Host-pathogen interactions of different strains of *Mycobacterium tuberculosis*

By

Ms. Pampi Chakraborty

Enrolment No.-LIFE01200604003

BHABHA ATOMIC RESEARCH CENTRE, MUMBAI

A thesis submitted to the
Board of Studies in Life Sciences
In partial fulfilment of requirements
For the degree of

DOCTOR OF PHILOSOPHY

Of

HOMI BHABHA NATIONAL INSTITUTE

April, 2014

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfilment of requirements for an advanced degree at Homi Bhabha National Institute (HBNI) and is deposited in the Library to be made available to borrowers under rules of the HBNI.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the Competent Authority of HBNI when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

(Pampi Chakraborty)

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

(Pampi Chakraborty)

List of Publications arising from the thesis:

a. Published:

1) Drug resistant clinical isolates of Mycobacterium tuberculosis from different genotypes exhibit differential host responses in THP-1 cells. **Pampi Chakraborty**, Savita Kulkarni, M.G.R. Rajan, K. B. Sainis, PLoS ONE, 1213, 8(5): e62966. doi: 10. 1371 / journal. pone.0062966

b. Manuscript under preparation:

1) *Mycobacterium tuberculosis* strains from modern and ancient lineage induce distinct patterns of immune responses in human macrophages, dendritic cells and whole blood. **Pampi Chakraborty** *et al.*
2) Infection with different *Mycobacterium tuberculosis* genotypes in a BALB/c mouse model exhibit differential pattern of cytokine expression, bacterial load and lung pathology. **Pampi Chakraborty** *et al.*

c. Papers presented in Conferences:

1. **P. Chakraborty**, S. Kulkarni, M.G.R. Rajan, K.B. Sainis (**2008**) To study host-parasite interactions by standardizing infection model using THP-1 cell line and different strains of *M.tuberculosis.* DAE-BRNS 4th Life Sciences Symposium on Recent Advances in immunomodulation, stress and cancer, Mumbai .
2. S. P. Kulkarni, **P. Chakraborty**, K. B. Sainis. (**2009**) The immune subversion of XDR strains of *M.tuberculosis* isolated from Mumbai. 36th Annual conference of Indian Immunology Society, Bangalore
3. S. Kulkarni, M.Makwana, P. Gupta, **P.Chakraborty**, M.G.R. Rajan. (**2010**) Study of polymorphisms in *Mycobacterium tuberculosis* using TbD1 deletion analysis and spoligotyping. DAE-BRNS Life Science Symposium on Current Trends in Biology and Medicine, Mumbai.
4. **P.Chakraborty**, S. P. Kulkarni**,** M.G.R. Rajan, K. B. Sainis. (**2011**) Study of immune responses in THP-1 cells infected with strains of *Mycobacterium tuberculosis* from different lineages prevalent in India. Keystone symposium Tuberculosis: Immunology, Cell Biology and Novel Vaccination Strategies. Vancouver, Canada**.**
5. **Pampi Chakraborty**, Savita Kulkarni, M. G. R. Rajan, K. B. Sainis. (**2011**) Differential immune response to different genotypes of *M.tuberculosis* is a strain dependent phenomenon'. 37th annual conference of Indian Immunology Society, Jammu, February 7-9,.

6. Savita Kulkarni , Sriparna Das, **Pampi Chakraborty**, M. G. R. Rajan. (**2011**) Over-expression, purification and immunological characterization of recombinant 38kda antigen of *M.tuberculosis.* 37th annual conference of Indian Immunology Society, Jammu.
7. **P. Chakraborty**, S. P. Kulkarni , M.G.R. Rajan, K. B. Sainis. (**2012**) Different strains of *M.tuberculosis* exhibit lineage specific immune responses to subvert the host immunity, irrespective of the type of host they infect. 5th Congress of the Federation of Immunological Societies of Asia Oceania, New Delhi.
8. Pramod Kumar Gupta, **Pampi Chakraborty**, M. G. R. Rajan, Savita Kulkarni.(**2012)** Modulation of maturation and activation of murine bone marrow derived dendritic cells (BMDCs) by different clinical isolates of *M.tuberculosis.* U.S.-India Joint Workshop on tuberculosis at International Centre For Genetic Engineering And Biotechnology, New Delhi
9. Sujay Gaikwad, **Pampi Chakraborty**, Savita Kulkarni, M.G.R Rajan. The lipid profiles of different genotypes of M.tuberculosis and its correlation to in vitro immune response induced by the same stains. 39th Annual Conference of Indian Immunology Society (IMMUNOCON 2012), 9-11 Nov, 2012.Varanasi.
10. Gupta P.K., Kulkarni S., Rajan M.G.R., **Chakraborty P**.,Singh P.K., Kumar S., Sainis K.B. G1-4A (2013) an immunomodulatory polysaccharide from Tinospora cordifolia inhibits the survival of multi drug resistant strains of Mycobacterium tuberculosis. Keystone symposium on 'Host response in Tuberculosis' Whistler, British Columbia Canada, Canada.

Pampi Chakraborty.

Dedicated
To my
Loving parents & parents in-law

Acknowledgements

I feel highly fortunate and proud to have Prof. Krishna B. Sainis, Distinguished Scientist, Director, Bio-Medical group, BARC as my guide. I wish to express my deep gratitude to my guide for his guidance, advice, patience, time and support, even from the days of training school (course work). Indeed, I am honoured to be one of his students. It would not have been possible for me to carry out and complete the work without his kind endurance and help.

I am deeply indebted to Dr. (Mrs.) Savita Kulkarni, SO 'G', Leader, Molecular Biology and Tuberculosis group, Radiation Medicine Centre, who has been very encouraging and supportive throughout my tenure. She has introduced me to the world of Tuberculosis. I found her standing beside me, in many ups and downs in my research and personal life. Her constant faith and confidence in me motivated me to work with new enthusiasm. I thank her for giving me the chance to work in her group and always being approachable.

I am greatly indebted to Dr. M. G. R. Rajan, Head, Radiation Medicine Centre, B.A.R.C., also a member of my doctoral committee, for giving me the opportunity to work in this department, for providing the necessary facilities and support.

It is my great privilege to acknowledge Prof. Mohandas K. Mallath, Prof. M Seshadri, and Prof. (Mrs.) Shubhada Chiplunkar as members of my HBNI doctoral committee for providing valuable suggestions for the completion of this work.

I express my sincere thanks to Mr. Avik Chakraborty, my colleague and husband for giving critical suggestions while doing the experiments and always helping in troubleshooting in every possible way.

I also wish deepest sense of gratitude to Mr. Mukti Kanta Ray and Mr. Pramod Kumar Gupta for helping me in several experiments. I thank my lab colleagues Mr. Sujay Gaikoad and Mr. J. Kumarswamy for their moral support and valuable guidance. I also like to thank Dr. Praful Kumar Singh and Mr. Santosh Kumar for their immense support during animal experiments in ICGEB. Further, I am thankful to Dr. Yogita Pawer, Dr. Uma S Bhartiya, Mrs. Smita G, Mrs. Chandrakala G, Mrs. Yogita R, Mr. Sushanta Kumar Roy and Mr. S. Lohar for their help and encouragement. I am also thankful to Mr. U. Sheikh for providing me the required lab help day in and out. I am also thankful to all RMC colleagues and staff members for their support at different times.

I am deeply indebted to my teachers, parents and parents in law who enlighten the way of my life with their efforts, care and love to shape and mould my scientific career. I wish to acknowledge to my brother, Ankan, brother in law, Souvik, my daughter Mrittika and my friend Aditi for their moral support and encouragement.

I wish to sincerelyplace on record a note of heartiest gratitude to all those who directly or indirectly helped to bring this work to the present form.

Pampi Chakraborty.

Contents

SYNOPSIS

Host-pathogen interactions of different strains of *Mycobacterium tuberculosis*

INTRODUCTION:

Tuberculosis is the second leading cause of death among all infectious diseases worldwide. According to World Health Organization (WHO), India ranks the world's second most heavily affected country, with estimated 2 deaths every 3 minutes [1]. Emergence of Multi-drug resistance (MDR), extreme drug resistance (XDR) and failure of conventional vaccination with BCG have made the disease unmanageable. Tuberculosis is acquired through inhalation of droplets containing live *Mycobacterium tuberculosis* (MTB), the causative agent and the lung is the major site of the disease (pulmonary tuberculosis). The bacilli spread from the site of initial infection through the lymphatics or blood to other parts of the body such as bones, joints, the nervous system and the lymphatic system (extra-pulmonary tuberculosis).

The internalization of MTB by alveolar macrophages and alveolar epithelial cells is the first event in the host-pathogen interaction which decides outcome of the infection. Infecting bacilli are either killed or remain 'viable but latent' inside the host macrophage for decades. In the infected individuals who develop active disease, bacilli appear to evade or subvert the host's protective cellular immune responses. Within 2 to 6 wk of infection, cell-mediated immunity (CMI) develops and there is an influx of lymphocytes and activated macrophages into the lesion resulting in granuloma formation [2, 3]. The CMI response is necessary to control MTB infection and it is also responsible for the pathology associated with tuberculosis. Activated macrophages and T-cells secrete cytokines- like interleukin-1 (IL-l), tumour necrosis factor (TNF-α) and interferron- gamma (IFN-γ)- which play essential role in protection against TB [4]. In TB infection, Th1-type cytokines seem essential for protective immunity whereas Th2-type cytokines inhibit the in-vitro production of IFN-γ, as well as the activation of macrophages, and may, therefore, weaken host defence.

Development of active TB is believed to be determined by multiple factors of host, like genetic background including Major Histocompatibility Complex (MHC) and immune status of the individual. MHC,Toll Like Receptor-2 (TLR-2) Vitamin D receptor, IFN-γ and IL-12R gene polymorphism of the host were shown to be linked with the susceptibility of the disease. The impact of the strain variation for human disease has been well established for a number of

bacterial pathogens, but it is not still well established for tuberculosis. It was believed until recently that MTB exhibits a clonal population structure and therefore, was regarded as an organism with little relevant genetic variation. However, after the discovery of molecular typing methods for MTB, different types and subtypes of bacteria were revealed. Further, the genotypes were also shown to be restricted to a specific geographic location, for example, it was shown that MTB from South India were devoid of sulpholipids and less virulent and more susceptible to oxidative stress as compared to one from Great Britain [5]. Similarly, the F11 strain represents the largest proportion of all isolates from tuberculosis patients in the Western Cape of South Africa [6].

More recently, it has been established that differences the genotypes of the MTB strain are responsible for the differences in virulence, antibiotic susceptibility, relapse and prevalence [7-9]. The precise pathogenesis of TB and the factors determining the highly variable outcome of infection are only partly understood. Besides, a majority of the studies related to pathogenesis of TB were using the laboratory strains *H37Rv, H37Ra, M. bovis BCG*, Erdman and *CDC1551*. As the laboratory strains can suffer from artifacts because of their adaptation to the laboratory, a study using clinical isolates may be advantageous as they may represent the behavior of wild type pathogen. In view of this, in present study, clinical isolates of specific genotypes were selected for evaluating the host-pathogen interactions at the cellular and molecular level. This will facilitate understanding of the mechanisms of different pathways used by pathogen to subvert host immunity. Such an understanding may help in future for discovery of new drug targets.

OBJECTIVES

The highly variable outcome of MTB infection varies from life-long asymptomatic infection to severe disease and is determined by the battle between the host immunity and the virulence of the MTB strain. Influence of MTB genomic diversity on disease outcome is still unexplored. In view of this the main objective of the of present study was to evaluate the effect of genetically diverse strains of MTB on *in vivo* as well as *in vitro* host responses.

The study involved

1. **Selection of MTB clinical isolates from different genotypes and complete characterization with respect to drug resistance, MIRU-VNTR and TbD-1.**
2. **Analysis of consequences like intracellular growth, induction of cytokine and apoptosis of infected cells by different MTBstrains in monocyte leukemic cell line,**

THP-1

3. ***Ex vivo* infection of monocyte derived macrophages (MDM), monocyte derived dendritic cells (MDDC) and whole blood from healthy individuals, with different strains of MTB and assessment of cytokine responses.**
4. ***In vivo* infection study in BALB/c mice with aerosols of different strains of MTB.**

ORGANISATION OF THE THESIS

The work reported in the thesis is organized into five chapters: Chapter 1 deals with general introduction about the scientific information available related to the present work and its objectives. The characterization of the selected strains using different molecular typing methods is described in Chapter 2. Chapter 3 presents the results obtained for the innate immune parameters such as apoptosis, intracellular growth, phagocytosis and cytokine responses in THP-1 cells, infected with strains of MTB from different genotypes. To evaluate the innate immune responses in different hosts, *ex vivo* infection with different clinical isolates was carried out in macrophage, DCs and whole blood isolated from healthy individuals and the results are described in Chapter 4. The results of the responses to *in vivo* infection in BALB/c mice with different strains are given in Chapter 5. The overall discussion and summary of the work will be presented at the end of all chapters.

EXPERIMENTAL

All MTB strains were grown in Middelbrook 7H9 medium till in their mid log phase and then harvested either for DNA extraction or for preparation of single cell suspension. The genomic DNA of all strains was extracted by CTAB method and utilized for strain typing. Spoligotyping, PCR for Spoligotyping of MIRU-VNTR, RD1, TbD1 loci, and mutation analysis and antibiotic sensitivity assays were performed to characterize the clinical isolates [10, 11]. THP-1 cells, maintained in RPMI were infected with MTB for four hours at a multiplicity of infection (MOI) of 10. After the initial period of co-culture, the extracellular bacteria were washed off thoroughly and fresh medium was added for desired time. The internalization of bacteria was scored by ZN acid fast and Rhodamine-auramine staining under visible and UV light microscope respectively. The post-infection cytokine and chemokine expression were monitored by quantitative real time PCR and ELISA. The intracellular growth of the bacterium was monitored by the radiorespirometry technique [12] and counting Colony Forming Units (CFU). Apoptosis in infected host cells was assessed by apoptosis ELISA kit, flowcytometric analysis (PI and Annexin V staining) and by monitoring apoptosis related protein expression by Western Blot. MDM and MDDC were generated from peripheral blood

mononuclear cells (PBMC) of healthy individuals and GM-CSF and IL-4 were used for the generation of DC. Finally, BALB/c mice were infected by aerosolization using different strains of MTB and intra-pulmonary bacterial growth was monitored as CFU in lungs [13]. Both the infected and uninfected lungs were evaluated by histopathology. In all the experiments, uninfected cells were considered as negative control and H37Rv/ H37Ra and *M.bovis* BCG were used as laboratory reference strains.

RESULTS & DISCUSSION:

Characterization of *Mycobacterium tuberculosis* Strains:

The primary difficulty in trying to link genomic diversity to phenotypic diversity in MTB strains is the lack of appropriate tools to index and classify strains. MTB is a genetically monomorphic organism and some of the genotyping tools applied to other bacterial pathogens are uninformative for this pathogen. One single molecular technique is not sufficient to describe the strains properly. Hence, several techniques were used in present study for this purpose.

The strains obtained from different hospitals were first plated on TCH (thiophene-2-carboxylic acid hydrazide) containing Middlebrook medium and stained with Zeal-Nielson to reconfirm the species. Further, spoligotyping was performed for these strains. The most recent international spoligotyping database version, SpolDB4 [14] contained about 39,295 patterns distributed into 1,939 STs (shared-types, which are patterns shared by 2 or more clinical isolates), and 3,370 orphans (patterns reported for a single isolate). After comparing the spoligo patterns obtained for different clinical isolates, three strains from different clades, EAI5, LAM6 and Beijing having SIT numbers 763, 64 and 1 respectively were selected.

Recent developments in comparative genomics revealed differences in the presence or absence of regions of differences (RDs) and tuberculosis-specific deletion 1 (TbD1) between the strains of MTB. These genomic landmarks have been described as potential m arkers for understanding the virulence and historical origins respectively. RD1 may encode a novel secretion system, but the mechanism by which this region affects virulence is unknown. TbD-1 region harbours mmpL6 gene and its presence or absence could help to identify various lineages into ancestral strains or modern strains. Among our strains, EAI-5 represented ancient strain having intact TbD-1 region (2.6kb) and rest of the strains, including H37Ra and H37Rv exhibited truncated TbD-1 region confirming their modern lineage. The truncated TbD1 region

is due to deletion of an internal DNA segment which was further confirmed by nested PCR. All the strains exhibited presence of an intact RD1 region which confirms their probable virulent nature.

The MTB genome contains several repetitive elements; only a few are polymorphic and widely studied. A large number of genotyping tools for MTB fingerprinting exist, of which spoligotyping and MIRU-VNTR (*Mycobacteria*l interspersed repetitive units- Variable Number of Tandem Repeats) typing are the most commonly used. The development and application of the MIRU-VNTR typing for MTB became an important methodological achievement towards a better understanding of the genotype of the pathogen. The lengths of MIRU repeat units are in the range of 50-100 bp, and it belongs to the "minisatellite" VNTR category and located mainly in intergenic regions dispersed throughout the MTB genome. The typing is based on the number of copies of repeated units in a locus for which polymorphism is observed. In MIRU-VNTR study, EAI strain showed different copy number for eight loci compared to LAM-6 and for 6 loci compared to Beijing strain. On the other hand, both modern genotypes, LAM-6 and Beijing, also differed from each other by six loci.

Drug resistance in MTB occurs by random, single step spontaneous mutations at low frequency. These mutations can alter one or more genes which affect the primary drug target or the transport system. Increasing drug resistance in MTB clinical strains is a matter of great concern for TB control and TB pathogenesis study. Hence, for precise characterization, the phenotypic and genotypic drug resistance pattern of the strains should be examined. The drug susceptibility test was performed on LJ slants containing both first and second line antibiotics at the standard concentrations as per the recommendation by WHO. Additionally, the presence of mutation was analyzed by sequencing in seven genes (*rpoB, katG, inhA, ahpC*, *rpsL, rrs* and *embB*) reportedly associated with resistance to first line anti-TB drugs (RIF, INH, STR and EMB). Among the three clinical isolates, EAI-5 and LAM-6 was poly drug resistant and the Beijing strain was XDR.

Analysis of different innate immune interactions of *Mycobacterium tuberculosis* strains with monocyte leukemic cell line, THP-1

The human acute monocyte leukemic cell line,THP-1, is a suitable alternative to peripheral blood monocyte models and it has proved to be the best system to study the intracellular growth of MTB, immunomodulatory effect and apoptosis after infection [15].

Further, THP-1 can serve as genetically identical and stable host for genetically diverse pathogen population.

Phagocytic index for different strains of M.tuberculosis

The extent of infection in THP-1 cells for H37Rv, H37Ra and three clinical isolates was scored based on the phagocytic index using ZN acid fast and phenolic auramine staining. All the strains showed comparable infectivity in terms of percentage of macrophages infected (~ 55% and 70 % after two hours and four hours of infection respectively). However, when the phagocytic index was divided into three different groups like 1-5, 6-10 and greater than 10 bacilli per cell, it was found that H37Ra had significantly high percentage of accumulation of bacteria (18±5%) in the category '>10 bacilli/cell' than both Beijing (7±3%) and LAM (11±3%) strains. The percentage of infected macrophages in '1-5 bacilli /cell' category was higher compared to '6-10' and '>10'bacilli/cell categories for all the clinical isolates.

Assessment of Mycobacterial growth in infected THP-1 cells using radiorespirometry technique.

The curves for intracellular growth were established by radiorespirometry technique for all strains growing in THP-1 cells. The CPM (counts per minute) values represented cumulative amount of $^{14}CO_2$ released by viable bacilli. The CPM values were comparable on day 0 for all MTB strains which confirmed the similar extent of infection by all the strains. The intracellular bacillary load was compared on fifth day samples and a gradual increase of intracellular bacteria was seen for all the strains till fifth day. H37Rv and LAM-6 showed significantly higher intracellular bacillary load compared to H37Ra on 5^{th} day of infection. On the other hand EAI-5 and Beijing showed intracellular growth at intermediate pace lower than H37Rv. The intracellular growth was also monitored by CFU assay and it gave similar result as by radiorespirometry.

iNOS, RNI and ROS assays with infected THP-1

Putative mechanisms involved in killing of MTB within the phagolysosomes of activated macrophages include the production of reactive oxygen species (ROS) or reactive nitrogen intermediates (RNI). Besides, these reactive intermediates also serve as secondary messenger molecules for cytokine-chemokine gene expression. It has been observed that several *Mycobacteria*l cell wall products, including sulfatides and lipoarabinomannan(LAM), are able to scavenge ROS[16]. In view of the information that, the cell wall composition varies with the clade of the MTB, iNOS mRNA expression, RNI production and ROS were evaluated using RT- PCR, Gris reagent and DCFDA respectively. It was observed that there exists a

differential expression of RNI and ROS in infected host for different strains. This indicated two possibilities that either the strains responded differently with the reactive species, or they could modulate the reactive intermediate production and manipulate the host immune response differently. The ancient EAI strain showed significantly higher iNOS expression, RNI and ROS than other strains; on the other hand, Beijing strain showed significantly lower production of iNOS than H37Rv.

Cytokine- chemokine profiles in THP-1 cells after infection with different strains

The gene expression for proinflammatory cytokines like TNF-α, IL-1β, IL-12 and anti-inflammatory cytokine IL-10, chemokines IL-8, and MCP-1 was assessed in infected THP-1 cells after 24 hours of infection. The expression of TNF-α, IL-12 and IL-1β was significantly ($P<0.005$) higher in THP-1 cells infected with EAI-5 than in cells infected with H37Rv, H37Ra and Beijing strains. However, the expression of these genes was comparable in LAM-6 and H37Rv infected cells. The infection with Beijing genotype showed unusually lower expression levels for all cytokine genes mentioned above. There was no significant difference in expression of IL-10 mRNA among the cells infected with different strains, except for Beijing which induced significantly lower amount of IL-10 compared to other strains ($P<0.05$). The most interesting observation about Beijing strain was that it induced lesser amount of both pro and anti-inflammatory cytokines in THP-1 cells in contrast to cells infected with other strains.

The levels of different cytokines measured by ELISA corroborated the mRNA expression pattern. TNF-α concentration in 48 hour post-infection supernatants of THP-1 cells were almost similar for H37Ra, H37Rv and LAM strains. However, Beijing genotype induced significantly lower and EAI induced significantly higher amount of TNF-α compared to the other three strains. Further, patterns for the secretion of other pro-inflammatory cytokines, IL-1β, IL-6 and IL-12, were similar to TNF-α, showing a highly significant correlation among them.

Apoptosis in THP-1 cells infected with different strains

Significant apoptosis was observed after fifth day of infection in THP-1 cells. The pattern of apoptosis in THP-1 cells after infection with different strains was monitored using Annexin V staining by flowcytometry after five and six days of infection. Among the five strains, H37Ra and EAI-5 induced significantly higher apoptosis compared that in uninfected and cells infected with Beijing strain. Further, the apoptotic response to LAM-6 was similar to that observed for H37Rv. The relative expression of pro-apoptotic protein, PARP was verified in uninfected cells and THP-1 cells infected with different MTB strains after 48 hrs of infection and it correlated well with the extent of apoptosis.

To understand the apoptotic pathway more precisely, the expression of *bcl-2*, *bax* and *caspase-3* genes in THP-1 cells infected by different strains were compared. For EAI infection, the *bcl-2* gene was down-regulated and *bax, caspase-3* were up-regulated, compared to other strains supporting the higher apoptosis observed for this strain. On the other hand, high amount of anti-apoptotic bcl-2 mRNA and low bax and caspase-3 mRNA expression were observed in case of Beijing strain supporting its lower apoptosis induction property.

As reported earlier [17], we observed a higher pro-inflammatory cytokine response for the non-Beijing strains and significantly lower induction of the same for Beijing strain. The ancient strain, EAI-5 consistently elicited a stronger pro-inflammatory response and higher apoptosis in the present study , like other ancient strains reported earlier [18]. On the other hand, LAM-6 behaved like typical laboratory virulent strain H37Rv. Both EAI and Beijing strains showed patterns of response typical for their respective genotype with no deviations due to drug resistance.

Assessment of immune responses after *ex vivo* infection of MDM, MDDC and whole blood from different healthy individuals with clinical isolates of *MTB*

The host defence in tuberculosis involves both mononuclear phagocytes and lymphocytes which are able to produce both pro-inflammatory and anti-inflammatory cytokines. The delicate balance between pro- and anti-inflammatory cytokines is considered to influence the outcome of disease [19]. The earlier part of the present study dealt with infection in THP-1 cells as the host, providing identical genetic background. To confirm our hypothesis that quality of immune responses towards MTB strains is property of particular lineage and they do not change with host belonging to different genetic background, blood from different healthy individuals was taken for infection study. The monocyte derived dendritic cells (MDDC) and monocyte derived macrophage (MDM) from different healthy individuals were infected with EAI-5 and Beijing strain that had shown opposite responses in earlier studies, along with BCG and H37Rv. The mRNA expression for TNF-α, IL-1β and IL-12 (proinflammatory cytokines), IL-10 (anti-inflammatory cytokines), IL-8, MCP-1 (chemokines) and TLR-2 was analyzed using SYBR Green Real time PCR, after 24 hours of infection.

The pro-inflammatory cytokine responses followed similar patterns as observed in previous experiments with THP-1. There were clear differences in the level of pro-inflammatory cytokines produced by a single host in response to different strains. Although the fold increase in the level of gene expression varied between individual hosts, EAI-5 consistently showed significantly higher levels of cytokines compared to other strains whereas

for Beijing it consistently remained the lowest in all individuals. The relative hierarchy of low and high responses was similar in the donors and a similar hierarchy was observed when the same strains were used to stimulate both, MDMs and MDDCs. It was observed that levels of mRNA for pro-inflammatory cytokines, TNF-α, IL-1 β and IL-12, were marginally higher in MTB-infected MDM as compared to one expressed in MDDC, except for IL-12. Further, IL-10 gene expression after infection was observed only in macrophages and was not at detectable level in MDDCs. Thus, though there was difference in the response of macrophages and DCs to MTB infection, the patterns with particular strain remain similar.

To evaluate how macrophages respond to infection with different MTB strains in the presence of lymphocytes, whole blood was taken for *ex vivo* infection. The whole blood cytokine assay is advantageous as it reflects *in vivo* conditions [20]. However, its drawback is that even with optimal standardization many cellular and humoral variables cannot be controlled. Further, plasma components, lipids or soluble cytokine receptors may influence results by enhancing or inhibiting cell stimulation and even inactivating or capturing secreted cytokines. In whole blood assay, lipopolysaccharide (LPS) and phytohaemagglutinin (PHA) were included as control stimuli for monocytes and T lymphocytes respectively. Additionally whole cell sonicates were also included to assess the effect of differential cell composition in immune response. The cytokines were monitored after 24 hours of infection in whole blood by ELISA. There was clear difference in the level of pro-inflammatory cytokines produced by a single host in response to infection with different strains and the relative hierarchy of low and high responses was maintained also in whole blood assay. The particular patterns of cytokine expression so far observed for specific strains were not observed with whole cell sonicate from those strains indicating that the cell wall composition might not be the only factor responsible for differential response and the interactions with live cells were necessary for the modulation of host immune response.

