, D.C. Small molecule enhancers of rapamycin-induced TOR inhibition promote autophagy, reduce toxicity in Huntington's disease models and enhance killing of mycobacteria by macrophages. *Autophagy.* 2007. 3(6), 620-2.

Filipe-Santos, O., Bustamante, J., Chapgier, A., Vogt, G., de Beaucoudrey, L., Feinberg, J., Jouanguy, E., Boisson-Dupuis, S., Fieschi, C., Picard, C. and Casanova, J.L. Inborn errors of IL-12/23- and IFN-gamma-mediated immunity: molecular, cellular, and clinical features. *Semin Immunol*. 2006, 18, 347–361.

Flynn, J. L., Chan, J. and Lin, P. L. Macrophages and control of granulomatous inflammation in tuberculosis, *Mucosal Immunology*. 2011. 4(3), 271–278.

Flynn, J.L. and Chan, J. Tuberculosis: latency and reactivation. *Infect Immun*. 2001. 69(7), 4195–4201.

Frieden, T.R., Sterling, T.R., Munsiff, S.S., Watt, C.J. and Dye, C. Tuberculosis. *Lancet.* 2003. 362(9387), 887-99.

Friedenstein, A.J., Deriglasova, U.F., Kulagina, N.N., Panasuk, A.F., Rudakowa, S.F., Luriá, E.A. and Ruadkow, I.A. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. *Experimental hematology.* 1974. 2 (2), 83–9.

Friedenstein, A.J., Gorskaja, J.F. and Kulagina, N.N. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol.* 1976. 4, 267–274.

Frucht, D.M., Fukao, T., Bogdan, C., Schindler, H., O'Shea, J.J. and Koyasu, S. IFN-gamma production by antigen-presenting cells: mechanisms emerge. *Trends Immunol*. 2001. 22, 556–560.

Fujimoto, T. and Parton, R.G. Not Just Fat: The Structure and Function of the Lipid Droplet. *Cold Spring Harbor Perspectives in Biology*. 2011. 3, a004838–a004838.

Galmiche, M.C., Koteliansky, V.E., Briere, J., Hervé, P. and Charbord, P. Stromal cells from human long-term marrow cultures are mesenchymal cells that differentiate following a vascular smooth muscle differentiation pathway. *Blood*. 1993. 82, 66 –76.

Garhyan, J., Bhuyan, S., Pulu, I., Kalita, D., Das, B. and Bhatnagar, R. Preclinical and clinical evidence of Mycobacterium tuberculosis persistence in the hypoxic niche of bone marrow mesenchymal stem cells after therapy. *Am J Pathol.* 2015. 185, 1924e34.

Garton, N.J., Christensen, H., Minnikin, D.E., Adegbola, R.A. and Barer, M.R. Intracellular lipophilic inclusions of mycobacteria in vitro and in sputum. *Microbiology.* 2002 148, 2951–2958.

Garton, N.J., Waddell, S.J., Sherratt, A.L., Lee, S.M., Smith, R.J., Senner, C., Hinds, J., Rajakumar, K., Adegbola, R.A., Besra, G.S., Butcher, P.D. and Barer, M.R. Cytological and transcript analyses reveal fat and lazy persister-like bacilli in tuberculous sputum. *PLoS Med.* 2008. 5, e75 0001-0012.

Gengenbacher, M. and Kaufmann, S.H. Mycobacterium tuberculosis: success through dormancy. *FEMS Microbiol Rev*. 2012. 36(3), 514-32.

Goldsby, R.A., Kindt, T.J., Osborne, B.A. and Kuby, J. *J. Immunology*. Fifth edn W. H. Freeman and Company; New York. 2003.

Gomez, J.E. and McKinney, J.D. M. tuberculosis persistence, latency, and drug tolerance. *Tuberculosis* (Edinb). 2004. 84(1-2), 29-44.

Gonzalez-Juarrero, M., Turner, O. C., Turner, J., Marietta, P., Brooks, J. V. and Orme, I. M. Temporal and spatial arrangement of lymphocytes within lung granulomas induced by aerosol infection with Mycobacterium tuberculosis. *Infection and Immunity*, 2001. 69(3), 1722–1728.

Gopinath, S.D., Webb, A.E., Brunet, A. and Rando, T.A. FOXO3 promotes quiescence in adult muscle stem cells during the process of self-renewal. *Stem Cell Reports*. 2014. 2(4), 414–426.

Greco, S., Girardi, E., Navarra, A. and Saltini, C. Current evidence on diagnostic accuracy of commercially based nucleic acid amplification tests for the diagnosis of pulmonary tuberculosis. *Thorax.* 2006. 61, 783-90.

Greenlund, A.C., Farrar, M.A., Viviano, B.L. and Schreiber, R.D. Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91) *EMBO*. 1994. 13, 1591–1600

Gupta, S and Chatterji, D. Stress responses in mycobacteria. *IUBMB Life*. 2005. 57(3), 149-59.