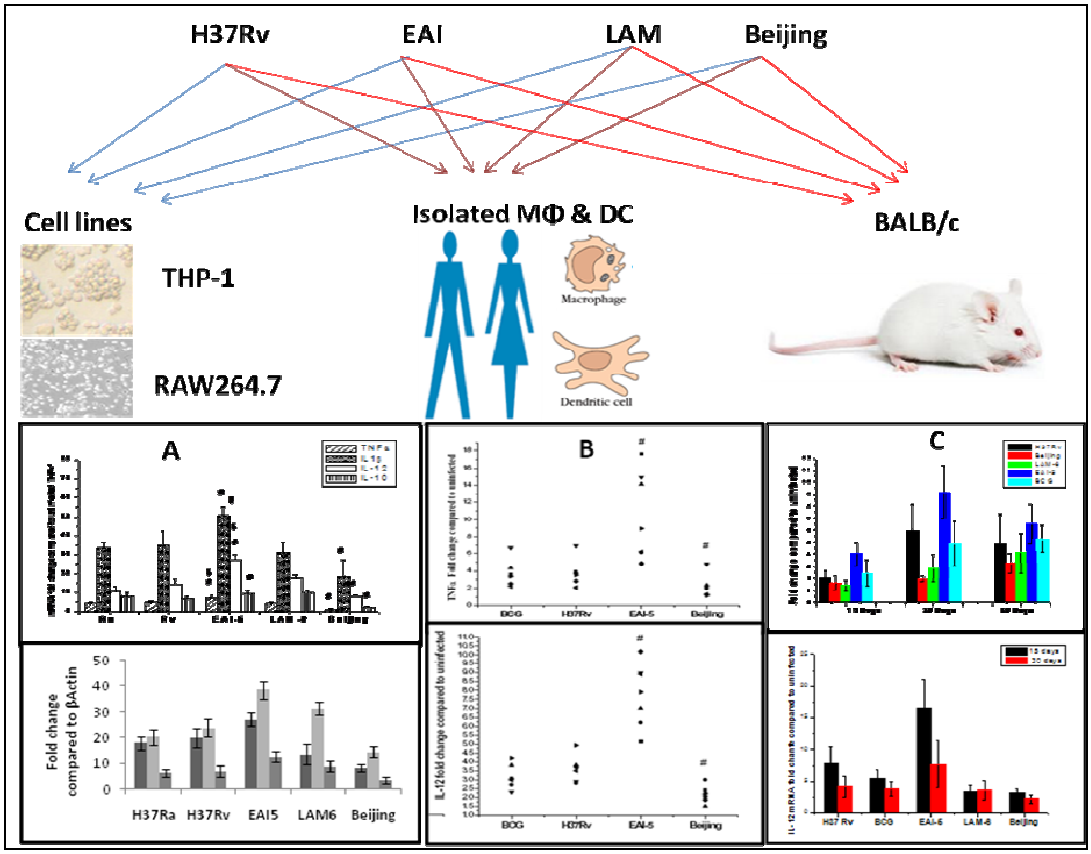


Figure : Cytokine induction in infected cells by different strains of MTB.

(A) Real time PCR was carried out to estimate the mRNA expression for TNF-α, IL-12 and IL-10, 24 hrs after infection of THP-1 cells and RAW264.7 respectively with *M. tuberculosis* H37Ra, H37Rv and three clinical isolates at MOI 10. **(B)** Real time PCR was carried out to estimate the mRNA expression for TNF-α and IL-12 after 24 hrs of infection in monocyte derived macrophage (MDM) with different strains. MDM from seven independent donors were averaged for scatter plot representation and clustered according to lineage. **(C)** Real time PCR was carried out to estimate the mRNA expression for TNF-α and IL-12 after 15 days of infection respectively in infected BALB/c mice. All these graphs showed relative mRNA expression corrected for total mRNA using the housekeeping β-actin gene. '#' sign indicates significant difference compared to H37Rv where p is at least ≤0.05.

Infection of mice using aerosols of different strains of *Mycobacterium tuberculosis*

For the study of growth kinetics of different MTB strains *in vivo*, BALB/c mice were infected by low-dose aerosol with EAI-5, LAM-6, Beijing, H37Rv and BCG. The precise number of bacilli delivered to the lungs was evaluated on the following day of aerosol exposure. The average infecting inoculums for each of the lineages across the experiments was kept constant. CFU in lung, spleen and liver, cytokine concentration, and lung histology were compared between strains on day 15, 30 and 60 post infections.

The growth of the MTB strains in lungs of infected mice was very similar during the first 15 days post infection. But Beijing, LAM and H37Rv strains showed higher bacterial growth in the lung of infected mice from day 30 onwards. Cytokine mRNA expression in the lungs of infected mice at 15 and 30 days post-infection was determined by using a quantitative PCR. Mice infected with EAI-5 showed increased levels of IL-12 and IFN-γ mRNA at 30 day post-infection. Whereas, on day 30, the expression levels of the mRNAs for Th1-type cytokines IFN-γ, TNF-α, and IL-12 were all lower in the lungs of Beijing-infected mice. It can be observed that due to the failure to elicit Th1 type response that is crucial for the control of *Mycobacteria*l infection, Beijing strain can grow rapidly in the lungs of infected mice and hence produced higher bacterial load.

Conclusion:

Three different clinical isolates of MTB from different genotypes were selected for the study and were used for *in vitro* as well as *in vivo* infection experiments. The *in vitro* study carried out in THP-1 cell showed that ancient EAI strain induced significantly higher proinflammatory response in the host the compared to modern clinical isolates. On the other hand, modern Beijing strain from lineage 2 consistently induced low levels of proinflammatory cytokines and IL-10 according to its lineage characteristic. Further, LAM-6 and H37Rv both belonging to lineage 4, showed similar proinflammatory response. Overall we noticed a lineage specific response and interestingly these patterns were not influenced by the drug resistant status of the strains.

Moreover, we observed a strain specific apoptotic response, with EAI-5 inducing significantly higher rate of apoptosis compared to Beijing strain and H37Rv ($P<0.05$). We observed a good correlation between all proinflammatory cytokine responses and apoptosis induced only by virulent strains after five and six days of infection. It was observed that for virulent strains, including H37Rv and clinical isolates, MTB accumulates less in number (less than 5) compared to avirulent strain H37Ra inside host and it has a relation with the induction of apoptosis of the host. In the present study, a positive correlation was observed between phagocytic index for '>10 bacilli/cell' and the percent apoptosis after five and six days of infection. It was evident that pro-inflammatory cytokines and initial higher accumulation of bacilli inside the host cell were the two determining factors for host cell apoptosis.

The induction of cytokines in human peripheral blood monocyte derived macrophages (MDM), monocyte derived dendritic cells (MDDC) and whole blood (WB) from six different

individuals, when infected by clinical isolates and laboratory strains also showed similar lineage specific patterns of immune response as observed earlier with THP-1 cells. It clearly established that the immune response was independent of the host and probably a property of the lineage of MTB. Further, the differential response pattern was not observed when bacterial sonicates were used instead of live *Mycobacteria*, demonstrating that pathogen's cell wall components might not be the only factor contributing to the induction of differential immune response, but it surely, was an outcome of complex interactions of host with live pathogen.

In vivo studies revealed that the strain exhibiting higher proinflammatory responses *in vitro* (EAI) induced higher Th1 type of response *in vivo* which is protective in nature. It is also reflected in the lower bacillary load in the lungs of mice infected with the same strain. Similarly, the Beijing strain which showed suppression of immune responses showed higher lung bacillary load in infected mice.

Thus, the study confirmed the differential immune subversion by different genotypes of MTB both *in vitro* as well as *in vivo* conditions. Further, it sheds light on an important fact that MTB strains exploit discrete pattern of interaction with host according to their genotype.

Significance of the work:

In the present study, *in vivo* and *in vitro* models of infection were used to investigate the impact of genomic diversity of the MTB strains on the host. So far, this kind of study was conducted with laboratory strains and with very few clinical isolates from TB outbreaks. The present study was conducted with clinical strains isolated from TB patients from Mumbai, India and hence is first of its kind. Additionally, this is also the first report where all the important parameters of immune interactions like intracellular growth, phagocytic index, induction of cytokine and apoptosis were monitored in host infected with highly drug resistant, well characterized MTB lineages in a single study. We observed lineage-specific patterns of innate immune responses of the strains in *in vivo* and *in vitro* conditions which may have consequences in immunopathology of TB patients infected with different lineages of MTB. It is thus logical to hypothesize that infection with genotypically distinct MTB strains is associated with distinct immunomodulatory effects which may lead to potentially different patterns of disease progression. Further investigations to understand the mechanisms underlying strain-specific responses will be of importance in understanding of TB pathogenesis which may lead to a new treatment regimen.

References:

1. Dye, C., et al., *Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project.* JAMA, 1999. **282**(7): p. 677-86.
2. Russell, D.G., *Who puts the tubercle in tuberculosis?* Nat Rev Microbiol, 2007. **5**(1): p 39-47.
3. van Crevel, R., T.H.M. Ottenhoff, and J.W.M. van der Meer, *Innate immunity to Mycobacterium tuberculosis.* Clinical Microbiology Reviews, 2002. **15**(2): p. 294-309.
4. Cooper, A.M. and S.A. Khader, *The role of cytokines in the initiation, expansion, and control of cellular immunity to tuberculosis.* Immunol Rev, 2008. **226**: p. 191-204.
5. Subbaiah, T.V., D.A. Mitchison, and J.B. Selkon, *The susceptibility to hydrogen peroxide of Indian and British isoniazid-sensitive and isoniazid-resistant tubercle bacilli.* Tubercle, 1960. **41**(5): p. 323-333.
6. Victor, T.C., et al., *Molecular characteristics and global spread of Mycobacterium tuberculosis with a western cape F11 genotype.* Journal of clinical microbiology, 2004. **42**(2): p. 769-772.
7. Lan, N.T.N., et al., *Mycobacterium tuberculosis Beijing genotype and risk for treatment failure and relapse, Vietnam.* Emerging Infectious Diseases, 2003. **9**(12): p. 1633.
8. Bifani, P.J., et al., *Global dissemination of the Mycobacterium tuberculosis W-Beijing family strains.* Trends Microbiol, 2002. **10**(1): p. 45-52.
9. Dormans, J., et al., *Correlation of virulence, lung pathology, bacterial load and delayed type hypersensitivity responses after infection with different Mycobacterium tuberculosis genotypes in a BALB/c mouse model.* Clin Exp Immunol, 2004. **137**(3): p. 460-8.
10. Brosch, R., et al., *A new evolutionary scenario for the Mycobacterium tuberculosis complex.* Proceedings of the national academy of Sciences, 2002. **99**(6): p. 3684.
11. Supply, P., et al., *Automated high-throughput genotyping for study of global epidemiology of Mycobacterium tuberculosis based on Mycobacterial interspersed repetitive units.* J Clin Microbiol, 2001. **39**(10): p. 3563-71.
12. Ganatra, R.D., et al., *Modifications in biphasic liquid-scintillation vial system for radiometry.* The Journal of Nuclear Medicine, 1980. **21**(5): p. 480-483.

13. Lopez, B., et al., *A marked difference in pathogenesis and immune response induced by different Mycobacterium tuberculosis genotypes.* Clin Exp Immunol, 2003. **133**(1): p. 30-7.
14. Brudey, K., et al., *Mycobacterium tuberculosis complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology.* Bmc Microbiology, 2006. **6**(1): p. 23.
15. Theus, S.A., M.D. Cave, and K.D. Eisenach, *Intracellular macrophage growth rates and cytokine profiles of Mycobacterium tuberculosis strains with different transmission dynamics.* J Infect Dis, 2005. **191**(3): p. 453-60.
16. Chan, J., et al., *Lipoarabinomannan, a possible virulence factor involved in persistence of Mycobacterium tuberculosis within macrophages.* Infection and immunity, 1991. **59**(5): p. 1755-1761.
17. Wang, C., et al., *Innate immune response to Mycobacterium tuberculosis Beijing and other genotypes.* PLoS One. **5**(10): p. e13594.
18. Portevin, D., et al., *Human macrophage responses to clinical isolates from the Mycobacterium tuberculosis complex discriminate between ancient and modern lineages.* PLoS pathogens. **7**(3): p. e1001307.
19. Rook, G.A.W. and R. Hernandez-Pando, *The pathogenesis of tuberculosis.* Annual Reviews in Microbiology, 1996. **50**(1): p. 259-284.
20. De Groote, D., et al., *Direct stimulation of cytokines (IL-1 [beta], TNF-[alpha], IL-6, IL-2, IFN-[gamma] and GM-CSF) in whole blood. I. Comparison with isolated PBMC stimulation.* Cytokine, 1992. **4**(3): p. 239-248.

List of Figures:

List of Tables:

Abbreviations:

%	-	Percent
^{0}C	-	Degree Celsius
µg	-	Microgram
µl	-	Microliter
µM	-	Micromolar
β-ME	-	Beta-mercaptpethanol
AFB	-	Acid fast bacilli
Ag	-	antigen
AIDS	-	acquired immunodeficiency syndrome
APS	-	ammonium persulphate
BCG	-	Bacillus Calmette Guerin
bp	-	basepairs
BSA	-	Bovine serum albumin
CFP-10	-	Culture filtrate protein-10
CFU	-	Colony forming unit
CSF	-	Cerebrospinal fluid
CTAB	-	N-acetyl-N,N,N-trimethyl ammonium bromide
DC	-	Dendritic cells
DMSO	-	Dimethyl sulphoxide
dNTP	-	deoxyribonucleoside triphosphate
DOC	-	diotyl sulfosuccinate
DR	-	Direct repeats
DTH	-	Delayed type of hypersensitivity
DTT	-	dithiothreitol
ECL	-	Enhanced chemiluminiscence
EDTA	-	ethylenediaminetetraacetate acid
EMB	-	ethambutol
ELISA	-	Enzyme linked immunosorbent assay
EPTB	-	Extrapulmonary tuberculosis
ESAT-6	-	Early secreting antigenic target-6
EtBr	-	ethidium bromide
FACS	-	Fluorescence- activated cell sorter
FBS	-	Foetal bovine serum
G+C content	-	Guanine + Cytosine content
G	-	Gram
HIV	-	Human immunodeficiency virus
HRP	-	Horse raddish peroxidase
HSP	-	Heat shock protein
IFN-γ	-	Gamma interferon
INH	-	Isoniazid
kb	-	Kilobases
kDa	-	Kilodalton
LB	-	Luria Bertani
LJ	-	Löwenstein–Jensen
MAC	-	*M.avium* complex
MDM	-	Monocyte derived macrophage
MDDC	-	Monocyte derived dendritic cells
MDR	-	Multi-drug resistant

MHC	-	Major histocompatibility complex
MIRU	-	Microbacterial interspersed repetitive units
MOTT	-	*Mycobacteria* other than tubercle bacilli
MTB	-	*Mycobacterium tuberculosis*
ng	-	Nanogram
ROS	-	Reactive oxygen species
RNI	-	Reactive nitrogen intermediates
ROI	-	Reactive oxygen intermediates
OD	-	Optical density
ORF	-	Open reading frame
PAGE	-	Polyacrylamide gel electrophoresis
PAS	-	Para-aminosalicylic acid
PBMC	-	Peripheral blood mononuclear cells
PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction
pg	-	Picogram
PGRS	-	Polymorphic GC-rich repetitive sequences
PMSF	-	Phenylmethyl sulfuryl fluoride
PPD	-	Purified protein derivative
RFLP	-	Restriction fragment length polymorphism
RIF	-	Rifampicin
RNase	-	ribonuclease
rRNA	-	ribosomal RNA
RT	-	Room temperature
SDS	-	Sodium dodecyl sulphate
SSC	-	Standard saline citrate
SSPE	-	Standard saline phosphate EDTA
STR	-	streptomycin
TB	-	Tuberculosis
TBS	-	Tris buffered saline
TE	-	Tris EDTA buffer
TEMED	-	N,N,N',N'-tetramethylethylene diamine
Th1	-	T helper 1 cells
Th2	-	T helper 2 cells
TLRs	-	Toll like receptors
Tris	-	tris (hydroxymethyl) aminomethane
TNF-α	-	Tumour necrosis factor alpha
U	-	unit(s)
UV	-	Ultraviolet
VNTR	-	Variable numbers of tandem repeats
v/v	-	Volume/volume
w/v	-	Weight/ volume
WHO	-	World Health Organization
WT	-	Wild type
XDR	-	Extremely drug resistant

Host-pathogen interactions of different strains of *Mycobacterium tuberculosis*

Chapter 1: General Introduction

1.1. Tuberculosis Epidemic

Tuberculosis (TB) has long been a major scourge for humanity, claiming millions of lives worldwide. While TB is preventable and curable, it has remained a significant cause of morbidity and mortality in resource poor nations like India. On the basis of tuberculin reactivity or Mantoux test it has been calculated that one-third of the world's population has been infected with *Mycobacterium tuberculosis* bacillus (MTB), the causative agent of the disease (Dye et al, 1999). Infecting bacilli are either killed or remain 'viable but latent' inside the host macrophage for decades. In the infected individuals who develop active disease, bacilli appear to evade or subvert the host's protective cellular immune responses. However, infection with immunodeficiency virus, treatment with corticosteroids, aging and consumption of alcohol increase the potential for reactivation of latent tuberculosis. Therefore, pathogenesis depends on bacterial factors and host factors which determine the disease outcome.

The term tubercle was coined by Franciseus Sylvius (1650) of Leyden. Jean-Antoine Villemin (1865) first established the infectious nature of tuberculosis, though he was unable to isolate the organism. In 1882 Robert Koch, a country doctor from East Prussia, announced to the Physiological Society of Berlin that he had identified and cultured the tubercle bacillus. He also described a staining technique for this bacillus and proposed a perfect experimental model for the infectious nature of the bacilli. In 1895 Roentgen discovered X-rays and exposure to X-rays was used in 1904 for the detection of this disease. Ever since, radiology and bacteriology have remained the main pillars of diagnosis of tuberculosis.

Tuberculosis is also an ancient disease. Bones of old pre-historic men dating back to 8000 BC found in Germany have shown a rare tuberculosis manifestation. Spines from

skeletons excavated in Egypt (2500 to 1000 BC) have also shown changes suggestive of tuberculosis. MTB was known as King's evil in England during the 11th and 12th centuries. Various other historical terms used for the description of tuberculosis are phthisis and consumption.

Global burden of Tuberculosis

In 2007, an estimated 9.27 million incident cases and approximately 1.75 million deaths due to TB occurred worldwide (WHO, 2009). After human immunodeficiency virus (HIV)/AIDS, TB is the second most common cause of death due to an infectious disease, and current trends suggest that TB will still be among the 10 leading causes of global disease burden in the year 2020 (WHO Fact Sheet, 2000). The global distribution of TB cases is skewed heavily toward low-income and emerging economies. The highest prevalence of cases is in Asia, where China, India, Bangladesh, Indonesia, and Pakistan collectively make up over 50% of the global burden. Africa, and more specifically sub-Saharan Africa, has the highest incidence rate of TB, with approximately 83 and 290 per 100,000, respectively (Figure1). TB cases occur predominantly (approximately 6 million of the 8 million) in the economically most productive 15- to 49-year-old age group (WHO Fact Sheet, 2000). Our understanding of TB epidemiology and the efficacy of control activities have been complicated by the emergence of drug-resistant bacilli and by the synergism of TB with HIV coinfection. In 1993, the World Health Organization (WHO) took an unprecedented step and declared tuberculosis a global emergency. It is estimated that between 2000 and 2020 nearly one billion people will be newly infected, 200 million will get sick and 35 million will die from TB if control is not strengthened further (WHO Fact Sheet 2000).

Tuberculosis in India

One fourth of the world's TB burden is borne by India. More than 2 million people develop active tuberculosis and more than 500,000 people die each year due to tuberculosis

in India. This means more than 1000 die each day or one every minute due to TB. TB causes more deaths per year than malaria, hepatitis, meningitis, nutritional deficiencies, sexually transmitted diseases, leprosy, and tropical diseases (WHO, 1997) put together. Prevalence of the disease is more than twice the incidence, indicating a failure of current treatment programmes. According to WHO estimates, India loses about Rs.1700 crores in economic output every year due to TB.

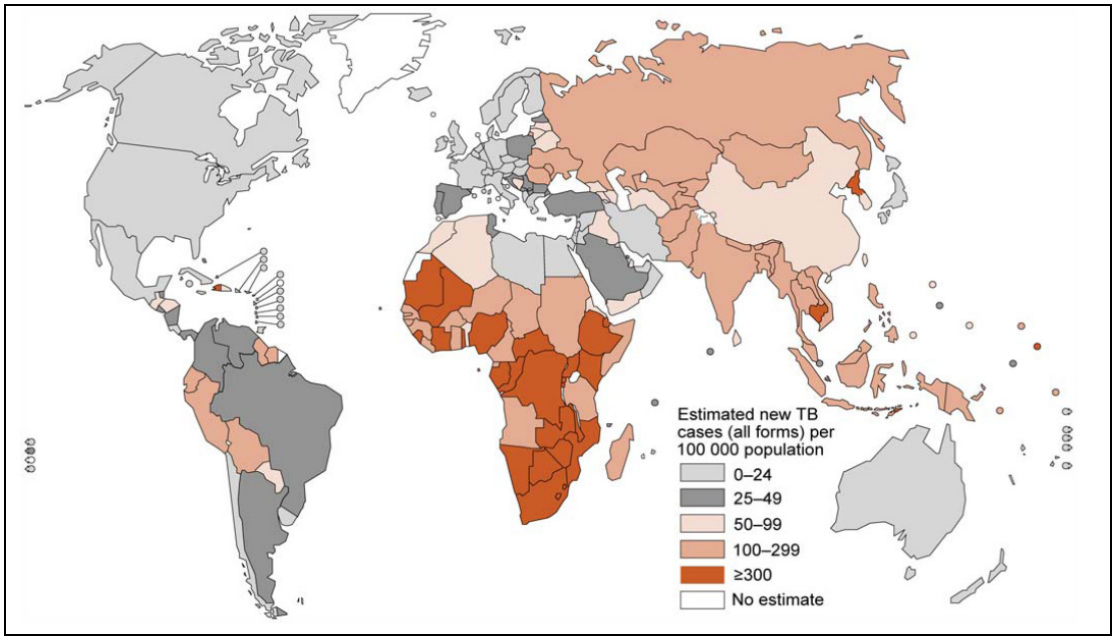


Figure 1: The global incidence of TB: The number of new TB cases per 100,000 population for the year 2007 according to WHO estimates

(Source: http://www.who.int/healthinfo/global_burden_disease/estimates_country/en/index.html)

1.2. The Disease – Tuberculosis:

The principal risk of acquiring TB infection is from breathing small droplet nuclei of the size 10 µm carrying 3 to 10 bacteria exhaled by a sputum positive pulmonary TB patient. The size of the droplet nuclei is crucial for tubercle bacilli to reach beyond the respiratory tract and penetrate into the terminal air passage, multiply and establish an infection, (Nardell, 1993). Droplet nuclei are stable and remain suspended in air for a long time. Good ventilation can remove droplet nuclei and exposure to sunlight kills tubercle bacilli and hence these can

prevent transmission of the disease. Two important factors, which determine an individual's risk of infection are, the concentration of droplet nuclei in contaminated air and the amount of time he/she is exposed to such air (Vijayan, 2002). The risk of infection progressing into disease varies with age, the risk being greatest in children below 3 years followed by elderly people and young adults.

Primary Tuberculosis

The development of pulmonary tuberculosis from its onset to the various clinical manifestations depends largely on the immune reactions of the host to the pathogen. There are two distinct effective immune responses for inhibiting the progression of the disease. First, a T-cell mediated macrophage-activating response, enhances the ability of the macrophage to kill or inhibit *Mycobacteria* (Orme et al ,1993) and the second, the tissue damaging response, is often produced during delayed-type hypersensitivity (DTH) reactions to tuberculin like products of the bacillus. The latter is used during the course of the disease to destroy macrophages within which the bacterium is multiplying (Dannenberg, 1994).

Using animal models various steps in the development of the primary disease have been understood. Among the animal models of the disease, tuberculosis in rabbits closely resembles the human disease. It has four major stages (Lurie M.B., 1928). The first stage that lasts for one week begins following the inhalation of the tubercle bacilli into an alveolus. The inhaled particle (one unit) should be small enough to reach the alveolar spaces and contain no more than three bacilli. Ten to 50 such units are required to establish an infection. It is believed that fewer than 10 live bacilli even can cause an infection (Nicas et al, 2005). The primary site of infection in the lungs, known as the "Ghon focus", is generally located in either the upper part of the lower lobe, or the lower part of the upper lobe (Kumar et al, 2007). The infected bacilli may persist or may be destroyed by the alveolar macrophages. The

outcome depends on the inherent microbicidal activity of the alveolar macrophages and virulence of the ingested bacillus.

Stage-2, a symbiotic stage (7 to 21 days) is one in which logarithmic bacillary growth and the early tuberculous lesion formation takes place. In favorable conditions, the bacilli grow logarithmically and simultaneously in infected macrophages. Monocytes derived from circulation are attracted to the infection site by various cytokines, initiating granuloma formation. The granuloma prevents dissemination of the *Mycobacteria* and provides a local environment for interaction of cells of the immune system. The initial granuloma formation is called primary tuberculosis.

Stage-3 starts after 3 weeks, lasts up to 8 weeks and is controlled by T cell immunity and DTH. This stage begins when logarithmic bacillary growth stops and the caseous necrosis develops at the centre of the granuloma due to DTH reaction produced by cytotoxic T cells. The killing of macrophages in the interior of the granuloma results in relatively large areas of necrosis, each surrounded by a layer of epitheloid leukocytes and multinucleated giant cells. These tubercules are surrounded by a cellular zone of fibroblasts, lymphocytes and blood derived monocytes. At the same time immunocompetent individuals develop a strong T cell immunity that activates macrophages and render them capable of destroying the bacilli. The caseous foci may calcify or ossify. The extent of macrophage activation determines the subsequent course of disease i.e. the strength of the host's T cell response determines whether an infection is arrested here or progresses to the next stage. With good T cell response, the infection may be arrested permanently at this point. The granulomas subsequently heal, leaving a small fibrous and calcified lesion. If T cell response is insufficient, dissemination of the organism occurs via intrapulmonary lymphatic route with extensive involvement of the hilar lymphnodes. Spillover from lymphatics to the bloodstream

enables the bacilli to reach almost all the organs of the body especially liver, spleen and kidney.

In Stage-4 progression of the disease occurs even in an immunocompetent host and this is caused by liquefaction and cavity formation. The factors that cause liquefaction are high levels of tuberculin reactivity and elevated hydrolytic enzymes like proteases, nucleases and lipases (Converse et al, 1996; Fink & Cookson, 2005). The liquefied material is an excellent growth medium for tubercle bacilli and the bacillus multiplies extracellularly, often reaching high numbers ($>10^8$). As the host is highly sensitive to large antigenic load of the bacillus which is quite toxic to the tissues and leading to necrosis, rupture of the walls of nearby bronchi forming a cavity. The walls of most cavities consist of an external zone of collagen, the cavity's capsule and a caseous liquefied internal zone where the oxygen content is high and nurtures the growth of the bacilli. By coughing, the patient aerosolizes this infectious material disseminating bacilli to other parts of the lung and to the outside world.

Post-primary tuberculosis (reactivation)

Even in a person who successfully fights his battle against TB but has bacilli in a dormant state inside granuloma, reactivation can take place that can lead to post-primary tuberculosis. The lesions in the granulomas are with necrosis and frequently occur at the apices of the upper lobes of lungs. These lesions (Assman's foci) undergo enlargement and liquefaction of caseous centre resulting in cavitation.

Clinical tuberculosis

The clinical expression of infection with *M.tuberculosis* largely depends on the site of involvement and is the most important factor influencing the clinical features of tuberculosis. In an immunocompetent host approximately 85% of the reported cases of tuberculosis are pulmonary and remaining 15% include extrapulmonary or both pulmonary

and extrapulmonary cases (Gangadharam et al, 1988). But in HIV infected patients, it was reported that 38% had only pulmonary TB, 30% had extrapulmonary TB and 32% had both pulmonary and extrapulmonary TB (De Viedma et al, 2002).

Pulmonary tuberculosis

The lungs are the most favored site of infection of MTB and the pathogen comes by respiratory route as described earlier. Lung infection may also occur via blood stream. This is known as a 'Simon focus' and is typically found in the top of the lung (Khan & Rahman, 2000). This hematogenous transmission can also spread infection to more distant sites, such as peripheral lymph nodes, the kidneys, the brain, and the bones (Herrmann & Lagrange, 2005). Cough is the most common symptom of pulmonary tuberculosis and hemoptysis (coughing blood) may result from rupture of a dilated vessel in the wall of an old cavity. In primary pulmonary TB occurring as a result of infection showing zonal infiltration of lymphocytes whereas progressive TB shows cavitation.

Extrapulmonary tuberculosis

Extrapulmonary tuberculosis (EPTB) includes a very wide range of conditions of diverse pathology and prognosis. The non-specific symptoms and results of investigations make diagnosis of EPTB difficult, delayed or missed, which may result in death or serious disability. Airway is almost invariably the portal of entry. If TB bacteria gain entry to the bloodstream from an area of damaged tissue, they can spread throughout the body and set up many foci of infection, all appearing as tiny, white tubercles in the tissues (Crowley & Crowley, 2010). This severe form of TB disease, most common in young children and those with HIV, is called miliary tuberculosis (TB/HIV Clinical manual, 2004). People with this disseminated TB have a high fatality rate even with treatment (about 30%) (Jacob et al, 2009). Metastatic foci thus established, may manifest into EPTB. Early forms of EPTB are cerebral and skeletal disease (via bloodstream) or lymph node, vertebral and pericardial

disease (via lymphatic). All parts of the body can be affected by the disease, though for unknown reasons it rarely affects the heart, skeletal muscles, pancreas and thyroid (Agarwal et al, 2005).