Gutierrez, M.C., Brisse, S., Brosch, R., Fabre, M., Omaïs, B., Marmiesse, M., Supply, P. and Vincent, V. Ancient origin and gene mosaicism of the progenitor of Mycobacterium tuberculosis. *PLoS Pathog*. 2005. 1(1):e5.

Harris, J., De Haro, S.A., Master, S.S., Keane, J., Roberts, E.A., Delgado, M. and Deretic, V. T helper 2 cytokines inhibit autophagic control of intracellular Mycobacterium tuberculosis. *Immunity*. 2007. 27, 505–517.

Harding, C.V. and Boom, W.H. Regulation of antigen presentation by Mycobacterium tuberculosis: a role for Toll-like receptors. *Nat Rev Microbiol*. 2010, 8(4), 296–307.

Haynesworth, S.E., Baber, M.A. and Caplan, A.I. Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. *Bone.* 1992. 13, 69–80.

Herbert, N., George, A., Baroness Masham of I, Sharma, V., Oliver, M., Oxley, A., Raviglione, M. and Zumla, A.I. World TB Day 2014: finding the missing 3 million. *Lancet.* 2014. 383, 1016-8.

Houben, E. N., Bestebroer, J., Ummels, R., Wilson, L., Piersma, S.R., Jiménez, C.R., Ottenhoff, T.H., Luirink, J. and Bitter, W. Composition of the type VII secretion system membrane complex. *Mol. Microbiol*, 2012. 86, 472–484.

Houben, R.M. and Dodd, P.J. The Global Burden of Latent Tuberculosis Infection: A Re-estimation Using Mathematical Modelling. *PLoS Med*. 2016. 13(10), e1002152.

Hsu, T., Hingley-Wilson, S.M., Chen, B., Chen, M., Dai, A.Z., Morin, P.M., Marks, C.B., Padiyar, J., Goulding, C., Gingery, M., Eisenberg, D., Russell, R.G., Derrick, S.C., Collins, F.M., Morris, S.L., King, C.H. and Jacobs, W.R. Jr. The primary mechanism of attenuation of bacillus Calmette–Guérin is a loss of secreted lytic function required for invasion of lung interstitial tissue. *Proc. Natl Acad. Sci. USA*. 2003. 100, 12420–12425.

Hunter, R.L., Jagannath, C. and Actor, J.K. Pathology of postprimary tuberculosis in humans and mice: contradiction of long-held beliefs. *Tuberculosis*. 2007. 87, 267–278.

Ignatov, D.V., Salina, E.G., Fursov, M.V., Skvortsov, T.A., Azhikina, T. and Kaprelyants, A.S. Dormant non-culturable Mycobacterium tuberculosis retains stable low-abundant mRNA. *BMC Genomics.* 2015. 16, 16:954.

Im, J.G., Itoh, H., Shim, Y.S., Lee, J.H., Ahn, J., Han, M.C. and Noma S. Pulmonary tuberculosis: CT findings: early active disease and sequential change with antituberculous therapy. *Radiology.* 1993. 186, 653-60.

In 't Anker, P.S., Scherjon, S.A., Kleijburg-van der Keur, C., Noort, W.A., Claas, F.H., Willemze, R., Fibbe, W.E. and Kanhai, H.H. Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. *Blood.* 2003.102(4), 1548-9.

Iseman, M.D. MDR-TB and the developing world--a problem no longer to be ignored: the WHO announces 'DOTS Plus' strategy. *The International Journal of Tuberculosis and Lung Disease*. 1998. 2(11), 867.

Jacobs, S.A., Roobrouck, V.D., Verfaillie, C.M. and Van Gool, S.W. Immunological characteristics of human mesenchymal stem cells and multipotent adult progenitor cells. *Immunol Cell Biol.* 2013. 91, 32e9.

Jamwal, S., Mehrotra, P., Singh, A., Siddiqui, Z., Basu, A. and Rao, K.V. Mycobacterial escape from macrophage phagosomes to the cytoplasm represents an alternate adaptation mechanism. *Sci. Rep*. 2016. 6, 23089.

Javazon, E.H., Beggs, K.J. and Flake, A.W. Mesenchymal stem cells: paradoxes of passaging. *Exp Hematol*. 2004. 32(5), 414-25.

Jiang, Y., Woronicz, J.D., Liu, W. and Goeddel, D.V. Prevention of constitutive TNF receptor 1 signaling by silencer of death domains. *Science.* 1999, 283, 543–546.

Jurkuvenaite, A., Benavides, G.A., Komarova, S., Doran, S.F., Johnson, M., Aggarwal, S., Zhang, J., Darley-Usmar, V.M. and Matalon, S. Upregulation of autophagy decreases chlorine-induced mitochondrial injury and lung inflammation. *Free Radic Biol Med*. 2015. 85(3), 83-94.

Kaplan, G., Post, F.A., Moreira, A.L., Wainwright, H., Kreiswirth, B.N., Tanverdi, M., Mathema, B., Ramaswamy, S.V., Walther, G., Steyn, L.M., Barry, C.E. and Bekker, L.G. *Mycobacterium tuberculosis* growth at the cavity surface: a microenvironment with failed immunity. *Infection and Immunity.* 2003. 71, 7099–7108.

Kanchana, M.V., Cheke, D., Natyshak, I., Connor, B., Warner, A. and Martin, T. Evaluation of the BACTEC MGIT 960 system for the recovery of mycobacteria. *Diagn Microbiol Infect Dis.* 2000. 37, 31-6.

Kaufmann, S.H. How can immunology contribute to the control of tuberculosis? *Nat. Rev. Immunol.* 2001. 1(1), 20-30.

Kaur, K., Kumari, P., Sharma, S., Sehgal, S. and Tyagi, J. S.DevS/DosS sensor is bifunctional and its phosphatase activity precludes aerobic DevR/DosR regulon expression in Mycobacterium tuberculosis. *FEBS J*. 2016. 283, 2949–2962.

Keane, J., Balcewicz-Sablinska, M.K., Remold, H.G., Chupp, G.L., Meek, B.B., Fenton, M.J. and Kornfeld, H. Infection by Mycobacterium tuberculosis promotes human alveolar macrophage apoptosis. *Infect Immun.* 1997, 65, 298–304.

Keane, J., Remold, H.G. and Kornfeld, H. Virulent Mycobacterium tuberculosis strains evade apoptosis of infected alveolar macrophages. *J Immunol*. 2000. 164, 2016–2020.

Khader, S.A., Rangel-Moreno, J., Fountain, J.J., Martino, C.A., Reiley, W.W., Pearl, J.E., Winslow, G.M., Woodland, D.L., Randall, T.D. and Cooper, A.M. In a murine tuberculosis model, the absence of homeostatic chemokines delays granuloma formation and protective immunity. *J Immunol*. 2009. 183(12), 8004–8014.

Khan, A., Mann, L., Papanna, R., Lyu, M.A., Singh, C.R., Olson, S., Eissa, N.T., Cirillo, J., Das, G., Hunter, R.L. and Jagannath, C. Mesenchymal stem cells internalize Mycobacterium tuberculosis through scavenger receptors and restrict bacterial growth through autophagy. *Sci Rep.* 2017. 7(1), 15010.

Kim, J.J., Lee, H.M., Shin, D.M., Kim, W., Yuk, J.M., Jin, H.S., Lee, S.H., Cha, G.H., Kim, J.M., Lee, Z.W., Shin, S.J., Yoo, H., Park, Y.K., Park, J.B., Chung, J., Yoshimori, T. and Jo, E.K. 2012. Host cell autophagy activated by antibiotics is required for their effective antimycobacterial drug action. *Cell Host Microbe.* 2012. 11, 457–468.

Krampera, M., Pasini, A., Pizzolo, G., Cosmi, L., Romagnani, S. and Annunziato, F. Regenerative and immunomodulatory potential of mesenchymal stem cells. *Curr Opin Pharmacol.* 2006. 6(4), 435-41.

Krampera, M., Marconi, S., Pasini, A., Galliè, M., Rigotti, G., Mosna, F., Tinelli, M., Lovato, L., Anghileri, E., Andreini, A., Pizzolo, G., Sbarbati, A. and Bonetti B. Induction of neural-like differentiation in human mesenchymal stem cells derived from bone marrow, fat, spleen and thymus. *Bone*. 2007. 40(2), 382-90.

Koch, R. 1882. Die Aetiologie der Tuberkulose. Berl. Klin. Wochenschr. 19:221-230. [Reprint, *Am. Rev. Tuberc*. 1932. 25, 285-323.

Kovarik, P., Stoiber, D., Novy, M. and Decker, T. Stat1 combines signals derived from IFN-gamma and LPS receptors during macrophage activation. *EMBO.* 1998. 17, 3660–3668.

Kulterer, B., Friedl, G., Jandrositz, A., Sanchez-Cabo, F., Prokesch, A., Paar, C., Scheideler, M., Windhager, R., Preisegger, K.H. and Trajanoski, Z. Gene expression profiling of human mesenchymal stem cells derived from bone marrow during expansion and osteoblast differentiation. *BMC Genomics.* 2007. 8, 70 12

La Rocca, G., Anzalone, R., Corrao, S., Magno, F., Loria, T., Lo Iacono, M., Di Stefano, A., Giannuzzi, P., Marasà, L., Cappello, F., Zummo, G. and Farina, F. Isolation and characterization of Oct-4+/HLA-G+ mesenchymal stem cells from human umbilical cord matrix: differentiation potential and detection of new markers. *Histochem Cell Biol.* 2009. 131(2), 267-82.

Le Blanc, K., Tammik, C., Rosendahl, K., Zetterberg, E. and Ringdén, O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol.* 2003. 31, 890–896.

Ling, D.I., Flores, L.L., Riley, L.W. and Pai, M. Commercial nucleic-acid amplification tests for diagnosis of pulmonary tuberculosis in respiratory specimens: meta-analysis and meta-regression. *PLoS One*, 2008. 3, e1536.