TB Diagnosis

The most powerful tool in any TB control program is prompt diagnosis and successful treatment of patients. There are several popular techniques used for this purpose.

Sputum smear microscopy. The use of stained-sputum microscopy for acid-fast bacilli still remains the most available, easy, inexpensive, and rapid diagnostic test for TB (Kent et al, 1985) specially in resource poor country like India (Steingart et al, 2006). But, the test is not totally specific and sensitive (Tuberculosis Prevention Trial, 1980). Further, diagnosis of TB by microscopy is difficult especially in children who rarely produce adequate sputum. Currently, the sensitivity of this test has improved considerably by the use of auramine-rhodamine/ fluorochrome method instead of the classic Ziehl-Neelsen stain which uses carbol-fuchsin (Wright & Wallace, 1998).

Cultivation of Bacteria. *Mycobacteria*l culture is the ultimate proof of *Mycobacteria*l infection and is often used as a reference method due to its high sensitivity and specificity (Schirm et al, 1995; Walker, 2001). However, it takes 4-6 weeks for MTB to grow on solid culture medium (e.g. agar based Middlebrook 7H10 or 7H11 or the egg-based Lowenstein-Jensson medium), and 3 weeks to grow in liquid 7H9 medium (Morgan et al, 1983). To increase the sensitivity and reduce the detection time some modifications are incorporated and new techniques have been developed.

BACTEC 460. The BACTEC 460 (Becton Dickinson, Sparks, Maryland) relies on radiometric detection of $^{14}CO_2$ as an indicator of bacterial growth. The Bactec vials contain Middlebrook 7H12 medium and fatty acid substrates labelled with 14C. Growing *Mycobacteria* release $^{14}CO_2$ as a metabolic end product. The gas is removed, analyzed and

the amount of radioactive ^{14}C released is expressed as a numerical value called the Growth Index (GI).

***Mycobacteria*l Growth Indicator Tube.** The *Mycobacteria*l Growth Indicator Tube (MGIT; Becton Dickinson, Sparks, Maryland, USA) introduced 15 years ago, is a rapid (4-13 days), high throughput method. It is based on fluorescence detection of *Mycobacteria*l growth in a tube containing a modified Middlebrook 7H9 medium together with fluorescence quenching-based oxygen sensor (a ruthenium pentahydrate substance embedded in silicone rubber) at the bottom of the tube (Reisner et al, 1995; Rusch-gerdes et al, 1999). As the bacteria grow and consume oxygen, the indicator fluoresces under ultraviolet light.

Biomarkers

TB diagnostic tests that rely on detection of host immunological markers currently in use include the tuberculin skin test (TST) (Huebner et al, 1993) and interferon gamma release assays (IGRAs) (Andersen et al, 2000; Pai et al, 2008).

Tuberculin skin test. The TST or Mantoux test or purified protein derivative (PPD) test has been used for almost a century as the standard test for the diagnosis TB infection and disease (Mendel, 1908). The TST test is based upon the type IV hypersensitivity reaction, in which a standard dose of 5 tuberculin units is injected intradermally into the forearm and read 48 to 72 hours later which is modified by Linnikova in 1939. The TST is based on the principle that T cells of individuals sensitized with *Mycobacteria*l antigens, produce IFN-γ and a variety of other cytokines that recruit and activate macrophages and other nonspecific inflammatory cells producing induration after an average 48 hours. The reaction is read by measuring the diameter of induration across the forearm, perpendicular to the long axis in millimetres. No induration is recorded as "0 mm", whereas reactions over 10 mm in size are considered positive in non-immunocompromised persons. The main drawback with the

clinical use of the TST is the lack of specificity due to cross-reactivity with proteins present in other *Mycobacteria*, such as BCG or mycobacterium other than tuberculosis (MOTT) (Farhat et al, 2006). Moreover, several factors such as age, poor nutrition, acute illness or immunosuppression induced by medication or HIV infection may contribute to false-negative results

Interferon gamma release assays. As a replacement for the Mantoux test, several other tests are being developed. IGRAs are based on the same principle as the TST, that T cells of individuals sensitized with *M.tuberculosis* produce IFN-γ when they re-encounter *Mycobacteria*l antigens. IGRAs quantify the amounts of antigen-specific IFN-γ in blood culture supernatants (Quanti FERONTB Gold, Australia) or determine the frequency of IFN-γ producing blood leukocytes (SPOT-TB assay, Oxford Immunotec, Oxford, UK) in response to specific MTB peptides such as early secretory antigenic target 6 (ESAT-6) and culture filtrate protein (CFP) 10 (Ravn et al, 2005). The only disadvantage of this assay is that immunocompromised patients show false negative due to lack of T-cells.

Molecular methods

Polymerase chain reaction has contributed to a more rapid and reliable diagnosis of pulmonary TB. This technique allows the amplification of specific target sequences of nucleic acids such as *hsp65*, *16SrDNA*, *38KDa, IS6110* etc. that can be detected through the use of nucleic acid probes; both RNA and DNA amplification systems are commercially available (Daley et al, 2007, Ling et al, 2008, Kulkarni et al, 2012).

Tuberculosis control

Vaccination. Albert Calmette and Camille Guerin produced an attenuated (BCG) strain by subculturing a strain from bovine tuberculosis for 13 years, about 230 times. This was used as a vaccine first in 1921.Though BCG is generally considered a vaccine

against Tuberculosis; it has also provided protection against leprosy in four major trials (Setia et al, 2006; Merle et al, 2010).

According to reports, BCG is ineffective in preventing infectious form of TB in adults but it gives protection against lethal form of tuberculosis like meningitis and disseminated disease (Rodrigues et al, 1993) especially in children. However, high coverage (>80%) of BCG vaccination does not contribute significantly to reduction in transmission. In view of the failure of the present BCG vaccine, a number of laboratories have attempted to develop better vaccines for TB.

Broadly, two approaches have been used to improve the TB vaccine. The first involves **subunit vaccines** and the other one is **live vaccines**. Recent results from several researchers have indicated that non-viable subunit vaccines based on soluble proteins such as ESAT-6, 85 KDa etc. with adjuvant can induce demonstrable level of specific immunity to challenge with *M.tuberculosis* (Brandt et al, 2000; McShane et al, 2005). DNA vaccination with naked DNA constructs for antigen 85 and *hsp60*, have been engineered. Both protein and DNA vaccine induce partial protection against experimental TB infection in mice but their efficacy has generally not been better than BCG (Kamath et al, 1999).

For the development of live TB vaccine, many of the groups preferred using BCG as their vector. BCG is good adjuvant and gives long lasting immunity without any side effects. So, modifying BCG vaccine is considered as a better approach in recombinant vaccine development. Several attempts have been made to improve immunogenicity of BCG either by enhancing its CD8+ T cell stimulating capacity or by endowing it with Th1 cell-inducing cytokines (Freidag et al, 2000). Among them, MVA85A (modified vaccinia Ankara 85A), has shown most promising result in Phase-I clinical trial, produces higher levels of long-lasting cellular immunity when used together with BCG (McShane et al, 2004). But, very recently the vaccine failed in a Phase-II clinical trial in South Africa that involved 2,794

healthy children (aged four to six months) (Tameris et al, 2013). All the other candidate vaccines are being evaluated only in animal experiments so far and hence it may take a long time to get a better vaccine in the market.

Chemotherapy. *M.tuberculosis* is a slow growing organism which grows intermittently or remains dormant for a prolonged period. The main goals of anti-TB chemotherapy are (1) to convert the sputum AFB positive individuals to negatives in the shortest time thereby reducing the transmission (2) to prevent the emergence of drug resistance and (3) to assure a complete cure without relapse. The World Health Organization and the International Union against Tuberculosis and Lung disease (IUATLD) recommended standardised TB treatment regimens, called directly observed treatment short course (DOTS) program in 1994 (Raviglione and Pio, 2002). A combination of four drugs referred to as first line drugs (Isoniazid, rifampicin, pyrazinamide and ethambutol) is used together in initial treatment for 6 months under close supervision. The combination of four or more drugs is required to kill several subpopulations of *M.tuberculosis* each of which has a distinctive metabolic status and therefore, varying susceptibility to anti-TB drugs. The subpopulation growing rapidly at the wall of the cavity due to favourable growth conditions of high oxygen content in a neutral pH which is particularly vulnerable to isoniazid. The second slow growing population in intracellular acidic environment is effectively killed by pyrazinamide. The third subpopulation present in caseous material when pH is neural but the oxygenation is poor is killed efficiently by rifampicin (Davidson, 1992). There are several different possible regimens recommended which vary depending on patient category. This short course regimen can cure 95-99% of susceptible TB cases among patients not infected with HIV as compared to 60-65% of cases with HIV.

Obstacles in TB control

Multi-drug resistant (MDR) tuberculosis:

Drug resistant tuberculosis is a form of TB in which MTB is resistant to one or more anti-tuberculosis drugs. The initial resistance is a mixture of primary resistance and unknown acquired resistance (7.5% in all new TB cases). Multi-drug resistance in MTB is defined as resistance to at least rifampicin and isoniazid with or without resistance to other anti-TB drugs. MDR tuberculosis usually occurs in chronic cases where the rate of acquired resistance is around 20%, in which resistance to both rifampicin and isoniazid occurs in 4-10% of cases. The prevalence rates of MDR, particularly in developing countries, are as high as 48%. Treatment of patients with MDR tuberculosis involves treatment with second line, reserve drugs like kanamycin, cycloserine, capreomycin, norfloxacin, which are toxic as well as very expensive. In India 1-3.3% of new TB patients have MDR TB and this will account for an estimated 20,000 new infectious cases of MDR-TB every year.

Factors that contribute to increased incidence of MDR-TB include the AIDS pandemic, populations with easy access to antituberculosis medications, deterioration of public health infrastructure, inadequate training of health care providers and above all non-compliance with medication. It has been reported that there is a close correlation between intravenous drug abuse and active TB, and between HIV infection and MDR-TB. A person suffering from HIV has decreased immunity which causes lesser effective anti-TB treatment and hence leading to a high mortality rate.

TB/HIV co-infection

Tuberculosis is one of the most common opportunistic infections in HIV patients. Globally, 36.1 million people suffering from HIV/AIDS and 70% of them live in sub-Saharan Africa. HIV fuels the TB epidemic as it profoundly reduces T cell immune response and effectively increases the chance of reactivation of a latent TB infection. In HIV infected patients, TB often presents with an atypical picture that confounds diagnosis. Approximately 4 million people are infected with HIV in India of which approximately half are infected with

M.tuberculosis. The increasing prevalence of HIV leading to AIDS epidemic in India represents a serious threat to TB control efforts. The proportion of tuberculosis patients among HIV seropositive individuals was shown to be 14.6%. One of the most worrisome aspects of the HIV/TB coinfection is the rapid spread of MDR-TB among HIV infected persons. Nosocomial transmission of TB is of grave concern especially where MDR-TB and HIV infection are prevalent (Pearson et al, 1992).

1.3. *Mycobacterium tuberculosis:* The Pathogen

Mycobacterium tuberculosis, an obligate aerobe is 1-4 μm long and 0.3-0.6 μm in diameter , nonmotile, rod-shaped bacterium distantly related to the *Actinomycetes*. Many non pathogenic *Mycobacteria* are components of the normal flora of humans, found most often in dry and oily locales. The bacterium is a facultative intracellular parasite, usually of macrophages, and has a slow generation time, 15-20 hours, and a physiological characteristic that may contribute to its virulence. It is not classified as either Gram-positive or Gram-negative because it does not have the chemical characteristics of either, although the bacteria do contain peptidoglycan (murein) in their cell wall. They are closer to Gram-positive organisms based on the phylogenetic analysis of 16S rRNA sequences (Pitulle et al, 1992) and it stains very weakly as positive. *Mycobacterium* species along with members of related genus *Nocardia* are classified as acid-fast bacteria. Cells of acid fast bacteria retain the fuchsin dye after acid extraction while other bacteria are decolorized. The cell wall of *Mycobacteria*, in addition to peptidoglycan is rich in waxes which include a group of compounds known as mycolic acid. The mycolic acid reacts with fuchsin (a basic red dye) and the mycolic acid fuchsin complex acts as a permeability barrier and impedes penetration of mineral acid. Genus *Mycobacterium* is broadly divided into two major categories:

Fast growers- they consist of strains of species which yield colonies on solid medium that are visible to the naked eye within less than seven days.

Slow growers- they require seven or more days to yield visible colonies.

Among the slow growers the *Mycobacteria* are grouped as:

- The ***Mycobacterium tuberculosis*** **complex (MTC)**: This complex comprises *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium canettii, Mycobacterium pinnipedii, Mycobacterium africanum and Mycobacterium microti. M.tuberculosis* complex *Mycobacteria* were termed as 'typical' and all other species of *Mycobacteria* (except *M.leprae*) were placed in the class of 'atypical *Mycobacteria*'. Other terms used for atypical mycobacterium were 'non-tuberculous' *Mycobacteria* (NTM) or *Mycobacteria* other than tubercle bacilli' (MOTT).
- The ***Mycobacterium avium*** **complex (MAC)**: The MAC consists of *Mycobacterium avium, Mycobacterium intracellulaire* and *Mycobacterium xenopi*. It is also referred to as *Mycobacterium avium* intracellulaire (MAI) complex.

Phenotypic identification of the MTB

Colony morphology : Colonies of primary MTB cultures invariably have a characteristic patterned texture, due to tight cording of the bacterial cells (Runyon, 1970), and this feature is often used to distinguish MTB from other *Mycobacteria*l species, such as *M. avium.* While virulent *M. avium* is associated with smooth and transparent morphotype, virulent MTB is mostly associated with rough morphotype although some study observed the opposite (Schaefer et al, 1970). Colony morphology conversion of *Mycobacteria* has been shown to be associated with changes in constituents of cell wall, such as glycolipids (Belisle & Brennan, 1989), lipooligosaccharides (LOSs), and mycosides (Daffe et al, 1991). Such changes in colony morphology are not usually observed among clinical isolates MTB within a few passages.

Biochemical tests: Biochemical indicators for the differentiation within the MTC include nitrate reduction on modified Dubos broth, niacin accumulation, growth in the presence of

thiophen-2-carboxylic hydrazide (TCH), catalase activity at room temperature, and growth characteristics on Lebek and on bromocresol purple medium (Kent & Kubica 1985).

Genome:

The biggest achievement in our knowledge about TB during the last decade was the availability of the complete genome sequence of the laboratory reference strain H37Rv (Cole et al, 1998). Its size is 4 million base pairs, with 3959 genes. Forty per cent of these genes have had their function characterised, with possible function postulated for another 44%. Within the genome, there are also 6 pseudogenes excluding insertion sequence elements.

It represents the second largest bacterial genome sequence currently available after that of *Escherichia coli*. The genome is rich in repetitive DNA, particularly insertion sequences, and in new multigene families and duplicated housekeeping genes. The characteristically high guanine plus cytosine content (65.5%) was found to be uniform along most of the genome, confirming the hypothesis that horizontal gene transfer events are rarely present in modern MTB (Sreevatsan et al, 1997). Only a few regions showed exception to this rule. A conspicuous group of genes with a very high G + C content (>80%) appears to be unique in *Mycobacteria* and belong to the family of PE and PPE (full form) proteins. In turn, the few genes with particularly low (50%) G + C content are those coding for transmembrane proteins or polyketide synthases. Fifty genes were found to code for functional RNAs.

Ribosomal RNA (rRNA) gene sequences are highly conserved among prokaryotes and have been used to establish phylogenic relationships among *Mycobacteria*. The information about 16S rRNA sequence clearly separates fast growers from slow growers among *Mycobacteria* and show high levels of similarity among the group (Cox & Katoch 1986). Pathogenic and closely related slow growing *Mycobacteria* have a single rRNA *(rrn*) operon whereas fast-growing species of mycobacterium have two operons. Most of the other bacteria have multiple copies of the operon e.g. *E.coli* has seven (Bercovier et al, 1986). The

rrn operon is situated about 1500 kb from *OriC* in *Mycobacteria* whereas other bacteria have one or more *rrn* operons near to *OriC.* The *rrn* operon is located downstream from a gene thought to code the enzyme UDP-N-acetylglucosamine carboxyvinyl transferase (UNAcGCT), which is important to cell wall synthesis (Gonzalez et al, 1996). The above facts explain the slow growth rate of MTB.

Genes involved in virulence: MTB does not have any classical virulence factors like other pathogenic bacteria. So, bacterial load in infected animals is popularly considered, as associated with virulence with this pathogen. A serine/threonine kinase, Pkn G, is suggested to be essential for the survival of *Mycobacteria* inside macrophages (Walburger, 2004). PknG is secreted within the *Mycobacteria*l phagosome and involved in blocking phagosome–lysosome fusion. However, *M.tuberculosis pknG* knockout mutants are impaired in growth both in the *in vitro* stationary phase and in mice (Sassetti et al, 2003; Cowley et al, 2004). It is also demonstrated that disruption of *M.tuberculosis hma* gene results in defective oxygenated mycolic acid synthesis causing deficiency in growth in mouse. Further, disruption in cell wall related genes often cause lower bacterial growth in infected animals, as cell wall lipid components are also considered as virulent factors for this pathogen. Glyoxylate cycle enzyme isocitrate lyase (ICL) is required for fatty acid catabolism and in the glycoxylate shunt pathway. A persistent infection of MTB requires intact *icl* gene expression, so it is considered as virulent factor of pathogen. Likewise, other virulence factors have been described: catalase-peroxidase, which protects against reactive oxygen species produced by the phagocyte; *mce,* which encodes macrophage-colonizing factor, phospholipases C, lipases, and esterases, which might attack cellular, or vacuolar membrane as well as several proteases. *M.tuberculosis* sigma factors which regulate gene expression in response to environmental stress are also shown to confer virulence (Hahn et al, 2005).

Classification and Phylogeny of *Mycobacterium*

MTB has evolved through single nucleotide substitutions, deletion and duplication events, so the population structure is strongly clonal (Gagneux and Small, 2007). The typical MTC members show more than 99.95% sequence similarity at the nucleotide level, with little or no evidence for the exchange of chromosomal DNA between strains. In spite of the close sequence similarity between members of the MTC complex, it is easy to distinguish the various members using molecular biology techniques.

The phylogeny for MTB complex was established for the first time when the strains were divided into three "major genetic" groups using mutations at katG463 and gyrA95 (Sreevatsan, et al, 1997) loci. Further, the diversity in MTB genome, especially in the human-adapted strains, was demonstrated by evaluating polymorphisms at different regions like, insertion elements, spacer elements in the direct repeat region and *Mycobacteria*l interspersed repetitive unit (Van Embden et al, 1993; Supply et al, 2001). Six phylogenetically distinct SNP cluster groups (SCGs) and five subgroups were identified based on 212 SNP markers (Filliol et al, 2000).

Comas and Gagneux, in 2009, in a robust phylogenetic study based on genomic deletion analysis, using large sequence polymorphisms (LSPs) and their geographical distribution, demonstrated that MTB strains could be grouped in six main lineages and 15 sublineages. It is possible that such clonal lineages may evolve specific virulence characteristics (Nicol & Wilkinson, 2008).

Lineage -1, Indo-oceanic group of bacteria, consists of East-African-Indian (EAI) and MANU1 strains which branched off from a common ancestor at an early stage of evolution are referred to as evolutionarily ''ancient'' lineages. The Indo-Oceanic lineage is almost entirely restricted to pulmonary TB patients originating from either the Indian subcontinent or Southeast Asia. This lineage was shown to induce higher Th1 response compared to Lineage-2 in the PBMC of infected person and their household contacts (Rakotosamimanana et al,

2010). The most widely studied strains of MTB belong to Lineage 2 (East Asia/Beijing). However, the clinical and epidemiological characteristics of Lineage 2 are not consistent. A few studies described an association of Lineage-2 with extra-pulmonary (Kong et al, 2005; Kong et al, 2007) or meningeal TB (Caws et al, 2008) whereas, another study confirmed no such association (Nicol et al, 2005). Further, a few groups also proved the HIV association with this lineage (Caws et al, 2006; Middelkoop et al, 2009); while others discard such hypothesis (De Jong et al, 2009). However, in most of the studies, it was confirmed that these strains induce lower levels of proinflammatory cytokines than H37Rv (Sohn et al, 2009; Tanveer et al, 2009), and also was associated with increased growth in human monocytes (Li et al, 2002). This lineage was also linked with higher levels of necrosis and lower levels of apoptosis, in infected macrophages (Sohn et al, 2009).

Lineage-3, mostly prevalent in India and East-Africa, consists of CAS strains induce less proinflammatory cytokines compared to H37Rv (Tanveer et al, 2009). CAS strains were shown to be associated with extrapulmonary disease (Lari et al, 2009). The Euro-American lineage or Lineage-4 was identified in patients from Europe, America, Caribbean, Middle East, and all subregions of Africa and .collectively accounted for 91% of all TB cases. It was also shown that Lineage-4 was predominantly involved in pulmonary than meningeal TB (Caws et al, 2008). Among the well studied strains, H37Rv/Ra, Erdman and CDC1551 belong to Lineage 4 (Euro-American). These strains grow more rapidly in liquid culture and generally induce high levels of TNF-α and IL-12 in the host (Sarkar et al, 2012).

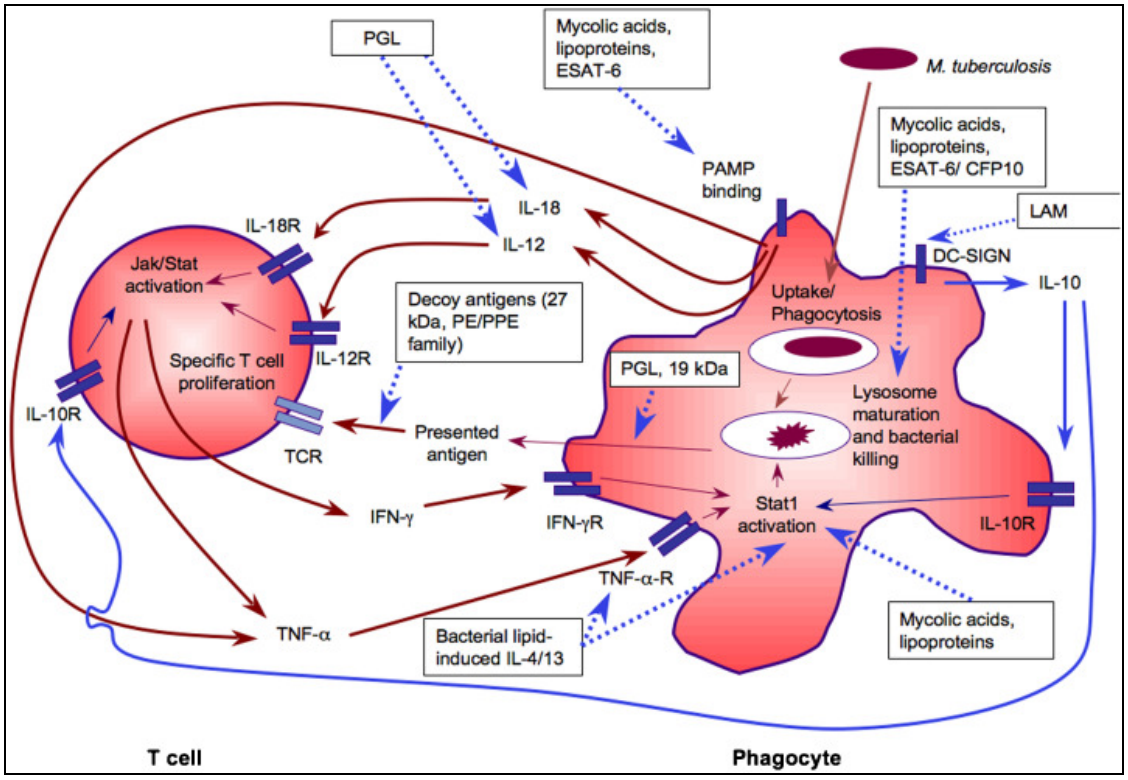


Figure 2: A simplified schematic diagram of host –pathogen interaction in Tuberculosis: The diagram is showing the interaction of the infected antigen-presenting cell and an antigen specific T cell after MTB infection. The key pathways in the host's immune response are shown as solid arrows that can suppress (red) or enhance (blue) bacterial growth, together with the known bacterial products (white boxes, dotted arrows) that can interfere with the host's response.
(Source: http://www.ncbi.nlm.nih.gov/pubmed/19400867)

Lineage-5 and Lineage-6 consist of *Mycobacterium africanum* which is prevalent in western Africa, where it causes up to 50% of smear-positive TB cases (De Jong et al, 2007).These strains are ancient and heterogeneous, lie in between *M. bovis* and MTB. On the basis of geographic origin and biochemical properties, *M. africanum* species has been subdivided into two major subgroups; those from West Africa are subtype I(Lineage-5), closer to *M. bovis*, while those from East Africa are closer to MTB and are subtype II (Lineage-6). Earlier animal studies suggested that *M. africanum* possessed lower virulence (Meyer et al, 2008) and induced mostly higher proinflammatory response in *ex vivo* infection. (Portevin et al, 2011) compared to MTB.

1.4. Host immunity to *M. tuberculosis*

Innate immunity

It is believed that the host innate immunity provides the initial resistance to infections with intracellular pathogens, such as *Mycobacteria*, before the adaptive immunity fully develops. The major cellular components involved in innate immunity include phagocytes; macrophages, neutrophils, dendritic cells (DCs); natural killer (NK) cells; γδ T cells, and soluble mediators released by these cells serve as a links to cell-mediated immunity (Figure-2). During the initial phase of infection, *Mycobacteria* are ingested by resident alveolar macrophages. However, *Mycobacteria* can also be ingested by alveolar epithelial type II pneumocytes (Bermudez & Goodman, 1996), found in greater numbers than macrophages in alveoli. Overall, phagocytic cells play a key role in restricting the multiplication and dissemination of intracellular pathogens, as well as initiation and direction of the adaptive immune response. In addition, DCs, known to be much better antigen presenters than macrophages (Tascon et al, 2000; Marino et al, 2004), play an important role in the early stages of infection through presentation of specific *Mycobacteria*l antigens to T cells (Wolf et al, 2008). A number of receptors are critical for MTB detection and uptake by phagocytes. Entry of *Mycobacteria* into phagocytic cells can occur through binding to multiple receptors. The pathogen recognition and uptake is described below:

Pathogen associated molecular patterns

The interaction between the MTB cell wall components and host cell surface receptors is of major importance in the pathogenesis of MTB infection (Figure-3). MTB contains a wide variety of bioactive lipids that have been implicated in the pathogenesis of the bacillus (Sartain et al, 2011). About 60% of the cell wall of MTB is composed of lipids including mycolic acids, trehalose containing lipids and several lipo-conjugates (Berg et al, 2007). In particular, the surface of MTB is dominated by a group of biosynthetically related

mannosylated lipoglycoconjugates, which mediate host cell recognition and entry through pattern recognition receptors (PRRs) (Torrelles & Schlesinger, 2010). The terminal mannose caps of ManLAMs from different MTB strains vary which determine their avidity for the mannose receptor (Schlesinger et al, 1996). ManLAM caps also bind to dendritic cell (DC)-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) on DCs (Tailleux et al, 2003a). Phosphatidyl-myo-inositol mannosides (PIM) are another major phospholipid component of the *Mycobacteria*l cell wall. They are defined as families by their number of mannose units (1–6) and as species by their number of fatty acids (2–4) (Khoo et al, 1995). Higher-order PIMs (PIM5f and PIM6f) only can be recognized by the mannose receptor. Conversely, lower-order PIMs and lipomanan (LM) are recognized by DC-SIGN and complement receptor 3 (CR3), independent of their degree of acylation. It was observed that virulent strains have significantly more higher-order PIMs and much less lower-order PIMs as compared to avirulent ones (Torrelles et al, 2006).

Internalization of MTB by host cells

Endocytosis of MTB involves different receptors on the phagocytic cell (Figure 3) which either bind to non-opsonized MTB or recognize opsonins on the surface of MTB. It includes complement receptor (CR) 1,3, 4, the mannose receptor, Toll like receptor (TLRs), CD14, Fc-γ receptor, scavenger receptor and surfactant protein (SP) receptors (Ernst, 1998; Stuart et al, 2005; Philips et al, 2005). Many of them function as pattern recognition receptors (PRRs), that recognise conserved features present on microbes (Janeway, 1989) and signal downstream to activate rearrangement to allow uptake via the plasma membrane (Aderem and Underhill, 1999).