Luca, S. and Mihaescu, T. History of BCG Vaccine. *Maedica (Buchar).* 2013. 8(1), 53-8.

Mac Donald, E.M. and Izzo, A.A. Tuberculosis vaccine development. In: Ribbon W (Ed.). Tuberculosis-expanding knowledge. *In Tech.* 2015.

Mackay, A.M. Beck, S.C. Murphy, J.M. Barry, F.P. Chichester, C.O. and Pittenger, M.F. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng.* 1998. 4(4), 415-28.

McDonough, K.A., Kress, Y. and Bloom, B.R. Pathogenesis of tuberculosis: interaction of Mycobacterium tuberculosis with macrophages. *Infect Immun.* 1993. 61(7), 2763-73.

Mitchison, D.A and Nunn, A.J. Influence of initial drug resistance on the response to short-course chemotherapy of pulmonary tuberculosis. *Am Rev Respir Dis.* 1986. 133, 423–430.

Miura, M., Gronthos, S., Zhao, M., Lu, B., Fisher, L.W., Robey, P.G. and Shi, S. SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci U S A*. 2003. 100(10), 5807-12.

Mizushima, N., Yoshimori, T. and Ohsumi, Y. The role of Atg proteins in autophagosome formation. *Annu Rev Cell Dev Biol*. 2011. 27, 107-32.

Mostowy, S., Inwald, J., Gordon, S., Martin, C., Warren, R., Kremer, K., Cousins, D. and Behr, M.A. Revisiting the evolution of Mycobacterium bovis. *J Bacteriol*. 2005. 187(18), 6386-95.

MRC. STREPTOMYCIN treatment of pulmonary tuberculosis. Br Med J 2, 1948**.** 769-782.

Munoz-Elias, E.J. and McKinney, J.D. Mycobacterium tuberculosis isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. *Nat Med.* 2005. 11, 638–644.

Murray, S. Challenges of tuberculosis control. *CMAJ*. 2006. 174(1), 33-4.

Nakahara, H., Dennis, J.E., Bruder, S.P., Haynesworth, S.E., Lennon, D.P. and Caplan, A.I. In vitro differentiation of bone and hypertrophic cartilage from periosteal-derived cells. *Exp Cell Res.* 1991. 195(2), 492-503.

Namatame, I., Tomoda, H., Arai, H., Inoue, K. and Omura, S. Complete inhibition of mouse macrophage-derived foam cell formation by triacsin C. *J. Biochem*. 1999. 125(2), 319–327.

Nayak, S. and Acharjya, B. Mantoux test and its interpretation. *Indian Dermatol Online J*. 2012. 3(1), 2-6.

Naik, S.K., Padhi, A., Ganguli, G., Sengupta, S., Pati, S., Das, D. and Sonawane, A. Mouse Bone Marrow Sca-1+ CD44+ Mesenchymal Stem Cells Kill Avirulent Mycobacteria but Not Mycobacterium tuberculosis through Modulation of Cathelicidin Expression via the p38 Mitogen-Activated Protein Kinase-Dependent Pathway. *Infection and Immunity*. 2017. 85 (10), e00471-17.

Nguyen, L. Antibiotic resistance mechanisms in M. tuberculosis: an update. *Arch Toxicol*. 2016. 90(7), 1585-604.

Orme, I.M., Robinson, R.T., Cooper, A.M. The balance between protective and pathogenic immune responses in the TB-infected lung. *Nat Immunol*. 2015. Jan; 16(1):57-63.

.

Pablos-Me´ndez, A., Raviglione, M.C., Laszlo, A., Binkin, N., Rieder , H.L., Bustreo, F., Cohn, D.L., Lambregts-van Weezenbeek, C.S., Kim, S.J., Chaulet, P. and Nunn, P. Global surveillance for antituberculosis-drug resistance. *N Engl J Med*, 1998. 338, 1641–1649.

Pai, M., Denkinger, C.M., Kik, S.V., Rangaka, M.X., Zwerling, A., Oxlade, O., Metcalfe, J.Z., Cattamanchi, A., Dowdy, D.W., Dheda, K. and Banaei, N. Gamma interferon release assays for detection of Mycobacterium tuberculosis infection. *Clin Microbiol Rev*. 2014. 27(1), 3-20.

Parrish, N.M., Dick, J.D. and Bishai, W.R. Mechanisms of latency in Mycobacterium tuberculosis. *Trends Microbiol*. 1998. 6(3).107-12.

Peyron, P., Vaubourgeix, J., Poquet Y., Levillain, F., Botanch, C., Bardou, F., Daffé, M., Emile, J.F., Marchou, B., Cardona, P.J., de Chastellier, C. and Altare, F. Foamy macrophages from tuberculous patients' granulomas constitute a nutrient-rich reservoir for M. tuberculosis persistence. *PLoS Pathogens*, 2008. 4. (11).

Phadwal, K., Watson, A.S. and Simon, A.K. Tightrope act autophagy in stem cell renewal, differentiation, proliferation, and aging. *Cell Mol Life Sci*. 2013. 70(1), 89–103.

Piatek, A.S., Tyagi, S., Pol, A.C., Telenti, A., Miller, L.P., Kramer, F.R. and Alland, D. Molecular beacon sequence analysis for detecting drug resistance in Mycobacterium tuberculosis. *Nat Biotechnol.* 1998. 16(4), 359-63.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S. and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. *Science.* 1999. 284,143–147.

Ponpuak, M., Davis, A.S., Roberts, E.A., Delgado, M.A., Dinkins, C. Zhao, Z. Virgin, H.W. IV, Kyei G.B. Johansen, T. Vergne, I. and Deretic V. Delivery of cytosolic components by autophagic adaptor protein p62 endows autophagosomes with unique antimicrobial properties. *Immunity*. 2010. 32:329–341.

Prattes, S., Horl, G., Hammer, A., Blaschitz, A., Graier, W.F., Sattler, W., Zechner, R., Steyrer, E. Intracellular distribution and mobilization of unesterified cholesterol in adipocytestriglyceride droplets are surrounded by cholesterol-rich ER-like surface layer structures. *J. Cell Sci*. 2000. 113, 2977–2989.

Prockop, D.J. Marrow stromal cells as stem cells for non-hematopoietic tissues. *Science.* 1997. 276, 71–74.

Pym, A. S., Brodin, P., Majlessi, L., Brosch, R., Demangel, C., Williams, A., Griffiths, K.E., Marchal, G., Leclerc, C. and Cole, S.T. Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. *Nat. Med*. 2003. 9, 533–539.

Rahman, M.A., Sobia, P., Gupta, N., Kaer, L.V. and Das, G. *Mycobacterium tuberculosis* Subverts the TLR-2 - MyD88 Pathway to Facilitate Its Translocation into the Cytosol. PLoS ONE. 2014. 9(1), e86886.

Ramaswamy, S. and Musser, J.M. Molecular genetic basis of antimicrobial agent resistance in Mycobacterium tuberculosis: 1998 update. *Tuber Lung Dis,* 1998. 79, 3-29.

Raghuvanshi, S., Sharma, P., Singh, S., Van Kaer, L. and Das, G. Mycobacterium tuberculosis evades host immunity by recruiting mesenchymal stem cells. *Proc Natl Acad Sci.* 2010. 107:21653e8.

Ramakrishnan L. Revisiting the role of the granuloma in tuberculosis. *Nat. Rev. Immunol*. 2012. 12, 352–366.

Reed, M.B., Gagneux, S., Deriemer, K., Small, P.M., Barry, C.E. 3rd. The W-Beijing lineage of Mycobacterium tuberculosis overproduces triglycerides and has the DosR dormancy regulon constitutively upregulated. *J Bacteriol.* 2007. 189: 2583–2589.

Roberts, D.M., Liao, R.P., Wisedchaisri, G., Hol, W.G. and Sherman, D.R. Two sensor kinases contribute to the hypoxic response of *Mycobacterium tuberculosis*. *J Biol Chem*. 2004. 279:23082–7.10.1074/jbc.M401230200.

Roach, D.R., Bean, A.G., Demangel, C., France, M.P., Briscoe, H. and Britton, W.J. TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. *J Immunol.* 2002. 168(9), 4620-7.

Russell, D.G. Who puts the tubercle in tuberculosis? *Nat Rev Microbiol*. 2007. 5, 39–47.

Russell, D.G. Mycobacterium tuberculosis: here today, and here tomorrow. *Nat Rev. Mol Cell Biol.* 2001. 2, 569e77.

Sassetti, C.M., and Rubin, E.J. Genetic requirements for mycobacterial survival during infection. *Proc. Natl Acad. Sci. USA* 100, 2013. 12989–12994.

Sandor, M., Weinstock, J. V. and Wynn, T. A. Granulomas in schistosome and mycobacterial infections: a model of local immune responses. *Trends in Immunology*, 2003. 24(1), 44–52.

Schultz, E., Gibson, M.C., Champion T. Satellite cells are mitotically quiescent in mature mouse muscle: An EM and radioautographic study. *J Exp Zool.* 1978. 206, 451–456.

Schroder, K., Hertzog, P.J., Ravasi, T. and Hume, D.A. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol*. 2004, 75, 163–189.

Schilling, T., Nöth, U., Klein-Hitpass, L., Jakob, F. and Schütze, N. Plasticity in adipogenesis and osteogenesis of human mesenchymal stem cells. Mol Cell Endocrinol. 2007. 271, 1–17.

Scordo, J.M., Knoell, D.L. and Torrelles, J.B. Alveolar Epithelial Cells in Mycobacterium tuberculosis Infection: Active Players or Innocent Bystanders?. J Innate Immun. 2016. 8(1):3–14.

Serbina, N.V. and Flynn, J.L. Early emergence of CD8 (+) T cells primed for production of type 1 cytokines in the lungs of Mycobacterium tuberculosis-infected mice. *Infect Immun.* 1999. 67, 3980–3988.