Mannose receptors: one of the most important receptor utilized for the entry of mycobacterium is the mannose receptor (Ezekowitz et al, 1990; Schlesinger, 1993). The best characterized receptor for non-opsonin-mediated phagocytosis of MTB is the macrophage

mannose receptor (MR), which recognizes terminal mannose residues on *Mycobacteria* (Schlesinger, 1993; Schlesinger et al, 1996). MR is a C-type lectin involved in the internalization of a variety of cargo (Ezekowitz et al, 1990) and a link between innate and adaptive immunity (Prigozy et al, 1997). The expression of mannose receptor is down regulated in activated macrophages which suggest that mannose receptor mediated uptake predominantly plays a role during the initial stages of *Mycobacteria*l infection.

Complement receptors: There have been accumulating evidences of the role of complement receptors (CR1, CR2, CR3 and CR4) in the phagocytosis for MTB (Schlesinger et al, 1990). Expression of CRs (particularly CR4) and MR increases when monocyte differentiates into macrophages. Among the complement receptors, CR3 is the most important as absence of CR3 reduce phagocytosis of MTB by approximately 70 to 80% by human macrophages and monocytes (Schlesinger et al, 1990; Schlesinger 1993).

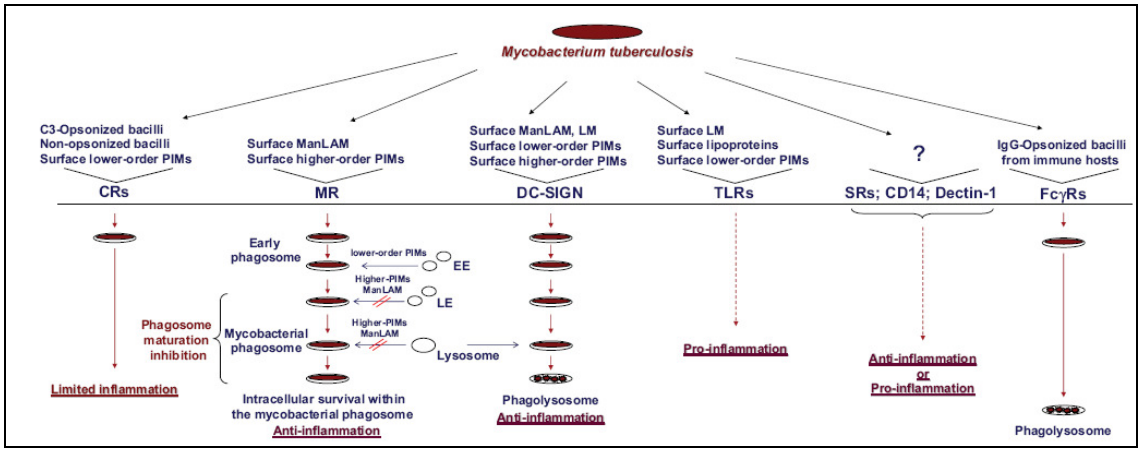


Figure 3: Host cell recognition and response to MTB.
*Mycobacteria*l cell wall components and opsonized bacilli associate with a subset of immune receptors and PRRs to initiate phagocytosis and the development of specific host cell responses. Interaction with specific receptors also leads to the activation of pro- and/or anti-inflammatory pathways in the host cell that influences intracellular survival of the bacterium. EE, early endosome; LE, late endosome.
(Source: http://www.wiley-vch.de/books/sample/3527318879_c01.pdf)

Surfactant proteins: Surfactant protein of the lung, especially SP-A and SP-D regulates the early interaction between *Mycobacteria* and macrophage. These proteins opsonise

mycobacterium and then the bacterial complex are internalized by SP receptors. SP-A thus increases the phagocytosis of *Mycobacteria* through a direct interaction of the protein with macrophage (Gaynor et al, 1995), which upregulates mannose receptor activity (Beharka et al, 2002). Interestingly, it has been reported that human immunodeficiency virus infected individuals have increased levels of Sp-A in the lungs, and this results in a threefold-greater attachment of MTB to alveolar macrophages (Downing et al, 1995). Surfactant proteins also inhibit MTB-induced NO production in IFN-γ primed murine macrophage (Pasula et al, 1999). SP-D opsonised MTB undergoes increased phago-lysosomal fusion and hence decreased intracellular survival (Ferguson et al, 2006; Harris et al, 2000). Again, it has been found to block the uptake of pathogenic strains of MTB in macrophages (Ferguson et al, 1999). It may therefore be hypothesized that the relative concentrations of different surfactant proteins also have a role in TB infection.

Toll like receptors (TLRs): Toll-like receptors (TLRs) are essential for microbial recognition on macrophages and dendritic cells (Visintin et al, 2001). Members of the TLR family are phylogenetically conserved mediators of innate immunity. These are transmembrane proteins containing repeated leucine-rich motifs in their extracellular domains, similar to other pattern-recognizing proteins of the innate immune system. The cytoplasmic domain of TLR is homologous to the signalling domain of IL-1 receptor (IL-1R) and links to IRAK (IL-1R-associated kinase), a serine kinase that activates transcription factors like NF-κB to signal the production of cytokines (Oddo et al, 1998). To date, at least 10 TLRs have been identified; of those TLR2, TLR4, and TLR9 seem responsible for the cellular responses to peptidoglycan and bacterial lipopeptides (Yoshimura et al, 1999), endotoxin of gram-negative bacteria (Schlesinger et al, 1990), and bacterial DNA (Gercken et al, 1994), respectively. MTB lysate or soluble *Mycobacterial* cell wall associated lipoproteins induce production of IL-12, a strong proinflammatory cytokine through TLRs (Brightbill et

al, 1999). MyD88 (myeloid differentiation protein 88), is a common signalling component that links all TLRs to IRAK (Oddo et al, 1998), found to be essential for MTB -induced macrophage activation (Underhill et al, 1999). A mutation of TLR2 specifically, although incompletely, inhibited MTB induced tumour necrosis factor alpha (TNF-α) production. It suggests that besides TLR2, other TLRs may be involved (Underhill et al, 1999). TLR2 was necessary for signalling by the *Mycobacteria*l LPS LAM (Means et al, 1999) and a 19-kDalipoprotein (Noss et al, 2001; Brightbill et al, 1999) .TLR-4 *Mycobacteria*l ligand is not well defined. Interestingly, *Mycobacteria*l infection and proinflammatory cytokines increase surface expression of TLR2 (Wang et al, 1999). Furthermore, the increased expression of CD14 and TLRs did not alter uptake of MTB in '*in vitro*' studies. Interestingly, a study showed that TLR2 activation directly led to killing of intracellular MTB in alveolar macrophages *in vitro* (Thoma-Uszynski et al, 2001). It may be anticipated that genetic polymorphism, or perhaps mutations, in the relevant TLR or the downstream signalling proteins will affect the performance of the innate host response to *Mycobacteria*.

Thus, there are multiple mechanisms for the uptake of MTB, which may lead to differences in signal transduction, immune activation, and intracellular survival of the pathogen. Virulent strains of MTB are phagocytosed through MR, while attenuated strains use other receptors (Schlesinger, 1993). It has been shown that entry through MR triggered less ROS production as there was less NADPH oxidase activation (Astarie-Dequeker et al, 1999). Moreover, it induced an anti-inflammatory signal to the phagocyte (Nigou et al, 2001).

Host intracellular trafficking and phago-lysosome formation

Once taken up, the bacteria begin to disrupt the mechanisms of phagosome maturation, creating an intracellular compartment that lacks the acidic, hydrolytic environment needed to kill the bacteria which is called early endosome. However, fusion with other vesicles and membrane remodelling and trafficking still occurs, allowing MTB to

acquire necessary nutrients and export its own proteins (Desjardins et al, 2005). Phagosome maturation is the process in which phagosome remodels through a series of independent events starting from its formation, complete fusion with lysosome and presentation to the surface of the phagocyte as phagocytic cup. Phagosome after internalization also shows transient access to the rapid recycling pathway, as defined by the classic marker of this pathway, transferrin (Hao and Maxfield, 2000). During the early stage of phagosome maturation, many of the early endosomal markers like 'early endosomal antigen 1' (EEA1), lysosomal hydrolases such as procathepsin D and Rab5 appear. Phagosome becomes more acidic through the accumulation of v-ATPases and GTPases that pump protons into the compartment and becomes hydrolytically competent through the acquisition of lysosomal enzymes. Lysosomal hydrolases are delivered to the endosomal network by both mannose – 6 –phosphate dependent and independent manner (Schweizer et al, 1996).

MTB interferes with phagosomal maturation by as yet not fully known mechanism. MTB utilise some putative transporters, iron-scavenging molecules and lipid-synthesizing molecules in preventing normal phagosome maturation. ESAT-6/CFP10 and SecA1/2 proteins on *M. tuberculosis* are considered as virulence factors that interfere with this process (Tan et al, 2006; Hou et al, 2008). This process is also dependent, to some extent on blocking of a calmodulin dependent Ca flux by multiple pathogen derived molecules (Russell, 2001; Connolly & Kusner, 2007). Lipids such as trehalose dimycolate can interfere with membrane trafficking, preventing phagosome maturation and surface expression of MHC molecules. Some phagosome-function-inhibiting lipids, such as mannose-capped lipoarabinomannan (ManLAM), appear to be mimics of host phosphatidylinositols, whose presence on the surface of the vacuole normally indicates a maturation state (Chua et al, 2004). Other molecules such as LRG-47 (MacMicking et al, 2003; Deretic et al, 2006) also interfere with tracking and control of the phagocytic vesicle. Finally, the expression by MTB of a

eukaryotic like serine/threonine protein kinase G (Pkn G) can inhibit phagosome–lysosome fusion. The abundance of known (and presumably unknown) genes involved in altering phagosome maturation and trafficking indicates that interfering with this is a major survival strategy for MTB.

ROI & RNI:

Putative mechanisms involved in killing of MTB within the phagolysosomes of activated macrophages include the production of reactive oxygen intermediates (ROI) or reactive nitrogen intermediates (RNI). In vitro, *Mycobacteria* seem resistant to killing by ROI such as superoxide and hydrogen peroxide (Chan et al, 1992). A possible explanation lies in the fact that several *Mycobacteria*l products, including sulfatides and LAM, are able to scavenge ROI (Pabst et al, 1988; Chan et al, 1991, Neill & Klebanoff, 1998). *In vivo*, it was found that p47phox knockout mice, which lack a functional p47 unit of NADPH-oxidase needed for superoxide production, suffer from increased early overgrowth of *Mycobacteria* in experimental infection (Cooper et al, 2000). Therefore, this supports a role for ROI in the killing of MTB. On the other hand, patients with chronic granulomatous disease, who have defective production of ROI, do not seem to display increased susceptibility to tuberculosis (Winkelstein et al, 2000). Additionally, there is a growing body of evidence suggesting that reactive oxygen species (ROS) can also act as signalling molecules and influence cytokine production (Yang et al, 2007). The role of RNI in tuberculosis also remains a matter of debate. In vitro, human alveolar macrophages infected with *M.bovis* BCG display increased inducible nitric oxide synthase (iNOS) mRNA (Nozaki et al, 1997), and inhibition of iNOS was followed by increased bacterial growth in the host (Nicholson et al, 1996). In tuberculosis patients, alveolar macrophages show increased production of iNOS as well. It is now well known that MTB lipid layers are important for ROI and RNI scavenging. As, the

composition of the cell wall and hence the lipids present vary with MTB strains, it may be possible that ROI and RNI response may vary.

Adaptive response

Failure of innate immune mechanisms to control the growth of MTB, is possibly related to insufficient production of NO and other immune mediators, after which adaptive immunity becomes important. The increasing immune pressure mounted by the adaptive immunity restores the immunological control. Innate and adaptive immunity are closely connected. Macrophages and dendritic cells, the primary cell types involved in the innate immune response to *Mycobacteria*, play a crucial role in the initiation of adaptive immunity.

Clearance of bacteria by macrophages is in part dependent on macrophage activation by the cytokine IFN-γ secreted by CD4+ T cells, CD8+ T cells and NK cells (Boom 1996; Flynn & Chan, 2001; Kaufmann, 2001; Wang et al, 2004; Feng et al, 2006; Ngai et al, 2007). Infected macrophages secrete pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6, as well as chemokines that lead to the migration of monocyte derived macrophages and DCs to the site of infection (Means et al, 1999; Flynn & Chan, 2001; Marino et al, 2004). The migration of cells to the site of infection results in the formation of granuloma, which functions to restrict further bacterial dissemination (Co Do et al, 2004).

DCs are central to the generation of acquired immunity after carriage of antigens to draining lymph nodes, where recognition by T cells can be maximized (Marino et al, 2004; Tian et al, 2005; Wolf et al, 2008). To optimally prime pathogen-specific Th1 responses, DCs require stimulation through TLRs (Sporri & Sousa, 2005) by the pathogen as well as host-derived factors such as type I and type II IFNs, cytokines, and chemokines (Kapsenberg, 2003). In these compartments, the stimulatory capacity of mature DCs ultimately leads to effector T cell differentiation and memory T cell expansion, which in turn, confer protection

against MTB in the lungs (Kaufmann, 2001; Kaufmann & Schaible, 2003). In contrast to macrophages, DCs have poor mechanisms to eliminate internalized *Mycobacteria* (Bodnar et al, 2001; Tailleux et al, 2003). Rather, it has been suggested that DCs offer a niche for long-term survival of intracellular bacteria (Bodnar et al, 2001; Tailleux et al, 2003). Presentation of *Mycobacteria*l antigens by macrophages and dendritic cells involves distinctive mechanisms. MHC class II molecules present *Mycobacteria*l proteins to antigen specific CD4+T cells. These antigens must be processed in phagolysosomal compartments in professional antigen-presenting cells. MHC class I molecules, expressed on all nucleated cells, are able to present *Mycobacteria*l proteins to antigen-specific CD8+T cells. This mechanism allows for the presentation of cytosolic antigens, which may be important as certain *Mycobacteria*l antigens may somehow escape the phagosome (Mazzaccaro et al, 1996). The importance of MHC class I-mediated antigen presentation has been shown in murine models (Sousa et al, 2000) and tuberculosis patients (Cho et al, 2000; Geluk et al 2000). Other than MHCs, nonpolymorphic MHC class I molecules such as type I CD1 (-a, -b, -c and -d) molecules, which are expressed on macrophages and dendritic cells, are able to present *Mycobacteria*l lipoproteins to CD1- restricted NKT cells. These cells are compelling candidates, being able to respond rapidly and subsequently to activate other cell types (Harada & Taniguchi, 2003). Their apparent self-reactivity and ability to quickly release large amounts of cytokines such as IFN-γ, is important in the initiation and regulation of various immune responses (Harada & Taniguchi, 2003). NKT cells are a subset of T cells that co-express a αβTCR (T cell receptor), but also express a variety of molecular markers that are typically associated with NK cells, such as NK1.1. Unlike conventional αβ T cells, their TCRs are far more limited in diversity and recognize lipids and glycolipids presented by CD1d molecules. *Mycobacteria* modulate expression of antigen presenting molecules in

macrophages, most likely through the production of antiinflammatory cytokines (Pancholi et al, 1993; Gercken et al, 1994).

Thus, all these mechanisms of antigen presentation enable the activation of a larger fraction of T cells. Several studies have shown that protective immunity to TB is dependent on the adaptive Th1 immune responses (Janis et al, 1989; Flynn & Chan, 2001; Kaufmann, 2001; Holtmeier and Kabelitz, 2005) mediated by macrophages, DCs, T cells and their interactions (Flynn & Chan 2001; Berrington & Hawn, 2007).

1.5. Cytokines, chemokines, and other soluble effector molecules in Tuberculosis

Tumour necrosis factor-α

Tumour necrosis factor-α (TNF-α) is a prototypic proinflammatory cytokine that is produced by monocytes, macrophages (Valone et al,1988) and dendritic cells (Henderson et al, 1997) when exposed to live bacteria or microbial products derived from MTB. TNF-α plays a key role in granuloma formation (Kindler et al, 1989; Senaldi et al, 1996), induces macrophage activation, and has immunoregulatory properties (Tsenova et al, 2005). In mice, TNF-α is also important for containment of latent infection in granuloma (Mohan et al, 2001). In tuberculosis patients, TNF-α production is present at the site of disease (Law et al, 1996) and systemic spill over of TNF-α may account for unwanted inflammatory effects like fever. TNF-α is produced as a trimeric surface molecule and is cleaved by TNF-α-converting enzyme to release the trimeric molecule from the cell. Severity of the disease associated with a selective increase of TNF-α in plasma (Bekker et al, 1998), and quick recovery is associated with a rapid decrease of this cytokine in plasma (Hsieh et al, 1999). In synergy with IFN-γ, TNF-α activates macrophages to produce nitric oxide synthase 2 (NOS2), allowing the macrophage to kill intracellularly replicating MTB (Ding et al, 1988). Mice deficient in TNF-α and TNF-α receptor (TNFR) show increased susceptibility to MTB and impaired granuloma formation following infection with MTB (Flesch et al, 1995). In humans, there is

evidence that TNF-α plays an important role in host defence against MTB as patients of Crohn's disease and rheumatoid arthritis receiving anti TNF-α antibodies are significantly susceptible to tuberculosis reactivation (including miliary and extrapulmonary) (Keane et al, 2001). Variation in both the TNF-α and TNFR genes were associated in linkage with *in vitro* TNF-α production as well as the development of active TB disease (Stein et al, 2007). The main polymorphisms that have been identified are found in the promoter region at −238 and −308 in Turkish, Chinese, Thai and Colombian populations (Wilkinson et al, 1999; Mege et al, 2006; Mohan et al, 2001). Although the majority of evidence suggests that the TNF-α promoter polymorphisms are not consistently associated with TB susceptibility, many of the studies have been insufficient in size to show minor differences. So, further studies are needed before possible the association with these common polymorphisms can be disregarded

Interleukin-1β

IL-1β is a proinflammatory cytokine that is produced in a myriad of infections and proinflammatory conditions (Dinarello, 1996). IL-1β is produced in the cell in an inactive precursor form (pro-IL-1β) and then is cleaved by Caspase-1 to an active form, which is secreted from the cell through an unknown mechanism. Conversion of pro-IL-1β to IL-1β can occur with numerous bacterial stimuli, including MTB, and can also occur when bacterial products are sensed within the cytoplasm by NLR proteins (i.e. NOD2), which contain a CARD domain important for Caspase-1 activation and inflammasome function. Once secreted, IL-1β acts primarily through IL-1R type I receptor, which further activates the expression of other proinflammatory cytokines. In some cases, however, IL-1β function is blocked by either binding to a membrane-bound decoy receptor (IL-1R type II) or by a soluble receptor antagonist (IL-1RA) that blocks the proinflammatory effects of IL-1β. Evidence that IL-1β plays an important role in the pathogenesis of MTB comes from studies

on IL-1 knockout animals. Animals that have both IL-1β and IL-1α deleted are unable to clear the *Mycobacteria* form granulomas as efficiently as wild type mice (Yamada et al, 2000). Furthermore, animals those lack the IL-1R type I have impaired survival and are unable to contain growth of the organism *in vivo* (Juffermans et al, 2000). This response may be because of impaired cell-mediated immunity, as measured by IFN-γ production. There is also evidence that susceptibility to clinical disease in an African cohort with MTB is associated with IL-1 gene cluster, specifically variation in the IL-1RA gene (Juffermans et al, 2000). In this study, other polymorphisms in the IL-1β and IL-1α gene did not show significant difference in association with TB susceptibility. Polymorphisms in non-coding regions of IL-1β have been linked to human variation in cytokine production and a −511 C allele in the promoter region of the gene is associated with protection from acquiring pulmonary TB (Awomoyi et al, 2005). Furthermore, studies carried out on emigrated Indians in London showed a relationship between polymorphisms in both IL-1β and IL-1RA and the functional ratio of expression and the acquisition of tuberculous pleurisy ($P = 0.028$), although multiple polymorphisms in either IL-1β or IL-1RA gene when analyzed individually were not associated with differences in TB resistance (Bellamy et al, 1998). The question whether variation in genes of the IL-1 cluster confer risk to clinical TB disease continues to be premature, and further studies are needed before substantial conclusions can be made.

IL-12/IL-12Rβ1

IL-12 is a heterodimeric, covalently linked cytokine comprised of two subunits (p40 and p35). The p40 subunit is present in both IL-12 and IL-23, while the p35 subunit is specific for IL-12. IL-12 is mainly secreted by hematopoietic phagocytic cells (monocytes, macrophages, and neutrophils) and dendritic cells, and promotes T-cell differentiation into T-helper 1 (Th1) cells and production of IFN-γ by signalling through IL-12Rβ1 and IL-12Rβ2 (Trinchieri G, 2003). IL-12 plays an important role in stimulating IFN-γ production and

establishing a potent Th1 response to intracellular pathogens such as MTB and *Salmonella*. Early studies showed that mice, when given exogenous IL-12, developed increased resistance to MTB (Cooper et al, 1997). Furthermore, mice deficient in IL-12p40 and IL-12p35 showed enhanced susceptibility to MTB infection (Khader et al, 2005). The role of IL-12 in *Mycobacteria*l disease has been firmly established by the presence of patients with uncommon polymorphisms or mutations that predispose to severe disseminated *Mycobacteria*l infection in a Mendelian fashion (Casanova & Abel, 2002). In addition to these Mendelian phenotypes, there is conflicting evidence that common variations in the IL-12Rβ1 gene confer susceptibility to MTB. In a Japanese cohort, increase in susceptibility to MTB was reported when three missense non-synonymous polymorphisms (M365T, G378R, and Q214R) were present (Akahoshi et al, 2003). These identical missense polymorphisms were present in a Moroccan cohort at high frequency, but no susceptibility was associated with these SNPs (Remus et al, 2004).

IL-6

IL-6 is detected early during *Mycobacteria*l infection at the site of infection (Law et al, 1996; Holland et al, 1998) and suppresses T cell responses (Van Heyningen et al, 1997). As it inhibits the production of TNF-α and IL-1β and promotes *in vitro* growth of *Mycobacterium avium* (Shiratsuchi et al, 1991), it can be said that IL-6 promotes infection. Other reports support a protective role for IL-6: IL-6-deficient mice display increased susceptibility to infection with MTB (Ladel et al, 1997), which seems related to a deficient production of IFN-γ early in the infection.

IFN-γ/IFN-γR

IFN-γ, the prototypic cytokine of the Th1 cell response, is a cytokine essential for the effective control of MTB in the host. IFN-γ is produced by CD4+ T cells, CD8+ T cells, and NK cells, and it stimulates a mycobactericidal response in macrophages characterized by the

induction of NOS (Flynn et al, 2001; Feng et al, 2006). Indeed, IFN-γ gene knockout (KO) mice are highly susceptible to *M.tuberculosis* (Cooper et al, 1993) and individuals lacking receptors for IFN-γ suffer from recurrent, sometimes lethal *Mycobacteria*l infections (Flynn et al, 1993; Holland et al, 1998). Th2-type cytokines inhibit the *in vitro* production of IFN-γ (Powrie & Coffman, 1993; Lucey et al, 1996) and may, therefore, weaken host defence. Mice that fail to produce IFN-γ have disseminated *Mycobacteria*l infection whether challenged by aerosolized route or intravenously (Cooper et al, 1993; Flynn et al, 1993). Furthermore, increased resistance to MTB following intravenous IL-12 administration was abrogated in IFN-γ gene-disrupted mice (Flynn et al, 1993). In humans, a series of uncommon genetic mutations in IFN-γR1 and R2 lead to Mendelian susceptibility to *Mycobacteria*l disease (MSMD) (Casanova & Abel, 2002). There have been inconsistent findings using IFN-γR1 microsatellite markers and association studies with TB susceptibility.

Common polymorphisms in the IFN-γ gene play a role in TB susceptibility. Three polymorphisms in the IFN-γ gene have been studied in various populations (A-1616G, T+874A, C+3234T) (Lio et al, 2002; Rossouw et al, 2003,Vidyarani et al, 2006;Cooke et al, 2006). Variation in the promoter region of the IFN-γ gene disrupts an NF-κB binding site (T+874A) and is associated with an increased frequency of TB in a study comparing 314 South Africans with pulmonary and meningeal TB versus 235 healthy controls (Rossouw et al, 2003). Overall, the evidence strongly indicates that IFN-γ is significantly associated with susceptibility to active TB disease.

Anti-Inflammatory Cytokines

Interleukin-10

IL-10 is a cytokine produced by macrophages, dendritic cells, B cells and regulatory T-cell subsets after binding of *Mycobacteria*l LAM (Dahl et al, 1996).In patients with tuberculosis, expression of IL-10 mRNA has been demonstrated in circulating mononuclear cells, at the

site of disease in pleural fluid, and in alveolar lavage fluid (Gerosa et al, 1999; Barnes et al,1993). *Ex vivo* production of IL-10 was shown to be upregulated in tuberculosis by some investigators (Torres et al, 1998), but this was not found by others (Lin et al, 1996). IL-10 antagonizes the proinflammatory cytokine response by down regulation of production of IFN-γ, TNF-α, and IL-12 (Gong et al, 1996), essential for protective immunity in tuberculosis. IL-10 transgenic mice with *Mycobacteria*l infection develop a larger bacterial burden (Murray et al, 1997). In human tuberculosis, IL-10 production was higher in anergic patients, both before and after successful treatment, suggesting that MTB -induced IL-10 production suppressed an effective immune response.

TGF-β

Monocyte and dendritic cells also produce TGF-β, an anti-inflammatory cytokine in response to *Mycobacteria*l products which seems to counteract protective immunity in tuberculosis. LAM from virulent *Mycobacteria* selectively induces TGF-β production (Toossi et al, 1995). Like IL-10, TGF-β is produced in excess during tuberculosis at the site of disease (Condos et al, 1998). TGF-β suppresses cell-mediated immunity: it inhibits T cell proliferation and IFN-γ production by T cells and in macrophages it antagonizes antigen presentation, proinflammatory cytokine production, and cellular activation (Epstein et al, 2000). In addition, TGF-β may be involved in tissue damage and fibrosis during tuberculosis, as it promotes the production and deposition of macrophage collagenases (Toossi et al, 1998) and collagen matrix. Naturally occurring inhibitors of TGF-β eliminate the suppressive effects of TGF-β on mononuclear cells from tuberculosis patients and in macrophages infected with MTB (Hirsch et al, 1997). TGF-β selectively induces IL-10 production, and both cytokines show synergism in the suppression of IFN-γ production.

IL-4

IL-4 interferes with intracellular MTB infection by the suppression of cytokine IFN-γ and macrophage activation. In mice progressive disease (Hernandez-Pando et al, 1996) and reactivation of latent infection (Howard et al, 1999) are both associated with increased production of IL-4. Similarly, overexpression of IL-4 intensified tissue damage in experimental infection (Lukacs et al, 1997). Conversely, inhibition of IL-4 production did not seem to promote cellular immunity: IL-4 double knockout mice displayed normal instead of increased susceptibility to *Mycobacteria* in two studies, suggesting that IL-4 may be a consequence rather than the cause of tuberculosis development (North, 1998).

Chemokines: MCP-1 and IL-8

CCL2, also called monocyte chemoattractant protein 1, is a β chemokine that is induced by monocytes infected with MTB (Lin et al, 1998). CCL2 causes chemotaxis of memory T lymphocytes, NK cells, and macrophages to sites of inflammation. In animal models, there is evidence that CCL2 may modulate disease severity. Mice that overexpress CCL2 showed high levels of CCL2 in all tissues, and these animals were susceptible to intracellular pathogens (Rutledge et al, 1995). Mice with targeted gene deletion of CCL2 failed to show increased susceptibility to MTB (Lu et al, 1998). CCL2 may cause decreased IL-12p40 production and skew the T-cell response away from a Th1 response (Chensue et al, 1996). In humans, variation in the promoter region of CCL2 causes significant changes in chemokine expression to IL-1β (Rovin & Saxena, 1999). Furthermore, genetic analysis in Brazilian patients has indicated that susceptibility to intracellular pathogens (*Leishmania*, MTB) are linked to chromosome 17q11–12, which codes for the CCL2 protein, along with many other chemokines and NOS2A (Jamieson et al, 2004). These authors, however, failed to show significant association of CCL2 promoter polymorphisms with increased TB susceptibility. In contrast, another study with both Mexican and Korean cohorts found increased susceptibility to TB in those with the A-2518G CCL2 promoter polymorphism

(Jamieson et al, 2004). Overall, elevated levels of CCL2 may favour Th2 cytokine response and decreased IL-12 production.