Shah, S. and Briken, V. Modular Organization of the ESX-5 Secretion System in Mycobacterium tuberculosis. *Front Cell Infect Microbiol.* 2016. 6:49.

Shah, N., Wright, A., Bai, G., Barrera, L., Boulahbal, F., Martín-Casabona, N., Drobniewski, F., Gilpin, C., Havelková, M., Lepe, R., Lumb, R., Metchock, B., Portaels, F., Rodrigues, M.F., Rüsch-Gerdes, S., Deun, A.V., Vincent, V., Laserson, K.F., Wells, C. and Cegielski, J. Worldwide Emergence of Extensively Drug-resistant Tuberculosis. *Emerging Infectious Diseases*. 2007. 13(3), 380-387.

Singh, A., Crossman, DK., Mai, D., Guidry, L., Voskuil, M.I., Renfrow, M.B. and Steyn, A.J. Mycobacterium tuberculosis WhiB3 maintains redox homeostasis by regulating virulence lipid anabolism to modulate macrophage response. *PLoS Pathog*. 2009. 5(8):e1000545.

Singh, C.R., Moulton, R.A., Armitige, L.Y., Bidani, A., Snuggs, M., Dhandayuthapani, S., Hunter, R.L. and Jagannath, C. Processing and presentation of a mycobacterial antigen 85B epitope by murine macrophages is dependent on the phagosomal acquisition of vacuolar proton ATPase and in situ activation of cathepsin D. *J Immunol* 2006. 177, 3250e9.

Silva Miranda, M., Breiman, A., Allain, S., Deknuydt, F. and Altare F. The tuberculous granuloma: an unsuccessful host defence mechanism providing a safety shelter for the bacteria? *Clin Dev Immunol*. 2012. 2012:139127.

Sirakova, T.D., Dubey, V.S., Deb, C., Daniel, J., Korotkova, T.A., Abomoelak, B. and Kolattukudy, P.E. Identification of a diacylglycerol acyltransferase gene involved in accumulation of triacylglycerol in Mycobacterium tuberculosis under stress. *Microbiology.* 2006. 2717–2725.

Siminovitch, L., Mcculloch, E.A. and Till, J.E. The distribution of colony-forming cells among spleen colonies". *Journal of Cellular and Comparative Physiology*. 1963. 62 (3), 327–36.

Smith, T., Wolff, K.A. and Nguyen, L. Molecular biology of drug resistance in Mycobacterium tuberculosis. *Curr Top Microbiol Immunol.* 2013. 374, 53-80.

Sordi, V., Malosio, M.L., Marchesi, F., Mercalli, A., Melzi, R., Giordano, T., Belmonte, N., Ferrari, G., Leone, B.E., Bertuzzi, F., Zerbini, G., Allavena, P., Bonifacio, E. and Piemonti L. Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. *Blood.* 2005. 106, 419–427.

Spaggiari, G.M., Capobianco, A., Abdelrazik, H., Becchetti, F., Mingari, M.C. and Moretta, L. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. *Blood*. 2008. 111(3), 1327-33.

Spaggiari, G.M., Abdelrazik, H., Becchetti, F. and Moretta, L. MSCs inhibit monocyte-derived DC maturation and function by selectively interfering with the generation of immature DCs: central role of MSC-derived prostaglandin E2. *Blood.* 2009. 113(26), 6576-83.

Stanley, S. A., Raghavan, S., Hwang, W. W. and Cox, J. S. Acute infection and macrophage subversion by Mycobacterium tuberculosis require a specialized secretion system. *Proc. Natl Acad. Sci.* USA, 2003. 100, 13001–13006.

Stead, W.W. The origin and erratic global spread of tuberculosis. How the past explains the present and is the key to the future. *Clin Chest Med.* 1997. 18, 65-77.

Sturgill-Koszycki, S., Schlesinger, P.H., Chakraborty, P., Haddix, P.L., Collins, H.L., Fok, A.K., Allen, R.D., Gluck, S.L., Heuser, J. and Russell, D.G. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. *Science*. 1994. 263(5147), 678-81.

Steingart, K.R., Henry, M., Ng, V., Hopewell, P.C., Ramsay, A., Cunningham, J., Urbanczik, R., Perkins, M., Aziz, M.A. and Pai, M. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. *Lancet Infect Dis,* 2006. 6, 570-81.

Sundaramurthy, V., Barsacchi, R., Samusik, N., Marsico, G., Gilleron, J., Kalaidzidis, I., Meyenhofer, F., Bickle, M., Kalaidzidis, Y. and Zerial, M. Integration of chemical and RNAi multiparametric profiles identifies triggers of intracellular mycobacterial killing. *Cell Host Microbe.* 2013. 13, 129–142.

Tauchi-Sato, K., Ozeki, S., Houjou, T., Taguchi, R. and Fujimoto, T. The surface of lipid droplets is a phospholipid monolayer with a unique Fatty Acid composition. *J Biol Chem*. 2002. 277(46), 44507-12.