Another chemokine produced by phagocytic cells and tissue cells after simulation with MTB is IL-8 (Riedel & Kaufmann, 1997). Increased levels of IL-8 are seen in the bronchoalveolar lavage fluid of humans with TB infection, and proportionately higher levels of IL-8 may be associated with increased mortality (Friedland et al, 1995). A polymorphism in the promoter region of IL-8, T-251A, is seen in high frequency in both Caucasian and African American populations. This polymorphism not only is associated with enhanced severity to bronchiolitis in infants with respiratory syncytial virus (Hull et al, 2000) but is also significantly with MTB infection (Ma et al, 2003).Further studies need to be carried out to identify the role IL-8 plays in MTB infection.

1.6. Genetic susceptibility to TB

A series of studies over the past 50 years suggest that host genetic factors influence susceptibility to TB (Casanova & Abel, 2002; Cooke et al, 2006). Although previous studies have uncovered some of the genes involved in human predisposition to *Mycobacteria*l infections, a comprehensive understanding of genetic susceptibility factors remains an elusive and important goal. There are four major lines of evidence to support a genetic basis for susceptibility to TB. First, studies in twins indicate that TB incidence rates among monozygotic twins are more than twice the rate of dizygotic twins (31.4 versus 14.9 TB cases per 100 twins for monozygotic and dizygotic twins, respectively, $P < 0.05$, binary variable multiple regression analysis) (Comstock, 1978). Second, several primary immunodeficiency disorders are associated with susceptibility to *Mycobacteria* in a Mendelian fashion attributable to rare single gene mutations with high penetrance. These disorders include severe combined immunodeficiency, hyper-immunoglobulin (Ig) E syndrome, chronic granulomatous disease, anhidrotic ectodermal dysplasia with immunodeficiency, hyper-IgM

syndrome, and Mendelian susceptibility to *Mycobacteria*l disease (MSMD) (Casanova & Abel, 2002). This latter group of disorders is more selectively associated with *Mycobacteria*l infection and sometimes also with *Salmonella* but not with excessive susceptibility to other pathogens. Most of the infections associated with these Mendelian disorders have been from BCG or environmental bacteria. However, some of these disorders are also associated with MTB susceptibility (Lienhardt et al, 2005). The third type of evidence for relationship host genetic makeup and TB susceptibility comes from the study of complex inheritance patterns, where the assumption is that genetic influence is polygenic and attributable to alleles that are common in the population with low penetrance for any single allele. Several genome-wide studies of susceptibility to TB have been performed with family-based linkage studies. These studies have identified several loci that include 2q35 in a Canadian population (Greenwood et al, 2000), 8q12–13 in a study from Morocco (El Baghdadi et al, 2006), 17q11.2 in Brazil (Jamieson et al, 2004), and 15q and Xq in populations from The Gambia and South Africa (Cervino et al, 2002). Fine mapping of the 15q locus in families from Africa (The Gambia, Guinea, and South Africa) suggests that Ube3a or a closely linked gene may contain the causative locus (Barreiro et al, 2006). Efforts to identify the genes underlying each of these associations are ongoing. The fourth line of evidence comes from candidate gene association studies. These studies evaluate whether common polymorphisms in candidate genes are associated with susceptibility to disease. The most common study design is a case–control format with comparison of polymorphism (single nucleotide, insertions, deletions, or microsatellite markers) frequencies between cases and controls. The strength of this study design is the capacity to enrol large cohort sizes. One disadvantage is the problem of population heterogeneity or admixture, where differences in ethnic composition of the cases and controls can lead to false associations that are not attributable to differences in disease susceptibility. A number of candidate genes have been identified in case–control studies for

their possible role in TB susceptibility. The most promising candidates that have shown consistent effects in their association with TB susceptibility in multiple studies include HLA (DRB1) and Slc11a1 (Bellamy, 2003). Other genes with strongly suggestive associations include IFN-γ, TIRAP/MAL, and CCL2. Advances in genomic technology and immunology have accelerated the candidate gene association studies in infectious diseases. Together, these studies are providing insight into human susceptibility to TB and the underlying mechanisms of pathogenesis from genetically regulated variation of macrophage function and the innate immune response.

1.7. Rationale of the study:

A hallmark of the natural history of tuberculosis has long been variability in the outcome of disease. Only 30% of exposed persons show evidence of infection and of those infected, only 10% become ill. Among those who become ill, there is variability in disease time course, severity, and anatomic distribution. MTB acquires genetic changes with time which confers the virulence property to the pathogen as well as the ability to modulate host immune response in its own favour. The precise pathogenesis of TB and the factors determining the highly variable outcome of infection are only partly understood.

It was initially believed that MTB complex constituted a genetically highly conserved group of bacteria with limited phenotypic differences, hence most of the earlier immunological studies have used a limited number of laboratory strains, such as H37Ra, H37Rv and BCG. In 1905, MTB strain H37 was cultivated from the sputa of a 19-year-old male with pulmonary tuberculosis. Later on, this isolated strain was used as standard laboratory virulent strain. In 1922, it was noted that this strain had changed the colony morphology and was not able to produce disease. This attenuated progeny came to be known as H37Ra, an avirulent standard laboratory strain. BCG strain, the world's most widely used vaccine has also been generated analogously from a *M. bovis* isolate. The clear phenotypic

differences between H37Ra and H37Rv, BCG and *M. bovis* have inspired several groups to seek the genomic differences in them. A 25 kb DNA fragment called *ivg* (*in vivo* growth-promoting locus) has been identified in H37Rv but not in the attenuated H37Ra (Pascopella et al, 1994; Brosch et al, 1999; Gordon et al, 1999). In addition, a 7.9 kb fragment containing genes coding for a putative sugar transferase, oxidoreductase, and a membrane protein are seen in the genome of H37Ra but not H37Rv (Brosch et al, 1999; Gordon et al, 1999). Interestingly, the introduction into H37Ra of genes that are restricted to H37Rv did not correct the attenuated phenotype of H37Ra (Pascopella et al, 1994; Brosch et al, 1999). Studies comparing H37Rv, H37Ra, and BCG have identified phenotypic differences *in vivo* and in vitro. For instance, the number of bacteria recovered from the lungs and spleens of infected guinea pigs after inoculation with H37Ra was100-fold less than with H37Rv at 3 weeks and declined rapidly thereafter (Alsaadi & Smith, 1973). Differences in virulence have been investigated in monocyte and macrophage models. Moreover, epidemiological data showed that differences in transmissibility and virulence among MTB strains are related to the genetic background of the organisms (Valway et al, 1998). Thus, pathogenesis in tuberculosis is driven by many components of the host immune system, pathogen and environment (Van der Spuy et al, 2009). Environmental and host factors clearly contribute to the clinical and epidemiologic behaviour of strains. But, the pathogen variability factor must also be carefully integrated into the investigative process. A coherent knowledge about the factors is still lacking.

The interaction of pathogen with its host cell is a very important area to study in order to know the different survival strategies followed by the pathogen. But, for MTB it is not very easy. First of all, accurately determining laboratory and clinically relevant phenotypes is a significant challenge. For example, early reports of the unusually high growth rate of CDC1551 in mice appear in retrospect to be largely due to the relative attenuation of the

comparison strains. One additional difficulty in trying to link genomic diversity to phenotypic diversity has been the lack of appropriate tools to index genomic diversity and classify strains. As, MTB is a genetically monomorphic organism, single genotyping tool is uninformative for this pathogen (Achtman, 2008; Comas et al, 2009). So, our limited understanding of the genetics of MTB makes it difficult to predict which genetic polymorphisms may be of consequence. Another problem is with the laboratory adaptation of the strains.

The fact that genetically distinct strains of MTB have been prevalent in different population makes it possible to surmise that the susceptibility of these populations may be linked to the immune response against those strains. Again, the genetically different MTB strains from different lineages have been shown to induce differential host responses in macrophages, cell lines and mouse models (Hoal-van Helden et al, 2001; Lopez et al, 2003) and are demonstrated to vary with respect to their virulence, pathology and bacterial load (Marquina-Castillo et al, 2009). It was shown that selected W-Beijing strains elicit less proinflammatory and Th1 type cytokines than the non-W-Beijing strains. Further, it was reported that CAS1 and Beijing strains, belonging to lineage 2, have lower growth rate and induce lower levels of proinflammatory cytokines in THP-1 cells (Tanveer et al, 2009) as well as macrophages from human PBMNC compared to standard laboratory strain H37Rv (Portevin et al, 2011). In contrast to these observations, another group detected higher induction of TNF-α by Beijing strain in human macrophages (Chacón Salinas et al, 2005). In a mouse model, genetically different MTB strains elicited dissimilar immune responses in the lung, which determines differences in pathology and mortality. The Beijing genotype induced the highest mortality compared to H37Rv and Canetti genotype (Lopez et al, 2003). Additionally, Keane *et al,* 2000 showed that apoptosis contributed to innate host defence and is influenced by the virulence of the strains. Avirulent or attenuated strains induced

significantly more apoptosis than virulent strains in alveolar macrophages (Keane et al, 2000). Further, it was discernible that depending on TNF-α and IL-10 ratio induced by them, MTB strains modulated the host cell survival and apoptosis (Rojas et al, 1999). It was also noticed that the apoptosis of host cell was also governed by phagocytic index of respective strains (Rajavelu & Das, 2007). Thus, studies published so far evaluated one or two individual parameters, however, comprehensive studies to correlate different parameters induced by well typed clinical isolates have not been reported. Further, it is noticed that there is no comparative evaluation of different genotypes of MTB isolated from India for the immunological responses and other parameters induced by them.

The aim of present study therefore,

- **To select and characterize clinical isolates of *Mycobacterium tuberculosis* collected from Mumbai, India by different molecular biology techniques**
- **To analyze the several innate host responses induced by different clinical isolates of *Mycobacterium tuberculosis* in THP-1 cells**
- **To assess the cytokine response induced by different clinical isolates of *Mycobacterium tuberculosis* in monocyte derived macrophages (MDM), monocyte derived dendritic cells (MDDC) and whole blood from healthy individuals**
- **To evaluate the host responses after infection with the same clinical isolates in BALB/c mice**

Chapter 2: Characterization of *Mycomacterium tuberculosis* strains by several molecular typing methods

2.1. Introduction

It has been proposed that strain diversity in human tuberculosis (TB) has some role in disease outcome. The distinctive epidemiological and clinical characteristics of different strains of MTB are now well established. However, whether and how MTB genomic diversity influences human disease in clinical settings is an unresolved question. A few of MTB strains have been identified for their wide dissemination and acquisition of drug resistance (Bifani et al, 2002; Lan et al, 2003; Parwati et al, 2010) while a few others tend to predominate in restricted areas (Gagneux & Small, 2007). Interestingly, several studies have established an association between human population and particular MTB lineage for a specific geographic region (Gagneux et al, 2006; Hershberg et al, 2008; Reed et al, 2009). Earlier study from our laboratory (Kulkarni et al, 2005) also showed that among the TB patients screened from Mumbai, 30% are infected with Central Asian families CAS1 and CAS2 strains, 17% with the ancestral East African Indian (EAI) family and 10% of the isolates belonged to the Beijing family. Further, it was shown that, in Far East Asia almost 50% population is infected by Beijing strain (Brudey et al, 2006) while, the F11 strain represents the largest proportion of all isolates from tuberculosis patients in the Western Cape of South Africa (Victor et al, 2004). In addition, it was reported that the C strain, a drug-susceptible and reactive nitrogen intermediates resistant strain showed a spread among intravenous drug users (Friedman et al, 1997) and few strains showed a higher risk for relapse (Burman et al, 2009). More recently, it was demonstrated that a large proportion of the Latin-American-Mediterranean (LAM) strains of MTB caused more cavitary TB compared to other strains (Lazzarini et al, 2008), while another study provided evidence of relationship between genotype and clinical phenotype in pulmonary and meningeal TB

infection (Thwaites et al, 2008). The volume of information available resulted in an increased emphasis on characterization and identification of MTB strains.

Majority of the studies related to pathogenesis of TB, published so far, use laboratory strains H37Rv, H37Ra, *M. bovis* BCG and Erdman. Since, the laboratory strains suffer from artifacts because of the adaptation to the laboratory culture condition, a study using clinical isolates may be advantageous as they preserve the behaviour of wild type pathogens. It is also known by now that strain-to-strain variation can have important phenotypic consequences including virulence, transmissibility and response to treatment.

In chapter 1, the basis for the classification of MTB strains into different lineages has been described. Several genotyping methods are employed for the characterization of clinical isolates. These include;

2.1.1. Spoligotyping (Spacer oligotyping)**:** This is considered the simplest, rapid and most cost-effective technique that has been used to define clinical and veterinary clades of MTB worldwide. This typing method is based on the DNA polymorphism present at one particular chromosomal locus, the 'Direct Repeat' (DR) region, which is unique to MTB complex bacteria. Hermans et al. in 1991 first sequenced this region in *M. bovis* BCG. The DR region consists of directly repeated sequences of 36 base pairs which are interspersed by non-repetitive DNA spacers, each 35 to 41 base pairs in length. The number of copies of the DR sequence in MTB complex strains was found to vary significantly. The vast majority of the MTB strains contain one or more *IS6110* elements in the DR region. In contrast to the DRs, the spacers are usually present only once in the DR region. One DR and its neighbouring non-repetitive spacer are termed "Direct Variant Repeat" (DVR). This DR locus has been designated the spacer interspersed DR (SPIDR) region and was recently renamed by Jansen et al., as the "clustered regularly interspaced short palindromic repeats" (CRISPR) region (Jansen et al, 2002). When the DR regions of several strains were compared, a particular

pattern of spoligotype was observed (Groenen et al, 1993). The differences between spoligotype patterns were shown to be due to deletions of spacer sequences in the CRISPR region by transposition of *IS6110* elements (Fang et al, 1998; Filliol et al, 2000, Van Embden et al, 2000; Legrand et al, 2001) and are probably mediated by homologous recombination (Groenen et al, 1993) or replication slippage (Hancock, 1995).

Although, other formats of this technique have been used in some studies (Van der Zanden et al, 2002; Brudey et al, 2004), the most popular version is that originally suggested by Kamerbeek and colleagues (1997), having 43 spacer region which is commercially available as a kit (Isogen Bioscience BV, Maarssen, Netherlands). The specificity and sensitivity of this technique were shown to be 98% and 96% respectively with the clinical samples. Using this technique, various international spoligotyping databases have been constructed and are being constantly updated (Sola et al, 2001; Filliol et al, 2002; Filliol et al, 2003; Brudey et al, 2004). The most recent version, the fourth database, SpolDB4 (Brudey et al, 2004) initially contained 39,295 patterns distributed into 1,939 STs (shared-types, which are a patterns shared by 2 or more patients isolates), and 3,370 orphans (patterns reported for a single isolate). An updated version at present database contains about 60,000 patterns distributed into 2300 STs (renamed SIT for "Spoligotype International Type").

Spoligotyping is being extensively used in recent years for epidemiological studies of tuberculosis (Kamerbeek et al, 1997; Soini et al, 2000) and shown to be useful in strain discrimination when used in association with *IS6110*-RFLP typing, VNTR DNA repeats typing (Filliol et al. 2000) and DRE-PCR (Sola et al. 1998). It can also be performed directly from nonviable MTB or bacteria found in tissues in paraffin-embedded blocks or in archaeological samples.

2.1.2. MIRU-VNTR: This is a PCR-based method, analyses multiple loci containing variable numbers of tandem repeats (VNTR) of different families of interspersed genetic elements

collectively called Mycobacterial interspersed repetitive units (MIRU) (Supply et al, 1997; Frothingham & Connell, 1998; Supply et al, 2000; Supply et al, 2001). The method relies on the calculation of the number of repeats from the size of the PCR amplicon and the most used version of this method (MIRU-VNTR) is based on the analysis of 12 loci (Mazars et al, 2001; Supply et al, 2001). A standardized format proposed recently comprised of 24 loci, 15 of which constituted a discriminatory subset and were defined based on higher variability within the different clonal complexes studied. This new panel has been evaluated widely (Oelemann et al, 2007; Allix 2008) and permits the accurate and high-resolution analysis. The new tool is now available with an online multifunctional MIRU-VNTR plus database (Allix et al, 2008) for strain comparison.

2.1.3. Antibiotic susceptibility Testing:

Drug resistance in *Mycobacteria* is continually increasing world-wide and especially there is an upsurge of extremely drug resistant strains of MTB which is an immense threat to mankind. In view of this, the World Health Organization (WHO) has published a new definition of MDR (multidrug resistant) and XDR (extreme drug resistant) TB. According to it, strains showing resistance to at least rifampicin and isoniazid, the first line anti-TB drugs are called MDR strains whereas, XDR strain is one which is MDR and resistant to any fluoroquinolone, along with at least 1 of the following 3 injectable drugs used in anti-TB treatment: capreomycin, kanamycin and amikacin (http://www.who.int/tb/xdr/taskforcereport_oct06.pdf).

Since drug resistance in MTB is a result of point mutations in various genes it could be responsible for the various phenotypic characteristics of the pathogen. It is believed that drug- resistant status confers 'unfit' nature to strains (Von Groll et al, 2010) leading to the reduced ability of the intracellular growth due to mutation in essential genes of the pathogen.

However, few strains with specific genotypes can develop compensatory mutations to overcome this situation (Gagneux 2009).

Drug resistance, can be monitored broadly by two different ways, phenotypic and genotypic methods. Genotypic methods have the advantage of a shorter turnaround time, no need for growth of the organism, the possibility of direct application in clinical samples, lower biohazard risks, and the feasibility of automation. On the other hand, phenotypic methods are conventional ones, in general simpler to perform, but time consuming. There are three conventional phenotypic methods for drug susceptibility testing based on solid media: the proportion method, the resistance ratio method and the absolute concentration method (Canetti et al, 1963; Canetti et al, 1969). More recent methods are based on liquid media including the BACTEC radiometric and the Mycobacterial Growth Indicator Tube (MGIT) methods. Genotypic methods involve two basic steps: nucleic acid amplification such as by polymerase chain reaction (PCR) of the sections of the MTB genome known to be altered in resistant strains; and a second step of assessing the amplified products for specific mutations correlating with drug resistance (García de Viedma, 2003; Palomino, 2005). Rifampicin resistance is related to mutation in highly conserved 81-bp hot spot region in Rifampicin Resistance Determining Region (RRDR), affecting codons 507-533 (Somoskovi et al, 2001) in 90-98% of strains. Spontaneous mutations (deletions/ substitutions/ insertions) occurring in the *rpoβ* gene result in replacement of the aromatic amino acids with non-aromatic amino acids in the target RNA polymerase enzyme. This result in poor bonding between rifampicin and the RNA polymerase (Ramaswamy & Musser, 1998), and activity of the enzyme (transcription) is preserved, thus explaining resistance to rifampicin in bacteria. The Ser315Thr mutation in *katG* is found most often, occurring in approximately 40% of all isoniazid-resistant strains (Ramaswamy & Musser, 1998; Marttila et al, 1998; Zhang & Telenti, 2000). The Ser315Thr mutation results in a catalase enzyme that cannot activate

isoniazid, but retains approximately 50% of its catalase-peroxidase activity sufficient to enable the organism to detoxify host antibacterial radicals (Rouse et al, 1996). Mutations in the promoter region of a gene that encodes an alkyl hydroperoxidase reductase (*ahpC*) have been found in approximately 10% of isoniazid-resistant isolates, but mutations in *katG* were also found in these isolates (Goble et al, 1993; Drobniewski &Wilson, 1998). In the current study seven genes were analysed for first line drugs e.g. *ropB* for Rifampicin (R), *katG, inhA* and *ahpC* for Isoniazide (H); *rpsL* and *rrs* for Streptomycin (S); and *emb* for Ethambutol (E).

The proportion method as phenotypic indicator and mutation analysis for genotypic indicator was performed for the current study.

2.2. Materials & Methods:

2.2.1 Sputum samples & decontamination (Modified Petroff's method):

The clinical isolates used in the study were selected from sputum and tissue samples of TB patients. The samples were kindly provided by Department of Microbiology, KEM hospital and Tata Memorial Hospital Parel, Mumbai. Specimens like sputum might contain normal flora and therefore, were decontaminated and concentrated by centrifugation before inoculating culture media for the isolation of *Mycobacteria*.

For a given volume of the specimen in a screw capped test tube, 2 volumes of 4% sterile NaOH was added and the tube was placed in an incubator at 37°C for 30 minutes with continuous agitation for homogenizing the mixture. Sterile distilled water was then added to fill up the test tube and it was centrifuged at 4,000 rpm for 20 minutes. The supernatant was carefully discarded and the sediment was used to prepare a smear for acid fast staining and for inoculating LJ medium. The medium was incubated at 37°C and was checked for the appearance of growth every day for the first week and later every week for a period of 8

weeks. At the end of 8 weeks, if there was no growth, the medium was discarded and sample was marked as negative for mycobacterium culture.

2.2.2 Isolation of MTB strains from clinical samples

Thiophene-2-carboxylic acid hydrazide (TCH) readily distinguished human MTB strains from *M .bovis* and differentiate it from "atypical" *Mycobacteria* or *Mycobacteria* other than tubercle bacilli (MOTT). We selected strains that were grown on TCH containing Middlebrook medium, which inhibits *M.bovis* at 1 and 5 µg/ml concentrations. Nitrate reductase assay was performed as a confirmatory test of this pathogen. A loopful of log phase culture of MTB was inoculated in nitrate buffer and kept at 37^0 C for 2 hours. Two drops of nitrate reagent were then added to the bottle and red coloration confirmed the bacterial viability.

2.2.3. Extraction of genomic DNA from MTB culture isolates using CTAB method

Two hundred µl of MTB cell suspension (in PBS) was first mixed with 50 µl of lysozyme solution and incubated at 37°C for 90 min. Then 75µl of 10% SDS/proteinase K mixture was added to it. The suspension was vortexed and incubated at 65°C for 10 min. One hundred µl of 5M NaCl was added to the suspension, followed by 100 µl of CTAB/NaCl solution ('A' in Appendix I) which was prewarmed to 65°C. The tubes were vortexed till the liquid inside became milky white and then incubated at 65°C for 10 min. Next, 750 µl of CHISAM [Chloroform: isoamyl alcohol (24:1) mix] was added to the suspension, tubes were vortexed for 10 seconds and centrifuged at RT for 5 min at 12000 rpm. The supernatant was gently (without disturbing the interphase) transferred into a fresh microfuge tube and 0.6 volume (400 µl) isopropanol was added carefully to precipitate nucleic acids. The tubes were placed at -20°C for 30 min and centrifuged for 15 min at RT at 12000 rpm. Most of the supernatant was removed leaving behind 20 µl above the pellet. One ml of chilled 70%

ethanol was added and the tubes were then inverted slowly. After centrifuging, supernatant was discarded and pellet was washed again with absolute ethanol. Finally, the pellets were dried at RT for 10 min and dissolved in 50 µl of 1X TE ('A' in Appendix I). The extracted DNA was stored at 4°C and used for PCR.

Estimation of the DNA concentration using Nanodrop

All DNA samples were diluted in 1X TE. The absorbance was measured in Nanodrop instrument by using only 2µl of sample. Nucleic acids absorb light at a wavelength of 260nm. For double stranded DNA, an optical density (OD) of 1 at 260nm correlates to a DNA concentration of 50ng/µl, so DNA can be easily calculated from OD measurements.

2.2.4. Spoligotyping and data presentation

Spoligotyping was performed with DNA samples from clinical isolates according to the well established protocol. Briefly it consisted of three steps: PCR amplification of DR region, hybridization of PCR products with spoligo-blots and analysis of the results.

Amplification of the DR regions from MTB strains was the very first step of spoligotyping. PCR was performed using 10 ng of chromosomal DNA isolated from each strain. Amplification of the spacer was accomplished by using the primers DRa and DRb which enable to amplify the whole DR region. The details of the sequences of forward and reverse primers used for PCR and PCR master mix are described in Table-A in Appendix-II. After 3 min of hot-start at 96°C, the tubes were subjected to denaturation at 96°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 30 sec for 40 cycles. This was followed by the final step of extension at 72°C for 5 min.

PCR products (20µl) were mixed with 150 µl of 2X SSPE / 0.1% SDS and heat denatured for 10 min at 99°C in heating block and cooled on ice immediately. The spoligotyping membrane was washed for 5 min at 60°C in 250 ml 2X SSPE / 0.1% SDS. The

membrane was placed on support cushion in miniblotter, so that the slots were perpendicular to the line pattern of the spacer-oligonucleotide already immobilized on the membrane. The residual fluid was removed from the slots of the miniblotter by aspiration. In the next step, the heat denatured PCR products were filled in the slots, with care to avoid air bubbles and were hybridized for 60 min at 60°C on a flat horizontal surface of hybridization oven (Amersham). After that, the samples were removed from the miniblotter by aspiration and the membrane was taken out from minoblotter using forceps. Then it was washed twice in 250 ml 2X SSPE / 0.5% SDS for 10 min at 60°C and cooled down. The membrane was incubated in 10 ml of 1:4000 diluted streptravidin-POD conjugate (2.5 µl of streptavidin-POD conjugate (500 v/ml) to 10 ml of 2X SSPE / 0.5% SDS) for 45 min at 42°C. Finally, after washing the membrane, it was incubated with 10 ml of ECL detection fluid for 1 min and it was covered with a saran wrap and was exposed to hyper film ECL for 20 min to 30 min. The membrane was reused after washing with1% SDS at 80°C for 30 minutes and kept in 20 mM EDTA pH 8 at 4^{o}C. The buffer compositions are given in part 'B' in Appendix I.

To simplify recording, the band pattern was converted to a series of 1s and 0s (means presence and absence of signal respectively). Since there are only 1s and 0s in the number, this is called a binary code. To simplify this even further, the 43-digit binary code was converted to a 15-digit octal (i.e., base 8, having the digits 0-7) designation by a two-step process. First, the 43-digit binary code was divided into 14 sets of three digits (spacers 1 through 42) plus one additional digit (spacer 43). Second, each 3-digit binary set is converted to its octal equivalent, with the final additional digit remaining as 1 or 0. The translation of binary numbers to octal numbers was done as follows: 000 = 0; 001 = 1; 010 = 2; 011 = 3; 100 = 4; 101 = 5; 110 = 6; 111 = 7. Each octal designation is unique, representing one specific banding pattern. From an octal designation, the binary code of the spoligotyping pattern can be re-created. The hybridization signal was analyzed on the basis of international

spoligotyping database (SpolDB4) which has been created and maintained at the Institute Pasteur de Guadeloup (IPG) (Brudey et al, 2006).

2.2.5 Amplification in TbD-1 and RD-1 region

The presence of TbD-1 and RD1 regions in the selected strains was confirmed by gene specific PCR as described earlier (Brosch et al, 2002). The primer sequence and reaction conditions are described in Tables D and E in Appendix-II.

2.2.6. MIRU-VNTR analysis

Genomic DNAs of MTB clinical isolates, extracted by CTAB method were used in this study (Supply et al, 2001). Polymerase Chain Reaction was carried out to amplify 12 MIRU Loci of three MTB clinical strains with the primers listed in Table C in Appendix II and PCR master mix was prepared as described in Table B in Appendix-II. The PCR thermal conditions were as follows: initial denaturation for 15 min at 95°C followed by 40 cycles of 95˚C for 1min, 59˚C for 1min and 72˚C for 1.5 min. Then, the PCR products were run in 2.5% agarose gel and PCR amplicon size of different loci was determined with respect to DNA ladders.

2.2.7 Drug sensitivity testing of the clinical isolates by Proportion Method

At first, 3-4 loopful moist bacteria were taken in sterile distilled water and were vortexed vigorously to produce a uniform suspension. This suspension was kept on the bench for 15-20 minutes to allow the coarser particles to settle down. Then a 10-fold dilution was made from the suspension, by carefully adding sterile distilled water to it (S1, 10^{-1}). Two further serial dilutions 10^{-2}(S2) and 10^{-3} (S3) were prepared in a similar manner. Finally, one loopful of the suspension from each dilution was cultured on drug free and drug containing (concentration described in 'C' Appendix-I) LJ slopes.

2.2.8 Detection of point mutation by sequencing

The Genomic DNA of each clinical isolate was diluted to 10 ng/10 µl and amplified in PCR for seven gene targets (*rpoB, KatG, rps, rrs, inhA, ahpC, emb*). The primer sequences and PCR conditions were listed in Tables D and E in Appendix-II. The PCR products were purified by ammonium acetate/ ethanol method. By keeping ethanol concentration relatively low and temperature high (room temperature), precipitation of the short DNA fragments was prevented.