Tse, W.T., Pendleton, J.D., Egalka, M.C. and Guinan, E.C. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation*. 2003. 75(3), 389-97.

Tufariello, J.M., Chan, J. and Flynn, J.L. Latent tuberculosis: mechanisms of host and bacillus that contribute to persistent infection. *Lancet Infect Dis*. 2003. 3(9). 578-90.

Ulrichs, T. and Kaufmann, S.H. New insights into the function of granulomas in human tuberculosis. *J Pathol.* 2006. 208(2), 261-9.

van Crevel, R., Ottenhoff, T.H. and van der Meer, J.W. Innate immunity to Mycobacterium tuberculosis. *Clin Microbiol Rev.* 2002. 15(2), 294-309.

van Ingen, J., Rahim, Z., Mulder, A., Boeree, M.J., Simeone, R., Brosch, R. and van Soolingen D. Characterization of Mycobacterium orygis as M. tuberculosis complex subspecies. *Emerg Infect Dis*. 2012. 18(4), 653-5.

van der Wel, N., Hava, D., Houben, D., Fluitsma, D., van Zon, M., Pierson, J., Brenner, M., and Peters, P.J. M. tuberculosis and M. leprae translocate from the phagolysosome to the cytosol in myeloid cells. *Cell*. 2007. 129(7), 1287-98.

Velayati, A.A., Masjedi, M.R., Farnia, P., Tabarsi, P., Ghanavi, J., Ziazarifi, A.H. and Hoffner, S.E. Emergence of new forms of totally drug-resistant tuberculosis bacilli: Super extensively drug-resistant tuberculosis or totally drug-resistant strains in Iran. *Chest*. 2009, 136, 420–425.

Vergne, I., Chua, J., Singh, S.B. and Deretic, V. Cell biology of Mycobacterium tuberculosis phagosome. *Annu Rev Cell Dev Biol*. 2004. 20, 367–394.

Voskuil, M. and Schlesinger, L.S. Toward Resolving the Paradox of the Critical Role of the DosR Regulon in Mycobacterium tuberculosis Persistence and Active Disease. *Am J Respir Crit Care Med*. 2015. 191(10):1103-5.

Wajant, H., Pfizenmaier, K. and Scheurich, P. Tumor necrosis factor signaling. *Cell Death Differ.* 2003, 10, 45–65.

Wakitani, S., Saito, T. and Caplan, A.I. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. *Muscle Nerve.* 1995. 18, 1417–1426.

Wang, S., Qu, X. and Zhao, R.C. Clinical applications of mesenchymal stem cells. *J Hematol Oncol.* 2012 30, 5:19.

Woodring, J.H., Vandiviere, H.M., Fried, A.M., Dillon, M.L., Williams, T.D., Melvin, I.G. Update: the radiographic features of pulmonary tuberculosis. *AJR Am J Roentgenol.* 1986. 146, 497-506.

World Health Organization. Molecular line probe assays for rapid screening of patients at risk of multidrug-resistant tuberculosis (MDR-TB): policy statement [Internet]. Geneva: *World Health Organization*, 2008. [cited 2015 Mar 1]. Available from. http://www.who.int/tb/laboratory/lpa_policy.pdf.

World Health Organization. Global Tuberculosis Control Report 2010. Summary. Cent Eur J Public Health. 18,237.

World Health Organization. "BCG Vaccine". Weekly Epidemiological Record 79(4), 27-38.

World Health Organization. Global tuberculosis report. 2018.

World Health Organization. Global tuberculosis report. 2013. World Health Organization. https://apps.who.int/iris/handle/10665/91355.

World Health Organization. Global tuberculosis report. 2013. https://reliefweb.int/sites/reliefweb.int/files/resources/9789241565516-eng.pdf

Xia, M., Gasser, J. and Feige, U. Dexamethasone enhances CTLA-4 expression during T cell activation. *Cell Mol Life Sci.* 1999. 55, 1649–56.

Yuk, J.M., Shin, D.M., Lee, H.M., Yang, C.S., Jin, H.S., Kim, K.K., Lee, Z.W., Lee, S.H., Kim, J.M. and Jo, E.K. Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin. *Cell Host Microbe.* 2009. 6, 231–243.

Zaman K. Tuberculosis: a global health problem. *J Health Popul Nutr*. 2010 Apr;28(2):111-3. doi: 10.3329/jhpn.v28i2.4879.

Zhang, Y. and Yew, W.W. Mechanisms of drug resistance in Mycobacterium tuberculosis. *Int J Tuberc Lung Dis*. 2009. 13, 1320- 30.

Zhang, S.Y., Boisson-Dupuis, S., Chapgier, A., Yang, K., Bustamante, J., Puel, A., Picard, C., Abel, L., Jouanguy, E. and Casanova, J.L. Inborn errors of interferon (IFN)-mediated immunity in humans: insights into the respective roles of IFN-alpha/beta, IFN-gamma, and IFN-lambda in host defense. *Immunol Rev*. 2008. 226, 29–40.