In 25 µl of PCR product 11 µl 8M NH_4Ac and 37.5 µl 95% ethanol (room temperature) were added and mixed properly with gentle shake in a vortex. Then the tubes were kept at room temperature for 15 minutes. After that, the mixture was centrifuged at 3300 rpm for 30 minutes. The caps were removed and the tubes were put upside down on a paper towel to remove the ethanol. Again, 50 µl of 70% ice-cold ethanol was added and after 2-3 minutes, spun for 1 min at 3000 rpm. The solution was removed gently and 25 µl of ddH_2O added and mixed gently. The purified PCR products were sent for automated sequencing with their respective primers.

2.3 Results:

2.3.1 Strain Selection

MTB clinical isolates were obtained either from hospitals directly, as isolated cultures or were isolated from clinical samples in the lab. Forty (40) samples were collected and 9 of them were already in a LJ slant, known to have resistance to at least one first line anti-TB drugs. Thirty one (31) sputum samples were decontaminated for further biochemical tests. All the samples were inoculated in TCH containing medium and nitrate reductase buffer (Figure 4) to confirm the strain as MTB. Only two samples didn't show positive results in these two assays, hence 38 samples were selected for further characterization.

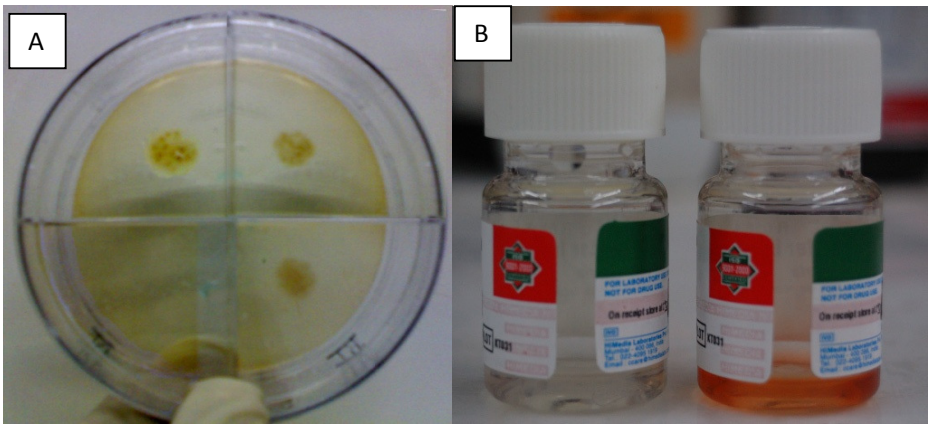


Figure 4 : Isolation of MTB

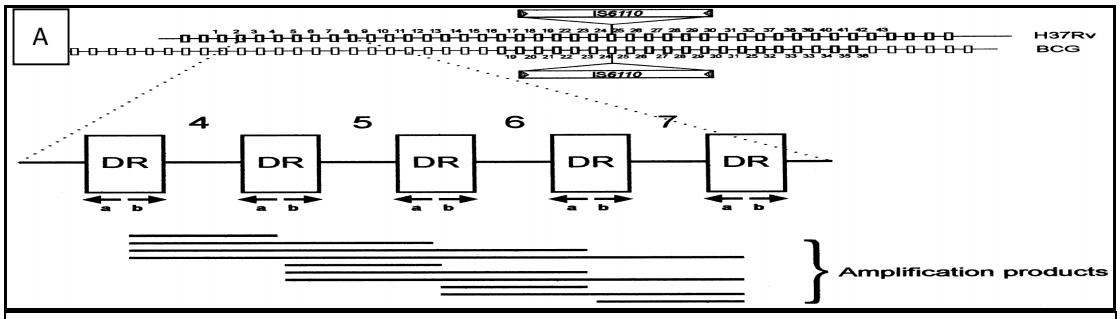
A. TCH test: To distinguish between MTB from other *Mycobacteria* this test was performed. In quadrants I to III TCH concentration was 0 µg/ml, 1µg/ml and 5µg/ml respectively and in quadrant IV was un-inoculated control. The growth of inoculums in the first three quadrants after 10 days, confirms it as MTB. *M. bovis* cannot grow in 0-10µg/ml of TCH containing medium. A representative photograph (left) is given above.
B. Nitrate reduction test: The ability of MTB to reduce nitrate is a confirmatory test. All the MTB clinical isolates showed red colour in presence of nitrate buffer and reagent (HIMEDIA). A representative photograph for the test is shown in the Figure above (right hand panel)

2.3.2 Spoligotyping of the strains

All the 38 MTB strains were spoligotyped (Figure 5) along with H37Rv and *M.bovis* BCG as a kit control. The octal spoligo codes were generated from the original binary film and the strains were identified. Among all the isolates, 26% (n=10) were Beijing strains, whereas LAM, S strains were 3% (single copy) (Figure 6). EAI, U, CAS, T and H strains also showed the representation. Total 12 single copy unique isolates were present among the strains. As the aim of the present study was to evaluate the immune interactions of the drug resistant MTB strains three drug resistant strains representing three different lineages viz: Myc431(Beijing); 4798A(LAM-6) and 5108R (EAI-5) were selected from the 9 drug resistant strains.

2.3.3 TbD-1, RD1 and MIRU-VNTR typing of the selected strains

The presence of TbD-1 and RD1 regions in the selected strains was confirmed by gene specific PCR (Figure 7). EAI-5 strain showed a 2.5kb TbD-1 region confirming its ancestral origin. On the other hand, all the three strains under study possessed intact RD1 region which confirmed their virulent nature. The 12 locus MIRU-VNTR analysis performed for the strains for their further classification is shown in Figure 8.



B

Samples	Spoligo Code	Strain Type
4798A	777777607560771	LAM-6
449	777777777760771	U
442	000000000003771	Beijing
Myc 431	000000000003771	Beijing
424	000000000003771	Beijing
414	000000000003771	Beijing
449	000000000003771	Beijing
5108R	777700777413700	EAI-5
379	702777740003771	CAS1 Delhi
364	743600000000000	Unique
373	777777777760771	T1
474	477777777720771	H3
404	777777777760771	T1
429	777777676000000	U like
H37	777747777760660	Unique
H15	000000000003771	Beijing
H425	000000000003771	Beijing
H428	741777740003771	Unique
H45	774377777413771	Unique
H24	000000000003771	Beijing
KEM 44	777746000003771	Unique
H58	777747777413661	Unique
204C	776377777760771	S
316A	400177777413071	EAI-3
343R	400177777413071	EAI-3
3497R	557767777720771	H3
SHAMULLA	000000000003771	Beijing
360K	557347437773771	Unique
901B	763777760003771	Unique
1691B	777746000003771	Unique
1804B	757747077760661	Unique
1785A	557747637600661	Unique
1801A	557000000003061	U
1774A	500000000000061	Unique
SANTOSH	777777777760761	T1
RAMBORI	000000000003771	Beijing
JOE	700377740003771	CAS2
370K	703777600001771	CAS
H37Rv	777767477760771	H
BCG	676777677777600	Bovis

Figure 5: Spoligotyping of MTB clinical isolates

A. Spoligotyping ('spacer-oligonucleotide-typing'), is based on the structure of the direct repeat (DR) locus. The DR locus is made up of a variable number of 36 bp repeats that are separated by unique inter-DR sequences of 35 to 41 bp. The primers of DRa and DRb regions amplify differential length of PCR amplicons which is hybridized with respective unique spacer sequence in a line blot.

B. *Mycobacteria*l DNA samples were spoligotyped and their spoligo patterns are presented in the table along with their spoligo code and strain type. Strain types were assigned as described in the international spoligotype database SpolDB4.

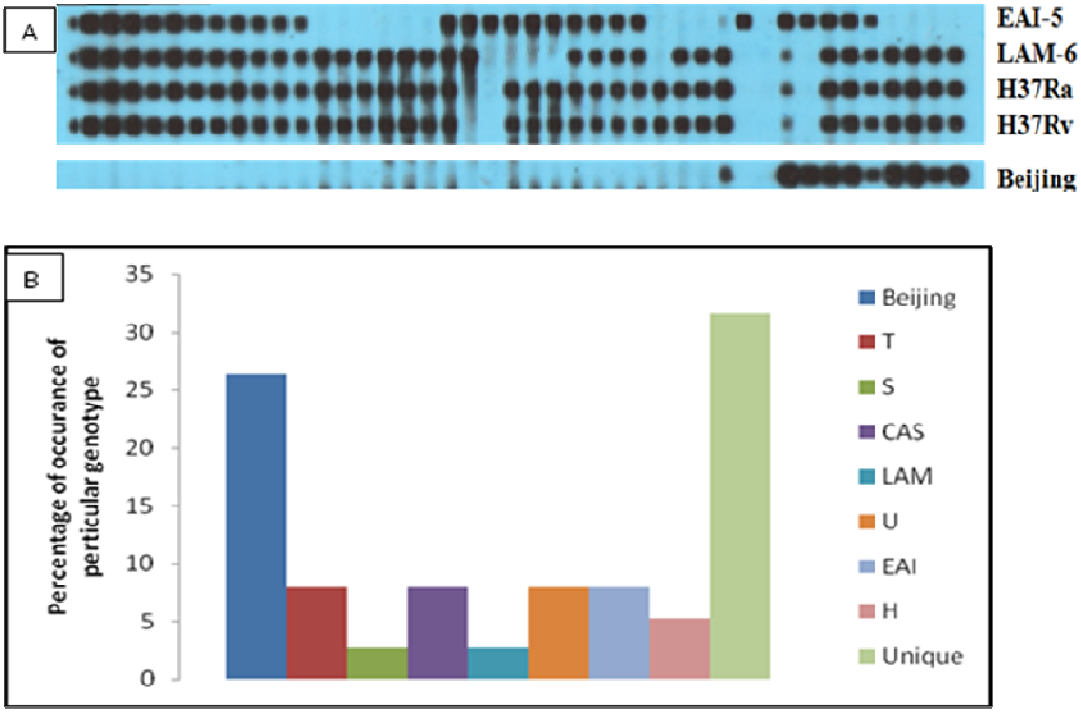


Figure 6: Percentage of different genotypes in clinical isolates of MTB

A. Percentage of different genotype of MTB among the clinical isolates used in this study (top).
B. Original X ray film showing the spoligo patterns of selected different strains are shown here. Spoligotype families as assigned as in the international spoligotype database SpolDB4.

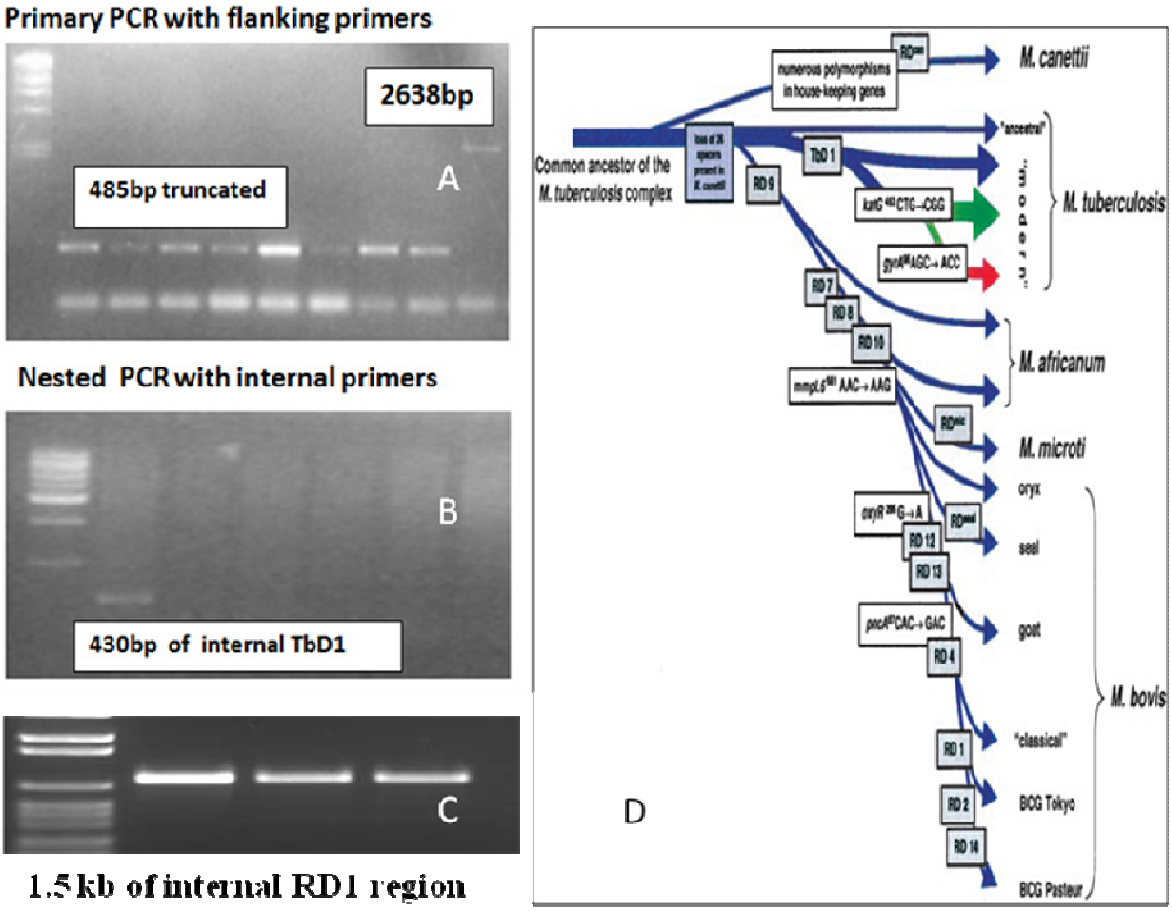


Figure 7: TbD-1 and RD1 PCR for clinical isolates

A. & B. TbD1 region consist of two genes *mmpS6* and *mmpL6*; in modern *M.tuberculosis* strains there is deletion of 2.1kb which means complete deletion of gene *mmpS6* and truncation of gene *mmpL6*. In TbD1 flank PCR, the ancestral strain gave an amplicon of 2638bp and for TbD1 internal PCR, an amplicon of 430bp. In modern strain because of TbD1 deletion when TbD1 flank PCR was done a 485bp amplicon was obtained and no amplification was seen with TbD1 internal PCR since the internal sequence is deleted.

C. RD1 amplicons obtained from strains that have the genomic region present or deleted. Sizes of amplicons in selected strains are same.

D. Scheme of the proposed evolutionary pathway of the tubercle bacilli illustrating successive loss of DNA in certain lineages (gray boxes). The scheme is based on the presence or absence of conserved deleted regions and on sequence polymorphisms in five selected genes. Blue arrows indicate that strains are characterized by katG463. CTG (Leu), gyrA95 ACC (Thr), typical for group 1 organisms. Green arrows indicate that strains belong to group 2 characterized by katG463 CGG (Arg), gyrA95 ACC (Thr).The red arrow indicates that strains belong to group 3, characterized by katG463 CGG (Arg), gyrA95 AGC (Ser), as defined by Sreevatsan et al. (1997) .

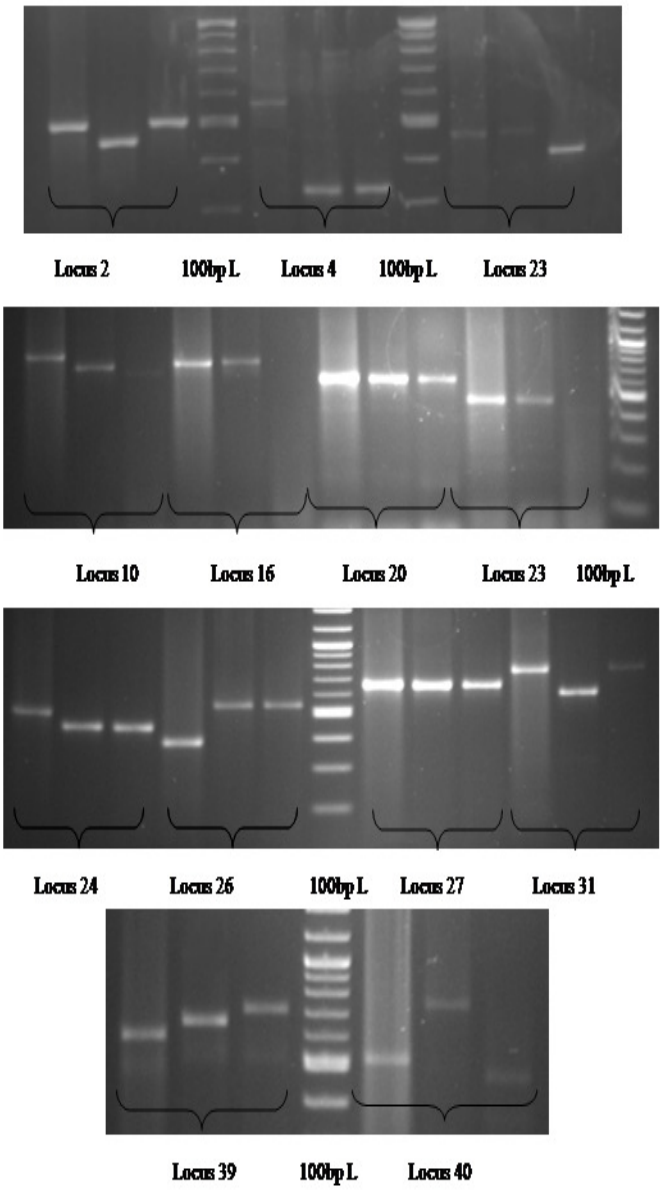


Table -1: MIRU-VNTR for three clinical isolates

Locus	No. of Alleles or repetition in VNTR locus		
	EAI-5	LAM-6	Beijing
2	2	1	2
4	5	2	2
10	4	3	2
16	3	3	3
20	2	2	2
23	6	6	5
24	2	1	1
26	2	5	5
27	3	3	3
31	5	3	5
39	1	2	3
40	3	8	3

Figure 8: MIRU-VNTR analysis: The MIRU-VNTR analysis was performed with three selected strains. Agarose gel (2.5%) electrophoresis of the PCR amplicons for 12 loci is displayed. The amplicon sizes confirm the repetitions of different loci present in the genome of the respective bacteria, which are shown in the table 1.

2.3.4 Antibiotic susceptibility test

When tested for resistance against 13 drugs including first line as well as second line drugs, clinical isolates Beijing, LAM and EAI were found to be resistant to 10, 6 and 8 drugs respectively as stated in Table 2. The phenotypic susceptibility of the strains was compared with the genotypes of the strains by sequencing (Table 3) and it was observed that in spite of being rifampicin resistant, LAM strain did not show any point mutations in *rpoB* gene (Figure 9). Interestingly, Beijing also did not show mutation in *katG, inhA* and *ahpC* genes which are responsible for isoniazide resistance.

Table 2: Antibiotic susceptibility test

The strains were sub-cultured and the drug susceptibility testing was performed using standard proportion method for 13 drugs which included first line and second line drugs . None of the selected clinical isolates were found to be sensitive to all four first-line anti-TB drugs (isoniazid, rifampicin, streptomycin and ethambutol).

	Resistant	**Sensitive**
EAI5	**Ethambutol, Streptomycin, Isoniazide, Pyrazinzmide, Cycloserin, Ethionamide, Kanamycin, Clarithromycin**	**Rifampicin, Ciprofloxacin,Amikacin,P-Amino salicylic acid, Rifabutin**
LAM6	**Rifampicin, Ethambutol, Streptomycin, Kanamycin, Pyrazinzmide,, Ethionamide**	**Isoniazide, Rifampicin, Clarithromycin, Ciprofloxacin, Cycloserine, Amikacin, PAS, Rifabutin**
Beijing	**Ethambutol, Streptomycin, Isoniazide, Pyrazinzmide, Rifampicin, Cycloserin, Ethionamide, Kanamycin, Clarithromycin, Rifabutin**	**A mikacin, P-Amino salicylic acid, Ciprofloxacin**

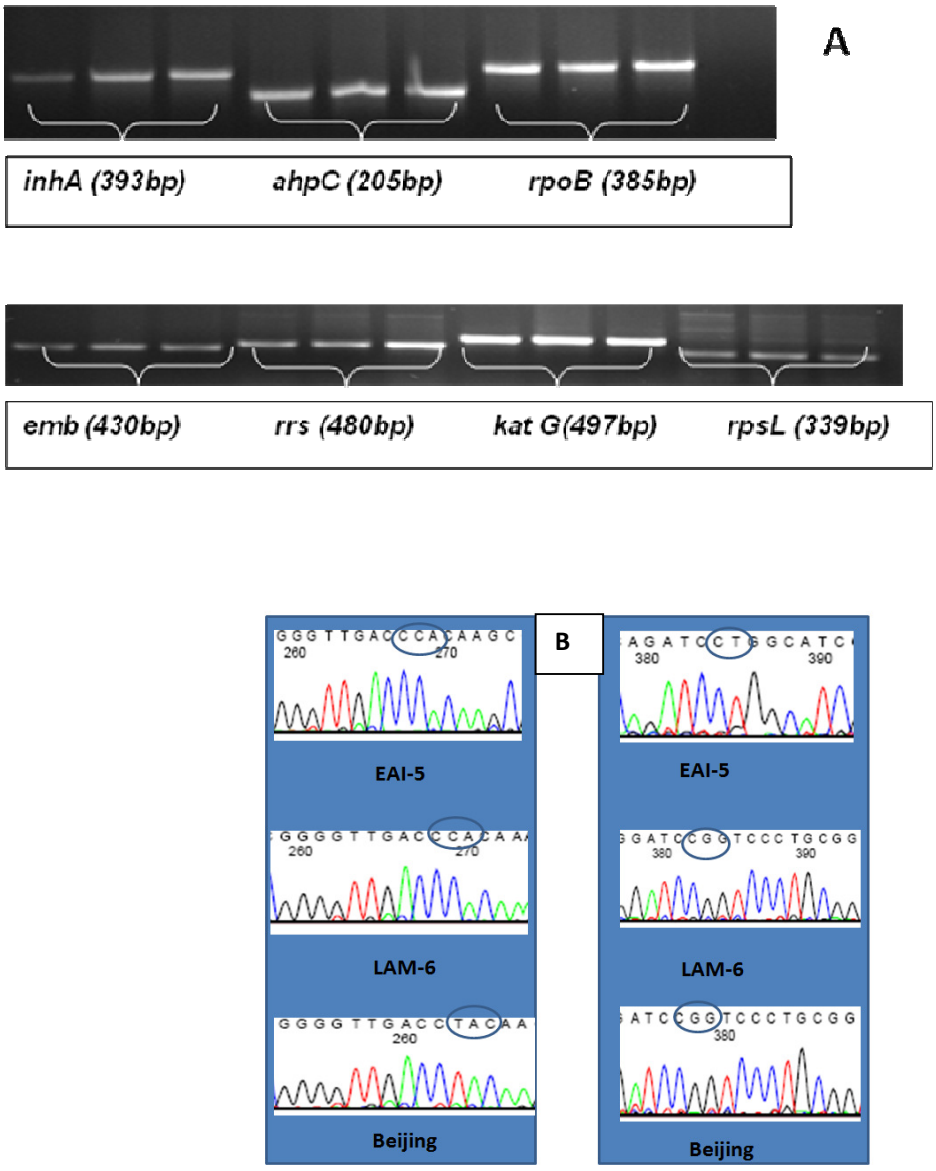


Figure 9: Amplification and sequencing of seven target genes for the detection of point mutations for the first line drugs

- **A.** The amplicons obtained for all seven targets :*rpoB, katG, ahpC, inhA, rps, rrs* (16SrRNA) and *emb* are shown in the figure A .
- **B.** Representative photograph of point mutation obtained for three different strains in *rpoB* gene in position 526 and *katG* gene in position 463.

Table 3 : Point mutation analysis forall seven genes of three clinical isolates:

In this table, 'wt' represents wild type sequence and others are mutant;
+ indicates'sequences matches' and – describes 'sequence does not matches' with the codon sequence described in the left column.

Codon Sequence	**EAI-5**	**LAM-6**	**Beijing**
rpoB 509-534 wt	+	+	-
rpoB 526 TAC	-	-	+
rpoB 526 GAC	-	-	-
ahpC wt (-45...-23)	+	+	+
rrs 491 wt	+	+	+
ahpC-wt2 (-20…-1)	+	+	+
rrs 513 wt	+	+	+
rpsL 88 wt (AAG)	+	+	+
inhA 1 wt (orf-inhA: -15-T)	+	+	-
embB 306 wt (ATG)	+	+	+
katG 2 wt (katG 463 CGG-Arg)	-	+	+
embB 306 CTG	-	-	-
katG 1m (katG 315 ACC-Thr)	-	-	-
embB 306 GTG	-	-	-
katG 2m (katG 463 CTG-Leu)	+	-	-
embB 306 ATA	-	-	-

2.4. Discussion:

A major difficulty in relating the genotypic diversity of the MTB strains with their phenotypic responses has been the lack of appropriate tools to index different clinical isolates. Therefore, the primary objective of the study was to characterize clinical isolates of MTB with different molecular biology techniques to obtain maximum genotypic information.

Initially after confirming the isolates from sputum samples as MTB strains, by TCH and nitrite assay, spoligotyping was performed to determine their lineage. Out of 38 clinical isolates, three isolates from three different major lineages were selected. LAM-6 is a part of the Euro-American lineage of MTB (Gagneux et al, 2006) which is described as the most successful group of strains of MTB in the world, with the LAM family alone believed to cause about 15% of TB worldwide (Gibson et al, 2008). These strains shared a spoligo pattern lacking spacers 23, 24, and 25 in addition to other common molecular characteristics and were designated the "Cameroon family" (Niobe-Eyangoh et al, 2004). The international spoligotyping database showed that the strain occurred 172 times among 1939 shared- type strains worldwide, mostly in West African countries and other regions of South Africa (Chihota et al, 2007). LAM is less prevalent in East African countries. In Tanzania, LAM and East-African Indian (EAI) families of strains were estimated to be about 22% and 17% respectively, while in Kenya and Uganda the only study on record showed that LAM strains were 11% and 6.7% respectively (Asiimwe et al, 2008). LAM is also less predominant in the Indian population (Figure 10).

The most prevalent strain in south India is East-African-Indian (EAI) strain, which belongs to Lineage 1, Indo-Oceanic lineage. Among the 38 strains, 3 strains were from this lineage and the drug resistant ones were chosen. The earlier study showed that the ancestral East African Indian (EAI) family, belonging to the MGG 1, represented one sixth (17%) of total isolates from Mumbai. The EAI family is also highly prevalent in Far-East-Asia

(33.8%), the Middle East (24.3%) and Central Asia and Oceania (22.9%). The EAI lineage is also more prevalent in South-East Asia, particularly in Myanmar (53%), Malaysia (Phyu et al, 2003), Vietnam and in Thailand (32%; Brudey et al, 2006). It is also believed that the EAI ancestral strains spread back from Asia to Africa through India concomitantly with human migrations (Mokrousov et al, 2005).