Zhou, S., Schuetz, J., Bunting, K., Colapietro, A., Sampath, J., Morris, J., Lagutina, I., Grosveld, G., Osawa, M., Nakauchi, H. and Sorrentino, B. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med.* 2001. 9, 1028e34.

Zink, A.R., Sola, C., Reischl, U., Grabner, W., Rastogi, N., Wolf, H. and Nerlich, A.G. Characterization of Mycobacterium tuberculosis complex DNAs from Egyptian mummies by spoligotyping. *J Clin Microbiol.* 2003. 41(1):359-67.

Zuñiga, J., Torres-García, D., Santos-Mendoza, T., Rodriguez-Reyna, T.S., Granados, J., and Yunis, E.J. Cellular and humoral mechanisms involved in the control of tuberculosis. *Clin Dev Immunol.* 2012. 2012:193923.

Zuk, P.A., Zhu, M., Ashjian, P., De Ugarte, D.A., Huang, J.I., Mizuno, H., Alfonso, Z.C., Fraser, J.K., Benhaim, P. and Hedrick, M.H. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell.* 2002. 13(12), 4279-95.

Publications

Publications

***Mycobacterium tuberculosis* Programs Mesenchymal Stem Cells to Establish Dormancy and Persistence**

Samreen Fatima, Shashank Shivaji Kamble, Ved Prakash Dwivedi, Debapriya Bhattacharya, Santosh Kumar, Anand Ranganathan, Luc Van Kaer, Sujata Mohanty, Gobardhan Das. (Under Revision), 2019

Luteolin enhances isoniazid treatment of tuberculosis by promoting central memory T cell responses

Dhiraj Kumar Singh, Ved Prakash Dwivedi, Sultan Tousif, **Samreen Fatima**, Luc Van Kaer, Anand Ranganathan, Gobardhan Das. (Under Revision), 2019

Allicin enhances antimicrobial activity of macrophages during *Mycobacterium tuberculosis* infection

Ved Prakash Dwivedi, Debapriya Bhattacharya, Mona Singh, Ashima Bhaskar, Santosh Kumar, **Samreen Fatima**, Parveen Sobia, Luc Van Kaer, Gobardhan Das. Journal of ethnopharmacology, 2019

Allicin enhances anti-mycobacterial activity of macrophages via SAPK/JNK signaling

Ved Prakash Dwivedi[1, #, *], Debapriya Bhattacharya[2, #], Mona Singh[2, #], Ashima Bhaskar[3], Santosh Kumar[1], Samreen Fatima[2], Parveen Sobia[4], Luc Van Kaer[5], Gobardhan Das[2, *]

1. Immunology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India.
2. Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi, India.
3. Signal Transduction Laboratory-1, National Institute of Immunology, New Delhi, India
4. College of Health Sciences, Laboratory of Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa.
5. Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN, USA.

# These authors contributed equally to the manuscript.

***Address for Correspondence:**
Prof. Gobardhan Das
Special Centre for Molecular Medicine,
Jawaharlal Nehru University,
New Delhi, India.
E-mail: gobardhan.das07@gmail.com
And
Dr. Ved Prakash Dwivedi
International Centre for Genetic Engineering and Biotechnology (ICGEB)
Aruna Asaf Ali Marg
New Delhi 110067
E-mail: vedprakashbt@gmail.com

Running Title: Allicin-mediated immunotherapy of tuberculosis

Keywords: *Mycobacterium tuberculosis*, T cells, Immunomodulation, Allicin

Turnitin Originality Report

Thesis by Samreen Fatima

From M.Phil/Ph.D 2019 (M.Phil/Ph.D)

- Processed on 11-Jul-2019 11:46 IST
- ID: 1150956872
- Word Count: 19571

Similarity Index
9%
Similarity by Source

Internet Sources:
5%
Publications:
6%
Student Papers:
6%

sources:

1 1% match (Internet from 03-Mar-2019)
https://www.frontiersin.org/articles/10.3389/fncel.2018.00480/full

2 1% match (Internet from 24-Jun-2019)
https://link.springer.com/article/10.1007%2Fs10616-014-9718-z

3 1% match (publications)
Sujata Mohanty, Sushmita Bose, Krishan Gopal Jain, Balram Bhargava, Balram Airan. "TGFβ1 contributes to cardiomyogenic-like differentiation of human bone marrow mesenchymal stem cells", International Journal of Cardiology, 2013

4 < 1% match (student papers from 21-Mar-2015)
Submitted to University of KwaZulu-Natal on 2015-03-21

5 < 1% match (Internet from 12-Jun-2019)
https://patents.justia.com/patent/20190002990

6 < 1% match (student papers from 12-Aug-2017)
Submitted to Jawaharlal Nehru University (JNU) on 2017-08-12

7 < 1% match (Internet from 02-Sep-2016)
https://www.scribd.com/doc/61996332/2005-ISSCR07Poster-Sessions

8 < 1% match (publications)
T. Fujimoto, R. G. Parton. "Not Just Fat: The Structure and Function of the Lipid Droplet", Cold Spring Harbor Perspectives in Biology, 2011