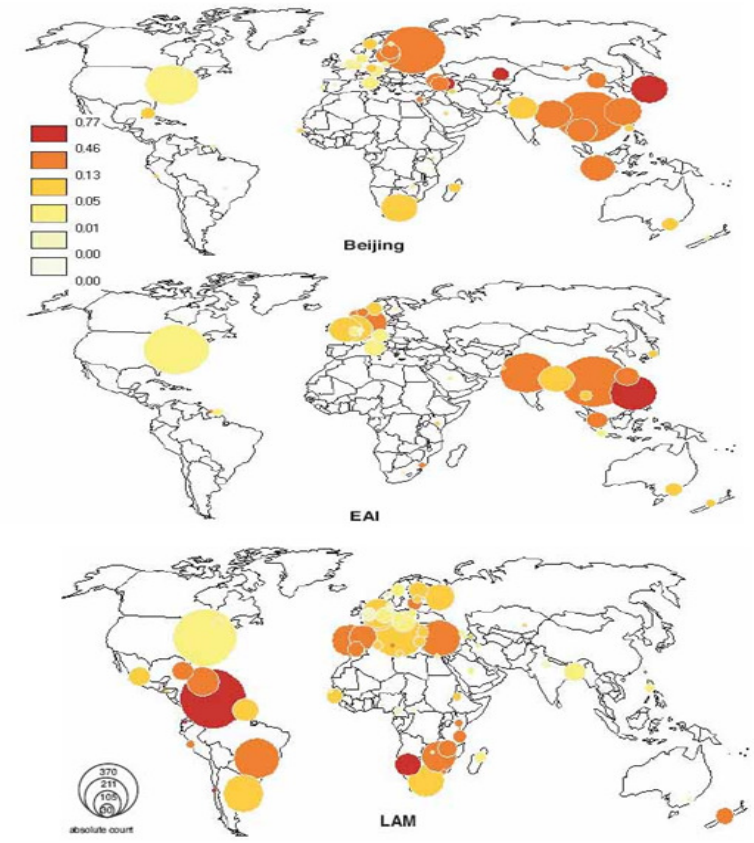


Figure 10: Occurrence of selected MTB strains in the World.
World map showing absolute (diameter) and percentage (colour) numbers of MTB strains within each country (Brudey et al, 2006). World Maps showing absolute (diameter) and percentage (colour) numbers of 3 genotype families within each country: Beijing; EAI (East-African Indian) and LAM (Latin-American and Mediterranean). These maps were built on an updated SpolDB4 on 2005 September 14th, on clusters of the 50 most frequent shared types (Beijing n = 4042, EAI n = 1684, LAM n = 3400). Maps were built using Philcarto (P. Waniez, version 4.38).
Source: http://www.biomedcentral.com/content/pdf/1471-2180-6-23.pdf

The MTB Beijing strains are widely known for their frequent appearance in outbreaks and association with drug resistance, suggesting that Beijing strains have enhanced fitness and the potential to spread. All Beijing-genotype strains share a number of independent genetic markers (Bifani et al, 2002). The spoligotype pattern for the Beijing family involves hybridization to a 9 spacer region between spacers 35 and 43 and absence of 1 to 34 at a stretch (Kremer et al, 2005). The Beijing genotype may represent a higher level of evolutionary development of MTB, indicative of a more recent common ancestor (Kremer et al, 2005). The Beijing family of strains is prevalent in Far-East-Asia, but also in Middle-East, Central Asia and Oceania (45.9%, 16.5% and 17.2% respectively) (Figure-11). The Beijing genotype may have been endemic in China for a long time (Qian et al, 1999), and emerging in some parts of the world, especially in countries of the former Soviet Union, and to a lesser extent in the Western world (Glynn et al, 2002). However, outbreaks in USA and France (Munsiff et al, 2003; Golesi et al, 2012) indicate that among all the strains, Beijing spreads much faster and is going to be a major threat to future world.

Recent developments in comparative genomics revealed the presence or absence of regions of differences (RDs) and tuberculosis-specific deletion 1 (TbD1) as potential markers for understanding the historical origins and genealogy of the present day *Mycobacteria*l pathogens. It is known from earlier studies that RD1 genomic region is present in virulent strains of MTB, and is missing from the vaccine strain *M. bovis* BCG, and its importance has been proved experimentally. Based on *in silico* analysis, it has been suggested that RD1 may encode a novel secretion system, but the mechanism by which this region affects virulence is unknown. Deletion of the 9.5 kb RD1 region from MTB results in attenuation similar to that of BCG in cultured macrophages and mice (Guinn et al, 2004). In another study, disruption of the RD1-encoded *ESAT-6* of *M. bovis* produced an attenuated phenotype in guinea pigs (Wards et al, 2000). Finally, on introduction of RD1 into BCG, the RD1-restored strain

persisted longer in immunocompetent mice and grew to higher numbers in SCID mice than the BCG Pasteur parent (Pym *et al*., 2002), though virulence was much reduced relative to MTB. In contrast to earlier reports, Rao *et al*, (2006) demonstrated that the deletion of RD1 does not correlate with a decrease in the virulence potential of MTB. Indian isolates (n = 30) examined by them were from diseased individuals and yet had lost the RD1 region. However, the extent of RD1 deletion in strains of other regions was comparatively less. Forty percent of Angolan isolates, 21% of Libyan and 2.6% of Peruvian isolates had deletions of RD1. All the clinical isolates obtained by us had intact RD1 region which showed their virulent nature undoubtedly.

In the course of evolution there have been many deletions in MTB genome leading to a variety of different species having different characters. Brosch et al (2002) have proposed an evolutionary tree for MTB complex, based on TbD1 deletion analysis. This region is intact in all other species of MTB complex except in some of MTB strains. Therefore, this deletion is named as Tuberculosis specific deletion. There are some strains of MTB which possess intact TbD1 region which belong to PGG group 1 (Sreevatsan et al, 1997) and are considered as ancestral strains and those strains in which there is TbD1 deletion belong to PGG group 2 and group 3, are considered as modern strains. TbD1 region consist of two genes *mmpS6* and *mmpL6*. In modern *M.tuberculosis* strains there is deletion of 2.1kb which means complete deletion of gene *mmpS6* and truncation of gene *mmpL6*. Based on TbD1 region, EAI-5 strain is an ancient strain. The presence of ancestral strains has been found to be 80% in South India (Gutierrez et al, 2006) and EAI strain represents one of them. LAM-6 and Beijing are modern strains.

Our clinical Isolates were screened for Multiple locus VNTR analysis using all "12 locus MIRU". This rapid and highly reproducible method clearly distinguished the majority of the isolates of Beijing, EAI, and Haarlem families from each other. The Beijing family

appeared the most homogenous family, which is consistent with the results obtained with *IS6110* RFLP and other markers. In addition, MIRU-VNTRs distinguished all strains except for two *M. bovis* BCG vaccine sister strains, in contrast to *IS6110* RFLP. In the present study, analysis of 12 loci MIRU-VNTR helped construct a dendrogram using NJ algorithim (Figure 11), in order to know the position of the selected strains among all the known well typed strains of similar kind. MIRU-VNTR analysis supports polyphasic typing by calculating a combined distance for different genotyping data. It allows the selection of the used genotyping methods, distance measures and weightings. The comparison with the reference database allows the identification of the phylogenetic lineage of new strains.

In the earlier studies, several attempts were made to correlate MIRU locus repetition unit with lineage of MTB strains. According to Rao *et al*, (2006), 7 repeats at MIRU Locus 26 is an identification signature for Beijing strain. However, some results contradicted these findings. Koksalan et al (2006) showed that in some geographic regions, such as the countries of the former USSR, Beijing genotype strains carried fewer than seven MIRU copies at locus 26 and are in striking predominance over strains with seven copies (Koksalan et al, 2006). In the present study, the Beijing strain posses only 5 copies instead of 7.

The drug resistant pattern obtained by proportion method depicted EAI-5 and LAM strain as resistant to eight and six drugs respectively whereas, Beijing strain was conventional XDR with resistance to ten drugs. It is well known that successful transmission of specific mutations, such as Ser315Thr in *katG* and Ser531Leu in *rpoB*, is associated with drug resistance status of MTB in several populations (Marttila et al, 2000). To confirm whether the drug resistant status of selected strains was due to a common mutation present in specific genes or not, sequencing of specific regions, mentioned in Table 2 for all the three strains was performed for first line drugs. EAI and LAM were resistant to isoniazide and rifampicin respectively, whereas Beijing was resistant to both the drugs. However, for LAM

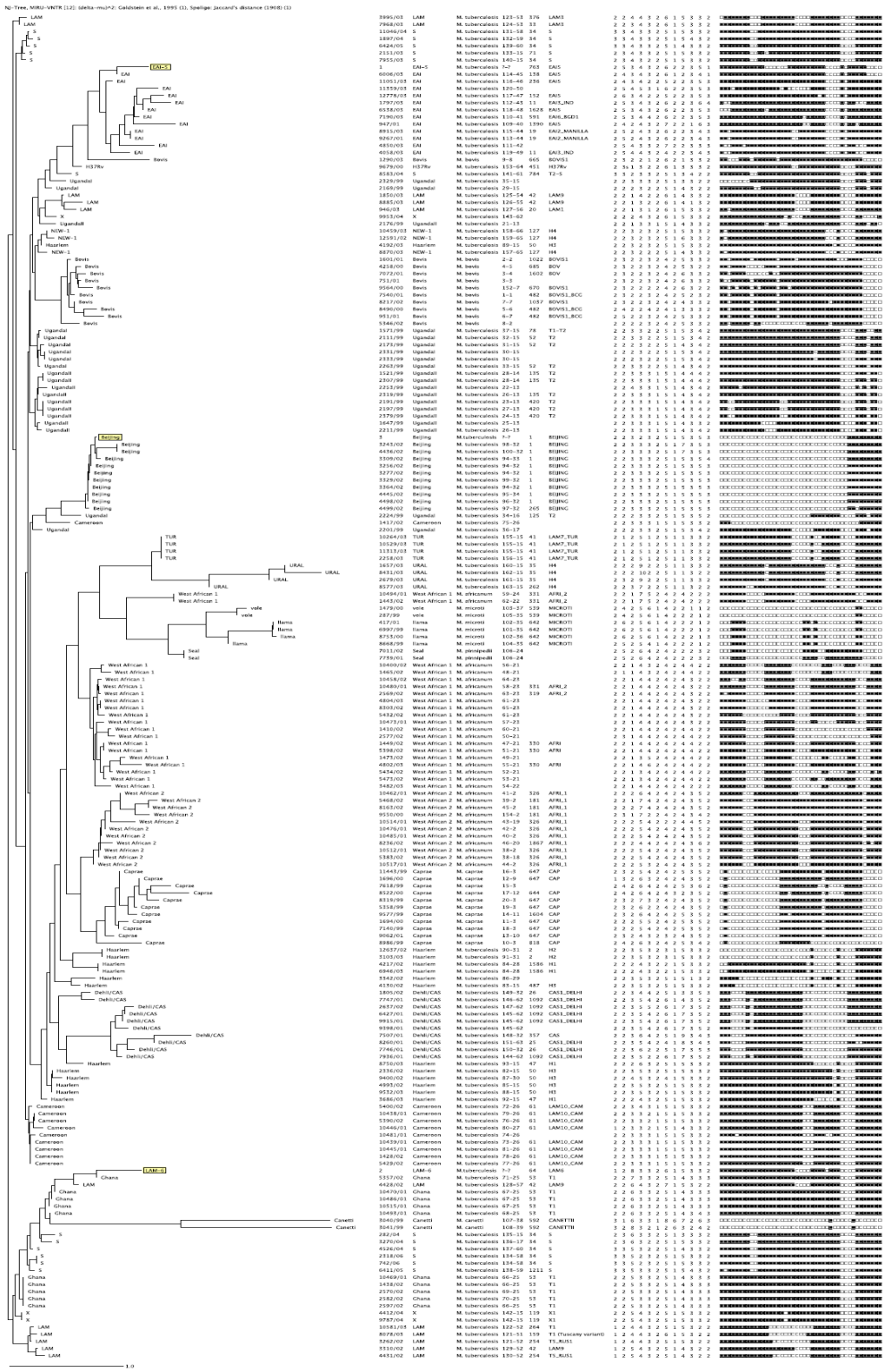


Figure 11: Neighbour-joining tree, based on 12 loci MIRU-VNTR typing and 43 spacer spoligotyping showing the phylogenetic relationship of strains used in the present study along with186 reference strains of MTB complex.

Table 4: Genotypes of M. *tuberculosis* strains used in the study:

Clades	Lineage	Spoligo code	SIT*	MIRU-VNTR #	TbD-1	RD1	Origin
EAI5	1- Indo-Oceanic	777700777413700	763	254326223513	+	+	Pulmonary
LAM6	4-Euro-American	777777607560771	64	123326153328	-	+	Pulmonary
Beijing	2- East Asian	000000000003771	1	222325153533	-	+	Extra-pulmonary

SIT = Spoligo-International-Type number
# 12 loci in the order = M2, M4, M10, M16, M20, M23, M24, M26, M27, M31, M39, M40

and Beijing strain, the phenotypes were not supported by the mutation in *rpoB* and *katG* codons as expected. A number of researchers have hypothesized different reasons for resistance in strains lacking mutations in respective genes, such as diminished antibiotic entry into bacteria due to changed membrane permeability (Abadi et al, 1996) or enzymatic degradation or modification of drug (Hetherington et al, 1995) and presence of certain kinds of mutations elsewhere in the gene e.g. at N-terminal part of *rpoB* (Heep et al, 2001). It is also possible that alteration of efflux pumps (Piddock, 2006) may protect the drugs from getting entry into the pathogen. Lastly, the possibility of involvement of other genes in drug resistance cannot be ignored (Tribuddharat & Fennewald, 1999).

Thus, three drug resistant clinical isolates from three different major MTB lineages were selected and well characterized by various molecular typing methods (Table 4). In the next set of experiments, the interactions of these different strains with different host cells were analysed to decipher their strategy of pathogenesis.

Chapter 3: Evaluation of various innate host responses induced by clinical isolates of *Mycobacterium tuberculosis* from different genotypes

3.1 Introduction:

Mycobacterium tuberculosis is one of the best examples of a pathogen which coevolved successfully with humans. It remains silent in 90% of the infection cases without causing overt disease (Bhowruth et al, 2008). Strain-to-strain variation of the pathogen may have impact on the molecular interactions with the host which dictate the overall pathogenesis of the infection and can have important consequences to future diagnostics and design of drugs and vaccines and patient management. Multiple factors regulate the course and outcome of infection by MTB and involve a complex interplay between the immune system of the host and survival strategies employed by the bacilli (Mischenko et al, 2004).

The bacterial cell wall composition differs with the genotype of the strains (Domenech & Reed, 2009) which is believed to be responsible for the initial recognition by the host (Torrelles et al, 2006). Virulent strains of MTB are phagocytosed specifically via mannose receptor whereas phagocytosis of avirulent strain H37Ra is mediated only through complement receptor which results in higher accumulation of virulent bacteria inside the host compared to avirulent one (Schlesinger,1993). On the contrary, one study has shown that, avirulent strain gets accumulated in higher number inside the host compared to virulent strain (Rajavelu & Das, 2007).

Intracellular bacterial growth is mostly influenced by induction of reactive oxygen and nitrogen species (Nozaki et al, 1997) and cytokines (Powrie & Coffman, 1993; Bekker et al, 2001). Human macrophages, both from primary cultures and from transformed monocytic cell lines such as THP-1, serve as a model host for early stages of infection and have been used to measure intracellular growth rates of MTB isolates (Manca et al, 1999; Zhang et al, 1999; Li Q et al, 2002, Theus et al, 2005). It was observed that isolates of MTB that caused

higher incidences of TB cases in central Los Angeles grew more rapidly in human macrophages than the strains that caused disease in only one patient (Zhang et al, 1999). This observation was confirmed in a separate study using activated THP-1 cells (Theus et al, 2005). Thus, these studies established that extensive spread of a particular MTB strain correlates with its capacity to replicate rapidly in human macrophages. Virulent strain H37Rv grew faster in human monocyte derived macrophage and mouse models compared to avirulent strain H37Ra (Zhang et al 1998; Jung et al, 2002). However, a study showed that avirulent H37Ra and virulent H37Rv grew at similar rates in human monocyte-derived macrophages, whereas H37Rv grew more rapidly in murine macrophages (Paul et al, 1996). Though sometimes contradictory, these findings underscore the importance of intracellular growth as a determinant of pathogenesis.

The genetically different MTB strains from different lineages have been shown to induce differential immune responses in macrophages, cell lines and mouse models (Manca et al, 2004; Chacón Salinas et al, 2005; Thus et al, 2005; Sinsimer et al, 2008; Tanveer et al, 2009; Portevin et al, 2011) and are demonstrated to vary with respect to their virulence, pathology and intracellular bacterial load (Marquina-Castillo et al, 2009). Maximum number of studies on innate immune responses has been performed with Lineage -2 (Beijing) strains so far, as they caused disease outbreak in different places. Further, it was reported that CAS1 and Beijing strains, belonging to lineage 2, induce lower levels of proinflammatory cytokines in THP-1 cells (Tanveer et al, 2009) as well as macrophages compared to that induced by standard laboratory strain H37Rv (Sohn et al, 2009; Portevin et al, 2011). A recent study has demonstrated that ancient Beijing strains (lineage 2) from Brazil (characterized by the absence of an insertion sequence 6110 within the noise transfer function region) induced similar levels of TNF-α and IL-10 as H37Rv whilst modern MDR Beijing strains (lineage 2) isolated in Russia, induced low TNF-α and high IL-10 in the THP-1 macrophage cell line

(Lasunskaia et al, 2010). In contrast, another study has found that Beijing strains (lineage 2), irrespective of subfamily, showed an immune phenotype of low level of TNF-α, IL-6, IL-10 and GRO-a (Portevin et al, 2011) production as compared to H37Rv and other genotypes of MTB in human macrophages. Despite these discrepancies, most of the studies confirmed the lower induction of cytokines by Beijing genotype. In contrast to Lineage-2, ancient strains from Lineage-1 repeatedly showed higher induction of pro-inflammatory cytokines in host cells (Portevin et al, 2011). H37Rv/Ra, Erdman and CDC1551 belong to Lineage 4 (Euro-American) which shows higher proinflammatory response compared to Lineage-2 strains (Manca et al, 1999; Bosio et al, 2000).

Apoptosis of the infected macrophages is another important innate immune response, increasingly understood to play a role in the host defence against MTB (Keane et al, 1997; Oddo et al 1998). Apoptotic *Mycobacterium avium* -infected macrophages were shown to be ingested by healthy macrophages that ultimately kill the intracellular bacteria (Fratazzi et al, 1997). In addition, the apoptotic death of macrophages was associated with a substantial reduction in bacillary viability in macrophages infected with either an attenuated or a virulent strain of MTB (Oddo et al, 1998). The attenuated H37Ra has been shown to induce a greater level of apoptosis in alveolar macrophages than the virulent H37Rv strain (Keane et al, 1997). Further, depending on the ratio of TNF-α and IL-10 induced by them, MTB strains modulated the host cell survival and apoptosis (Rojas et al, 1999). It has been suggested that the suppression of the death of host cells by the pathogen might represent a strategy by pathogen for survival. Infection of resting human monocytes with *Mycobacterium bovis* BCG increased monocyte viability by preventing them from undergoing apoptosis (Kremer et al, 1997). A number of recent studies have addressed a question, whether apoptosis is a virulence marker or not. However, the mechanisms of cell death, virulence and their relation with other innate factors are not well understood.

Thus, the studies published so far evaluated one or two individual parameters of innate immunity to MTB. However, comprehensive studies to correlate different parameters induced by well typed clinical isolates have not been reported so far. Further, it is noticed that there is no comparative evaluation of different genotypes of MTB isolated from India for the immunological responses and other parameters induced by them. The aim of present study, therefore, was to evaluate different parameters of innate immunity and their correlations, if any, in the monocyte leukemic cell line, THP-1 after infection.

3.2. Materials and Methods:

3.2.1. *Mycobacteria*l growth and single cell suspension

The MTB strains isolated from patients were plated on Lowenstein–Jensen (LJ) medium (HIMEDIA) and after confirmation of a pure culture and biochemical tests, single colony was added to Middelbrook 7H9 medium (HIMEDIA) to get mid log phase culture. The cells were harvested at this point and stored in glycerol at -70^{o}C. Before every infection study these cell stocks were grown into log phase and used. Thus the passage number was maintained at 5-6 for all the experiments. The single cell suspension was prepared as per the standard protocol with minor modification (Lambrecht et al, 1988). Briefly, the cell pellets were washed, suspended in PBS containing 0.2% Tween 20 and transferred to a hard glass test tube containing around 25 glass beads (3mm diameter). After 30 second of bath sonication and vigorous vortexing for 5 minutes, the suspension was kept undisturbed for half an hour. The cell count was monitored by taking optical density (OD) of the upper cell layer at 600nm and finally adjusted as required for infection experiments. The absence of clumps was confirmed by Ziehl–Neelsen Carbol Fuchsin (ZNCF) staining and the cell viability was evaluated by colony forming units (CFU) assay in each preparation.

3.2.2 Host cell culture and infection

THP-1 and RAW 264.7 cell lines were obtained from the National Centre for Cell Science, Pune, India. THP-1 was maintained as suspended cells in RPMI 1640 medium (GIBCO,USA) supplemented with 2 mM L-glutamine, 10mM HEPES buffer, 1.0 mM sodium pyruvate and 10% foetal bovine serum at 37ºC in 5% CO_2 humidified incubator. The cells were differentiated into macrophages by treatment with 20 nM/ml phorbol-12-myristate-13-acetate (PMA, Sigma). During the standardization process, three different concentrations (10, 20 and 40nM/ml) of PMA were used and 20 nM/ml PMA showed satisfactory result. After overnight incubation, the monolayer formed was co-cultured with different MTB strains at a MOI (multiplicity of infection) of 10 (10:1 bacilli/THP-1 cells), for 4 hours. The infection was also carried out at four different MOI (1, 5, 10 and 20) and MOI 10 was selected as it gave satisfactory results for monitoring all the different parameters. The infected cells were washed three times with PBS to remove extracellular bacilli.

RAW 264.7 is a macrophage-like, abelson leukemia virus transformed cell line derived from BALB/c mice. RAW cells were cultured in Dulbecco's modified Eagle Medium (DMEM) supplemented with 2mM L-glutamine, 1mM sodium pyruvate and10% fetal bovine serum (FBS), at 37ºC in 5% CO_2 humidified incubator. RAW monolayer formed was co-cultured with different MTB strains at a MOI of 10 (10:1 bacilli/RAW cells), for 4 hours. The infected cells were washed three times with PBS to remove extracellular bacilli.

3.2.3. Phagocytic Index

To determine the phagocytic index, the THP-1 cells were seeded on a sterile glass coverslip and infected with all MTB strains. After four hours of infection, the numbers of internalized bacilli were counted microscopically, using Ziehl Neelsen (ZN) acid fast and phenolic auramine staining. The reagents for microscopy are described in Appendix-I-E. At

least 300 consecutive macrophages were counted and grouped according to the number of intracellular bacteria.

3.2.4. Assay of *Mycobacteria*l growth

In general, the bacterial growth is measured by optical density (600nm) method. But, it has some disadvantages, e.g. clumping of the bacteria does not allow proper OD measurement. Therefore an alternative method, radiorespirometry, was used to assess bacterial growth. Radorespirometry is a sensitive and fast technique and gives sound results in 10^3 to 10^6 bacteria up to 10 days of assessment. Single cell suspensions of all the MTB strains were prepared and after monitoring their OD, the bacterial concentration was adjusted to similar value on the basis of OD for all the strains. Subsequently, 1ml aliquots were prepared and distributed in small 5 ml crew-capped tubes and kept in shaker incubator. After 5 and 10 days of incubation i.e. in their mid log phase, the bacterial cells were centrifuged and pellet was transferred to radiorespirometry vials. For each time point, a separate vial containing 1ml of bacterial suspension was kept in a tube. For each time point, 1ml of MTB suspension was centrifuged and suspended in 100 µl of PBS. The suspension was then transferred to a radiorespirometry vial with 1µCi ^{14}C acetate form BRIT (Boards of Radiation and Isotope Technology, Mumbai), India. The vial used for radiorespiromentry was an assembly of a small inner vial containing LJ medium (without glycerol) and an outer vial having Whatman paper1 dipped in alkaline scintillation cocktail, forming a hemi cylinder (Figure-15A). The paper was dipped in a mixture of liquifluor PPO-POPOP [2,5 diphenyloxazole-1,4-bis(5-phenyloxazoly) benzene] toluene concentrate and 4.0 N NaOH-methanol and dried before use. The generation of radioactive $^{14}CO_2$ was determined daily with a Perkin Elmer Liquid Scintillation Analyzer Tri-Carb 3100TR and data are presented as cumulative cpm (counts per minute) five days after vial preparation. The experiment was carried out in triplicate for all the strains.

3.2.5. Assay of intra-cellular growth

To determine intracellular growth of various MTB strains, at different time points, CFU assay and a modified radiorespirometric assay were used (Ganatra et al, 1980). In this experiment, monolayers of THP-1 cells were prepared in 24-well plates (10^5cells /well) and thereafter were infected with the MTB cell suspension in a ratio of 10:1 bacilli/THP-1 cells. Infected cells were incubated for 4 h at 37˚C in 5% CO_2, washed three times with PBS to remove extracellular bacilli and further incubated in medium for 1 to 5 days. After each incubation time point, cells were lysed with 1ml of water. After 15 minutes, subsequent to the microscopic confirmation of lysis, the lysate was properly mixed. One hundred micro-litres of the lysate was transferred into radiorespirometry vial with 1µCi ^{14}C acetate (BRIT, India) and 100µl was serially diluted and plated on complete 7H11 Middlebrook media (HIMEDIA) agar plate in triplicate for evaluation of CFU. The generation of radioactive $^{14}CO_2$ was determined daily with a Perkin Elmer Liquid Scintillation Analyzer Tri-Carb 3100TR and data are presented as cumulative cpm (counts per minute) obtained on day 5 of the assay. The values were compared for all MTB strains and were correlated with CFU.

3.2.6. RNI assay

Concentration of nitrite produced by RAW and THP-1 cells was determined as a measure of the production of NO by the method of Yamamoto et al, 1998. Briefly, 100 µl supernatant was removed from each culture well at 24 hr and 120 hr (for THP-1), centrifuged at 400 g for 10 min to make it cell free, incubated with 100µl of Griess reagent (1% sulphanilamide, 0.1% napthylethylenediamine dihydrochloride, 2.5% phosphoric acid) at room temperature for 10 min and absorbance was read at 543 nm in a spectrophotometer (Synergy, Bio-tek). The concentration of nitrite (NO_2) was determined by using sodium

nitrite as a standard. Nitrite release was reported as pM per 10^6 cells per well. Cell-free medium was used as blank for the assay.

3.2.7. ROI Assay

To determine ROI induced by various MTB strains after infection, DCFDA stain was used, as described in previous study (Ling et al, 2011). THP-1 cells were infected with various strains of MTB at a MOI 10. The cells were incubated with 10 μM H_2DCFDA (SIGMA) for 15 min at 37°C in 5% CO_2 prior to estimation of ROS production. The monolayers of the cells were then washed with clear media and the fluorescence intensity (excitation 485 nm; emission: 530 nm) was measured using a microtiter plate reader (Synergy). Experiments were done in triplicate and the average florescence intensity was calculated. Cells were also photographed using a fluorescence microscope (Olympus, Tokyo).

3.2.8. RNA extraction, cDNA synthesis and real-time reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from infected cells using RNA extraction kit (Qiagen) according to the manufacturer's instructions. Thereafter, cDNA was synthesized using 1μg of RNA using cDNA synthesis kit (Fermentas Cat.No.#K1622). Quantitative real-time RT-PCR was performed for TNF-α, IL-1β, IL-10, IL-12, TLR-2, MCP-1, IL-8 and β-actin (Tripathy et al, 2004; Boeuf et al, 2005) using SYBR Green master Mix (CAT # 600548, Stratagene) with the following amplification conditions: 95˚C for 10 min, for 40 cycles at 95˚C for 15s, respective annealing temperature for 30s and 72˚C for 30s. Wang et al (1999) demonstrated that NO level is directly correlated to iNOS mRNA expression in alveolar macrophages of TB patients. Hence, PCR with iNOS primers was also done along with that for the cytokines.

All the above mentioned primers along with annealing temperatures are listed in Table-F in Appendix-II. Melting curve analysis was performed for confirming the specificity of the real time PCR. Further, the Ct values for each gene amplification were normalized with respect to house-keeping gene, β-actin by $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) and the expression levels were presented as fold induction in comparison to uninfected THP-1 cells.

3.2.9. Cytokine estimation by ELISA (enzyme-linked immunosorbent assay):

Supernatants from infected cells were collected after 24 and 48 hours by centrifugation and were frozen at -70˚ C until further use. Quantification of TNF-α, IL-1β, IL-6, IL-12 and IL-10 was performed using commercial ELISA kits (BD OptEIA). The procedures are briefly described as follows: A 96 well plates were first coated with 100µl of capture antibody solution overnight at 4°C and then blocked with 200 µl of blocking buffer at RT for 30 min. Human cytokine standards and samples in a series of dilutions (100 µl per well) were added to wells in duplicates and the plate was incubated for 1 hr at RT. This was followed by addition of biotinylated anti-mouse antibody, (100 µl per well) and further incubation for 1hr at RT. Thereafter, 100 µl Streptavidin-HRP conjugate at 1:1000 dilution was added per well and the plates were incubated at RT for 30 min. In the end, 100 µl of the substrate (ABTS) buffer was added to each well. The plates were incubated in dark up to 30 min or till the colour developed and the reaction was stopped by addition of 50 µl of stop solution (2 N H_2SO_4). The OD was taken at 450 nm with λ correction at 570nm. All the dilutions were made in blocking buffer and each step of incubation was followed by extensive washing either 3 or 6 times as mentioned in the protocol. Description of all the solutions and buffers are listed in Appendix-II.

3.2.10. Assays for Apoptosis

Apoptosis ELISA: Induction of apoptosis in THP-1 cells by different strains was estimated by nucleosomal fragmentation ELISA (Cell Death Detection ELISAplus, Roche Applied Science, Indianapolis, IN). Briefly, 10^4 cells were plated per well in 96-well plates, with or without infection for 5 days and after removal of the supernatant, cells were lysed with the lysis buffer provided in the kit. After centrifugation of the lysate, supernatant was carefully transferred to pre-coated microtitre plate and subjected to ELISA as per the manufacturer's protocol. The absorbance values were normalized to those from uninfected cells to derive an enrichment factor as per the manufacturer's protocol (Roche Applied Science).

Expression of apoptosis related genes: Conventional PCR as well as SYBR Green quantitative real-time RT-PCR were standardized for apoptosis related genes, such as Bcl2, Bax, Caspase3 and housekeeping β-actin in infected THP-1 cells after 24 hours of infection. Primer pairs and their sequences are as shown in the Table-F in Appendix-II. Dissociation curve analysis was performed in Real Time PCR to check the PCR specificity. The Ct values for each gene amplification were then normalized with respect to house-keeping genes by $2^{-\Delta\Delta Ct}$ method and the expression values were presented as a fold induction.

Western Blot: Western blot was performed as previously described (Duan et al, 2001). In brief, infected THP-1cells were lysed, centrifuged and 80μg of protein was resolved in 10% SDS-polyacrylamide gel for one hour at 100V. The proteins were transferred to nitrocellulose membrane (Sigma) and after blocking for 2 h, were incubated with rabbit antibodies against PARP (Poly ADP ribose polymerase) or β-actin followed by biotinylated anti-rabbit secondary antibody (all antibodies from Cell Signalling, Beverly, MA, USA). After washing, the membranes were developed with chemiluminescence reagents (Roche) and exposed to x-ray film. The description of required reagents was described in Appendix-I-D.

Flowcytometric analysis: Infected THP-1 cells after 5 and 6 days of infection were washed with annexin-binding buffer (10 mM HEPES, 0.14 M NaCl, 2.5 mM $CaCl_2$, pH 7.4), 5µl of annexin V-FITC (Sigma) was added to 10^6 cells. Cells were gently mixed and incubated for 15 min at room temperature in dark. Thereafter, the cells were acquired in a flowcytometer (Partec Cyflow Space) and analyzed by Flowmax software version 2.0. The required reagents were listed in Appendix-I-F.

3.2.11. Statistical analysis:

Statistical analysis was performed using Sigmastat 3.5 and Microsoft Excel Statistical software. To determine differences between measurements from individual isolates, One Way Analysis of Variance was performed and $P \leq 0.05$ was considered as significant. For establishing correlation between pro-inflammatory cytokine productions, apoptosis, intracellular growth and phagocytosis, the Pearson correlation test was performed and $P \leq 0.05$ was considered as significant.

3.3 Results

3.3.1. Phagocytosis by different strains of *M.tuberculosis*

THP-1 cells were infected at different MOI and it was observed that internalization of MTB increased with higher MOI (Figure 12A). At lower MOI (1 and 5), the concentrations of induced cytokines were low. On the other hand at higher MOI (20) apoptosis was observed much earlier in the infected cells. Hence, at higher MOI, intracellular growth measurement would be erroneous as bacterial count would be reduced due to apoptosis. In order to keep the same MOI for all the parameters studied, MOI 10 was selected.

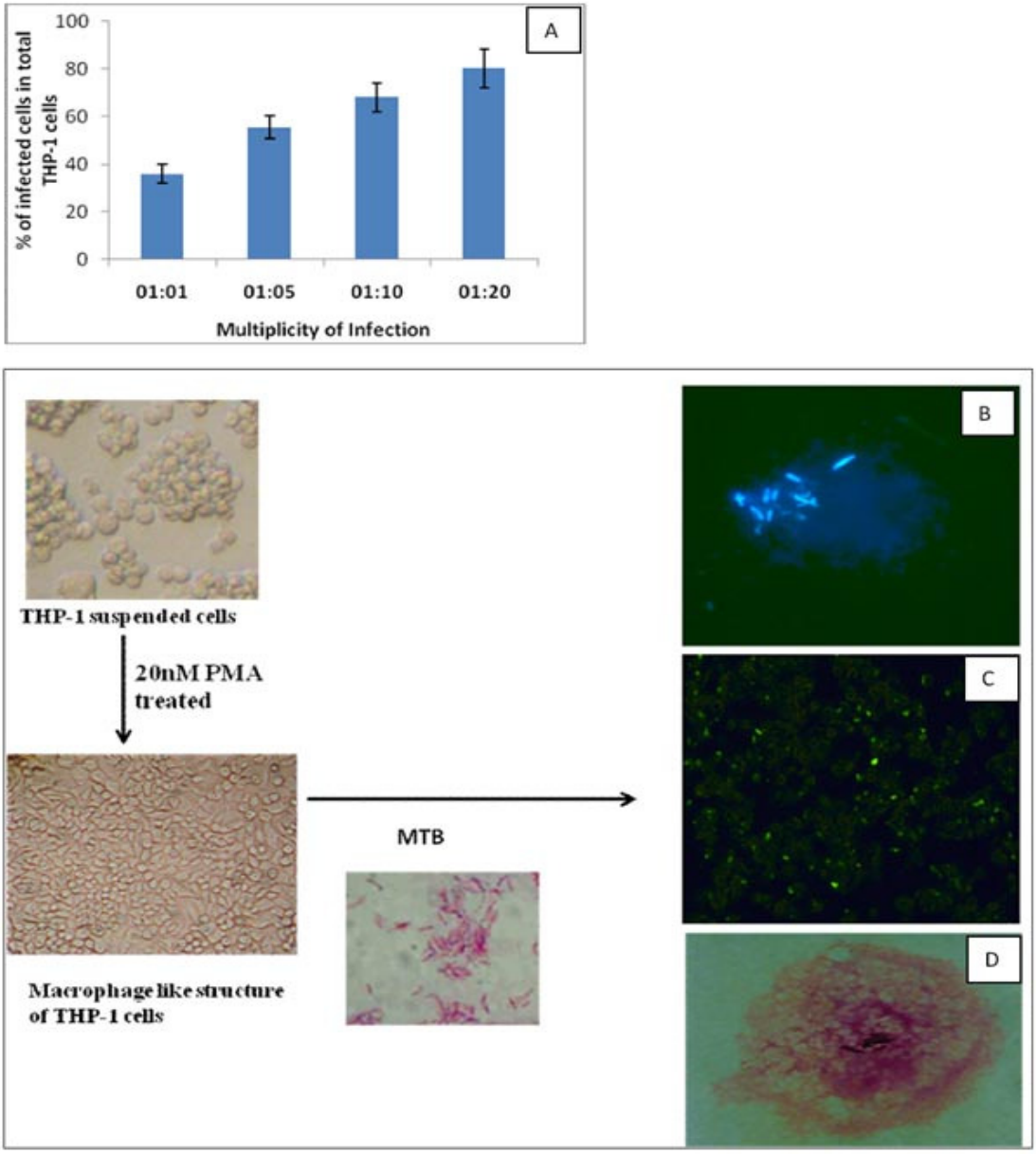


Figure 12 : MTB in infected THP-1 cells

(A)The bars represent per cent THP-1 cells infected with H37Rv at different multiplicity of infection after hours.
(B & C)The representative photographs show THP-1 cells infected with MTB and stained with auramine-$KMnO_4$ after 4 hours of infection, seen under UV light (BX60; M & M Olympus, Tokyo) at (B) high magnification (×1000) and (C) low magnification respectively(×400).
(D) THP-1 cells infected with MTB and stained with ZN acid fast stain after 4 hours of infection, seen under bright light (BX60; M & M Olympus, Tokyo) at a high magnification (×1000).

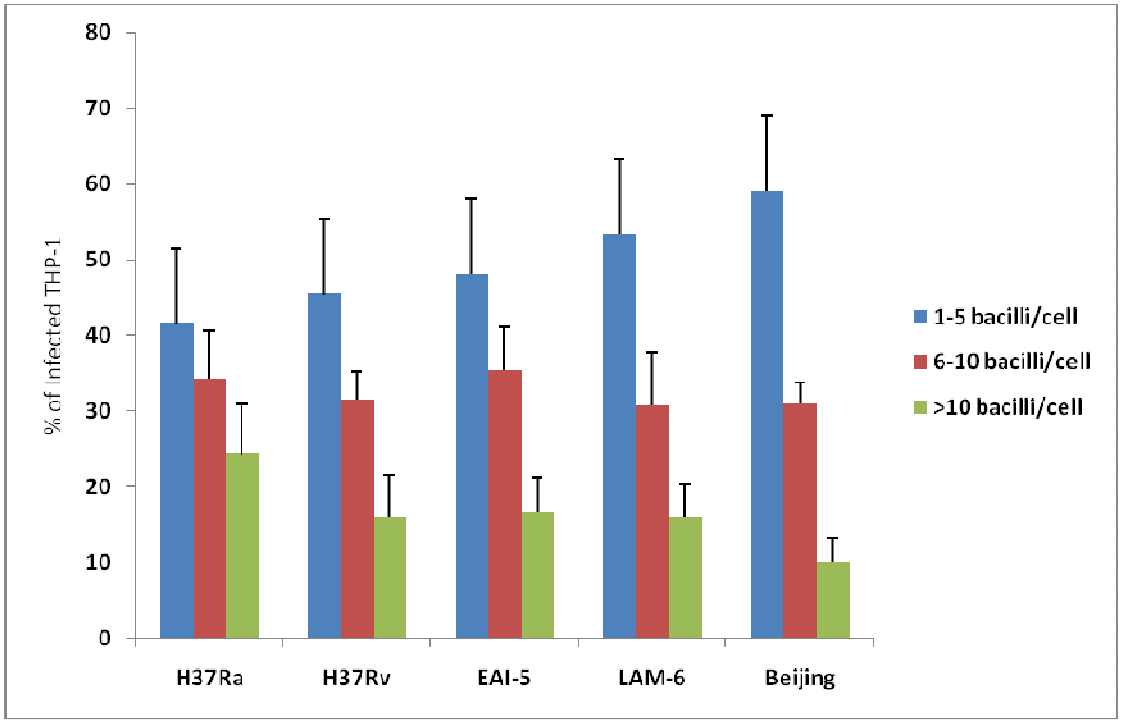


Figure 13 : Phagocytic index for infected THP-1 cells

THP-1 cells were seeded on a sterile glass coverslip and infected with H37Ra, H37Rv, EAI-5, LAM-6 and Beijing strains. After four hours of infection, the numbers of internalized bacilli were counted microscopically, using Ziehl Neelsen (ZN) acid fast and phenolic auramine staining. At least 300 consecutive macrophages were counted and grouped according to the number of intracellular bacteria. Phagocytic index was categorized into 1-5 bacilli, 6-10 bacilli and >10 bacilli per cell. Each bar represents the mean of five independent experiments.

The extent of infection in THP-1 cells for H37Rv, H37Ra and three clinical isolates was scored based on phagocytic index using phenolic auramine staining (Figure 12B and 12C) and ZN acid fast stain (Figure 12D). All the strains showed comparable infectivity in terms of percentage of THP-1 cells infected (65% to 70 %) after four hours of infection. However, when the phagocytic index was divided into three different groups like 1-5, 6-10 and greater than 10 bacilli per cell, it was found that H37Ra infected cells had significantly high percentage of cells accumulating bacteria (24±7%) in the category '>10 bacilli/cell' than cells infected by both Beijing (10±3%) and LAM (15±4%) strains. The percentage of infected THP-1 cells in '1-5 bacilli /cell' category was higher compared to '6-10' and '>10'bacilli/cell categories for all the strains (Figure 13).

3.3.2. Assessment of *Mycobacteria*l growth

Extracellular bacterial growth of the individual isolates was assessed in Middlebrook growth medium by radiorespirometry technique (Figure 14). LAM strain grew faster than other strains when compared on 10^{th} day after incubation ($p \leq 0.05$). There was no significant difference in growth of H37Ra and H37Rv strains. Beijing strain showed lower CPM values compared to LAM, H37Ra and H37Rv on the 10^{th} day of incubation.

3.3.3. Assessment of intracellular *Mycobacteria*l growth in infected THP-1 cells

The intracellular growth was monitored using both, radiorespirometry and CFU plating. Zero to 5 days post-infection, the THP-1 cells was lysed and the intracellular bacteria were subjected to radiorespirometry and CFU counting. Figure 15 represents the cumulative response obtained by radiorespirometry technique for intracellular bacterial load of different MTB strains at different time points. The cpm values in figure were obtained at 5 days after the MTB cells were added in radiorespirometry vial at each time point and represent

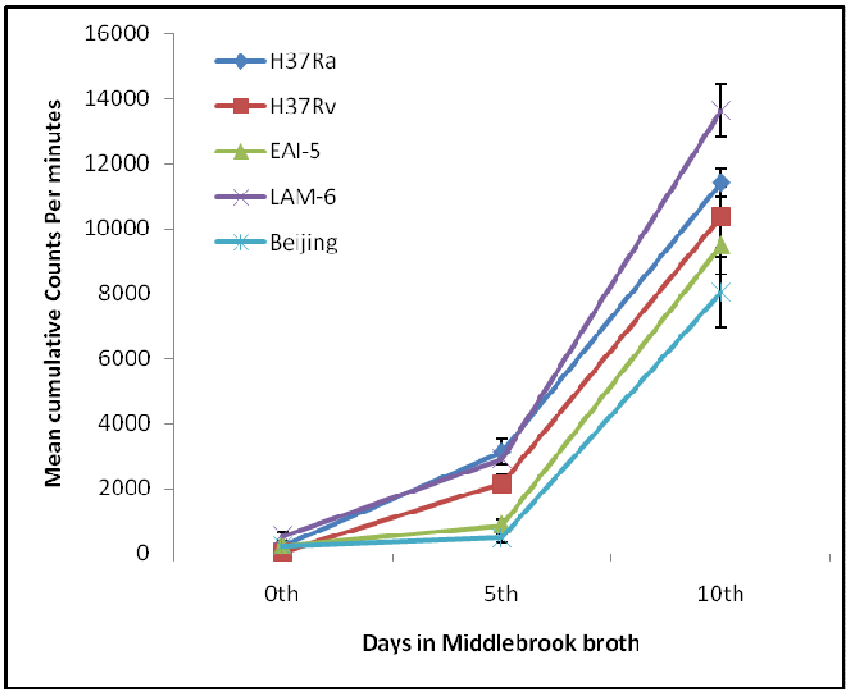


Figure 14 : Extra-cellular growth by different MTB strains in Middlebrook 7H10 medium by Radiorespirometry

After preparing single cell suspension of H37Ra, H37Rv, EAI, LAM and Beijing strains of MTB, it was suspended in middlebrook growth medium in the concentration of 10^3cells/ml for 10 days in a shaking incubator, in separate vials for each time interval. In each time point, 1ml of MTB suspension was centrifuged and th pellet was suspended in 100 µl of PBS. The suspension was then transferred to radiorespirometry vial and the counts were taken in a Liquid Scintillation counter (LSC) five days after vial preparation. The experiment was carried out in triplicate and the graph represents mean ± SD of all the samples.

collective amount of $^{14}CO_2$ released by viable bacilli. Figure 16 shows CFU counts obtained for different time points after 30 days of incubation. A gradual increase of intracellular bacilli was observed for all the strains, with H37Rv and LAM-6 showing significantly higher intracellular bacillary load compared to H37Ra. Though, percentage of infected THP-1 cells in three categories like 1-5, 6-10 and >10 bacilli per cell was different for each strain, in present study, the total number of intracellular bacteria on day zero were in the range of 6.6 x 10^4 to 7.1 x 10^4 (as per CFU assay), which were not significantly different to give differences in cpm values. The generation times of intracellular bacteria were calculated [**g= Generation time (Number of generations in time t)= (logN-logn)/ log2]** and avirulent strain H37Ra as expected, showed higher generation time compared to other virulent strains (Table-5).

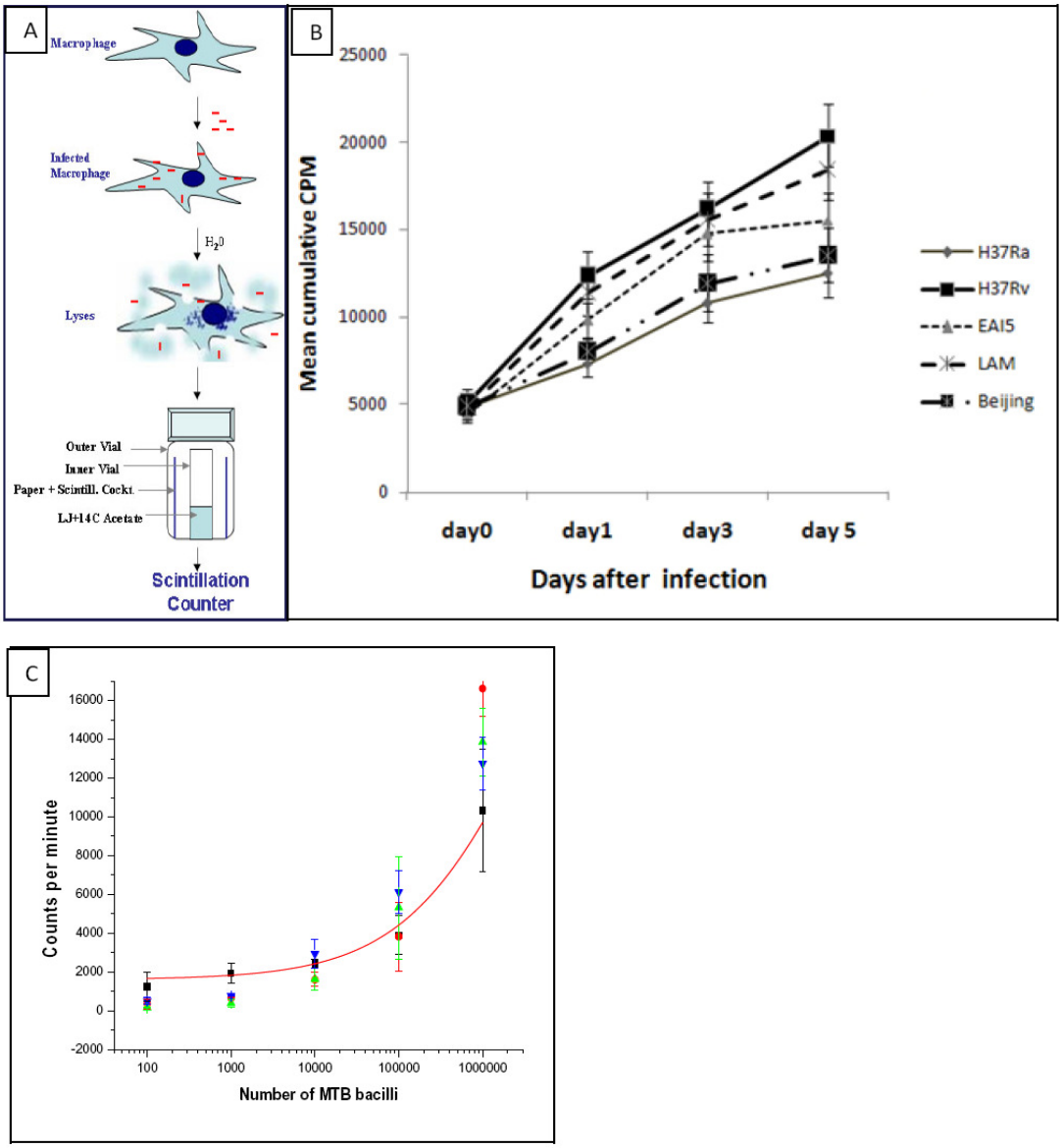


Figure 15: Intracellular growth by different MTB strains in THP-1 cells (Radiorespirometry)

(A) The picture shows, how intracellular bacterial load has been calculated.

(B) THP-1 cells were infected with H37Ra, H37Rv, EAI, LAM and Beijing strains of MTB at MOI of 10 for 4 hrs and after removing extracellular bacteria, the infected cells were incubated with medium for 1-5 days. After each incubation time, the infected cells were lysed. The lysates of infected cells were inoculated in radiorespirometry vial containing LJ medium with ^{14}C acetate. For radiorespirometry, the counts were taken in a Liquid Scintillation counter (LSC) five days after vial preparation. Three such independent experiments were carried out and the graph represents mean ± SEM.

(C) A standard growth curve was drawn with variable number of bacteria of different MTB strains and CPM were obtained ont 10^{th} day of vial preparation. Upto 5×10^4 MTB cells, any drastic change in the counts of all the strains after 10 days of vial preparation.were not observed

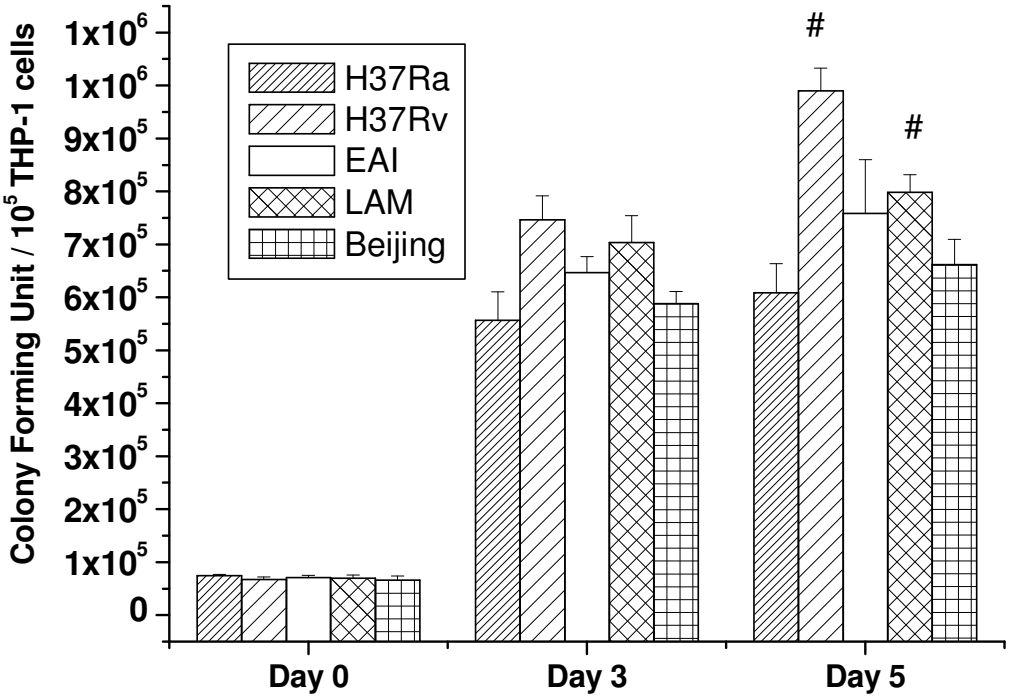


Figure 16: Intracellular growth by different MTB strains in THP-1 cells (CFU method)

THP-1 cells were infected with H37Ra, H37Rv, EAI, LAM and Beijing strains of MTB at MOI of 10 for 4 hrs and after removing extracellular bacteria, the infected cells were incubated with medium for 1-5 days. After each incubation time, the infected cells were lysed. The lysates of infected cells were serially diluted and plated for CFU assay. Colonies were counted after 30 days of plating. Three such independent experiments were carried out and the graph represents mean ± SEM. The symbol, # represents statistically significant difference with respect to H37Ra strain.

Table 5 : Generation time of all selected strains:

Strains	**Generation Time (Hr)± SE**
H37Ra	39.6 ± 2.42
H37Rv	30.9 ± 1.09
EAI	35.2 ±1.06
LAM	34.1 ± 1.7
Beijing	36.1 ± 1.3

3.3.4. ROI and RNI in MTB infected cells

ROI production was studied after initial infection in THP-1 cells using DCFDA reagent. Mycobacterial infection stimulated ROI production inside the host cell and the response was differentially modulated by different strains. Figure 17 depicts the results obtained for ROS production after 2 and 4 hours of infection respectively. Among all the strains, the ancient strain, EAI-5 showed higher levels of ROI in THP-1 compared to other strains. ROI levels inTHP-1 cells infected with all other strains were not significantly different.

RNI levels were estimated in two ways (Figure 18). Firstly, mRNA level for iNOS was estimated and secondly the actual nitrite concentration was measured in the supernatant of the infected THP-1 and RAW cells. In 24 hour post-infection RAW cells produced detectable levels of nitrite in culture supernatants. On the other hand, THP-1 did not show detectable levels of nitrite before five days post infection. Ancient strain EAI-5 again showed higher RNI response to host compared to other strains.

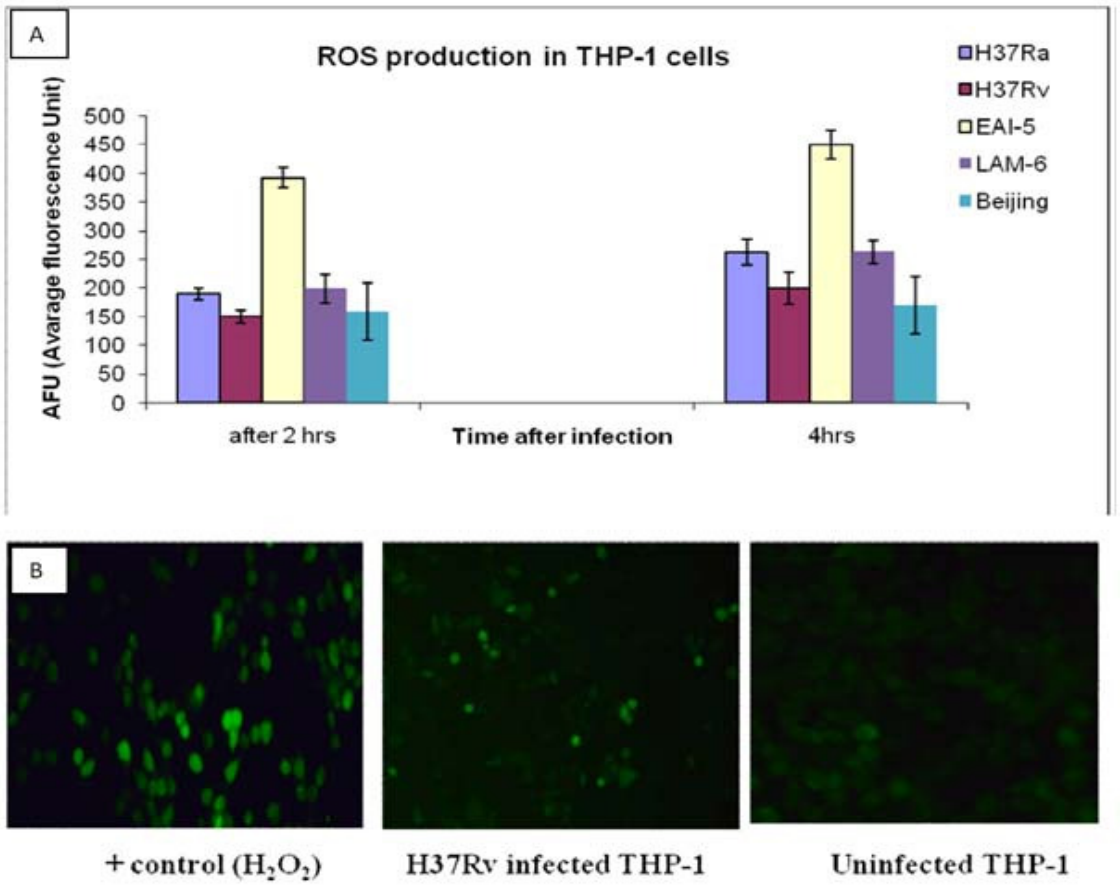


Figure 17 : ROS production in infected cells

(A) THP-1 cells were infected with various strains of MTB at MOI 10. The cells were incubated with 10 μM H_2DCFDA (SIGMA) for 15 min and washed with HBSS. The fluorescence intensity was measured in a fluorescent plate reader. Experiments were done in triplicate and the average florescencecount was calculated.

(B) Representative photographs of DCFDA stained THP-1 cells, H_2O_2 treated, infected with H37Rv and uninfected after 4 hours of infection, were given.

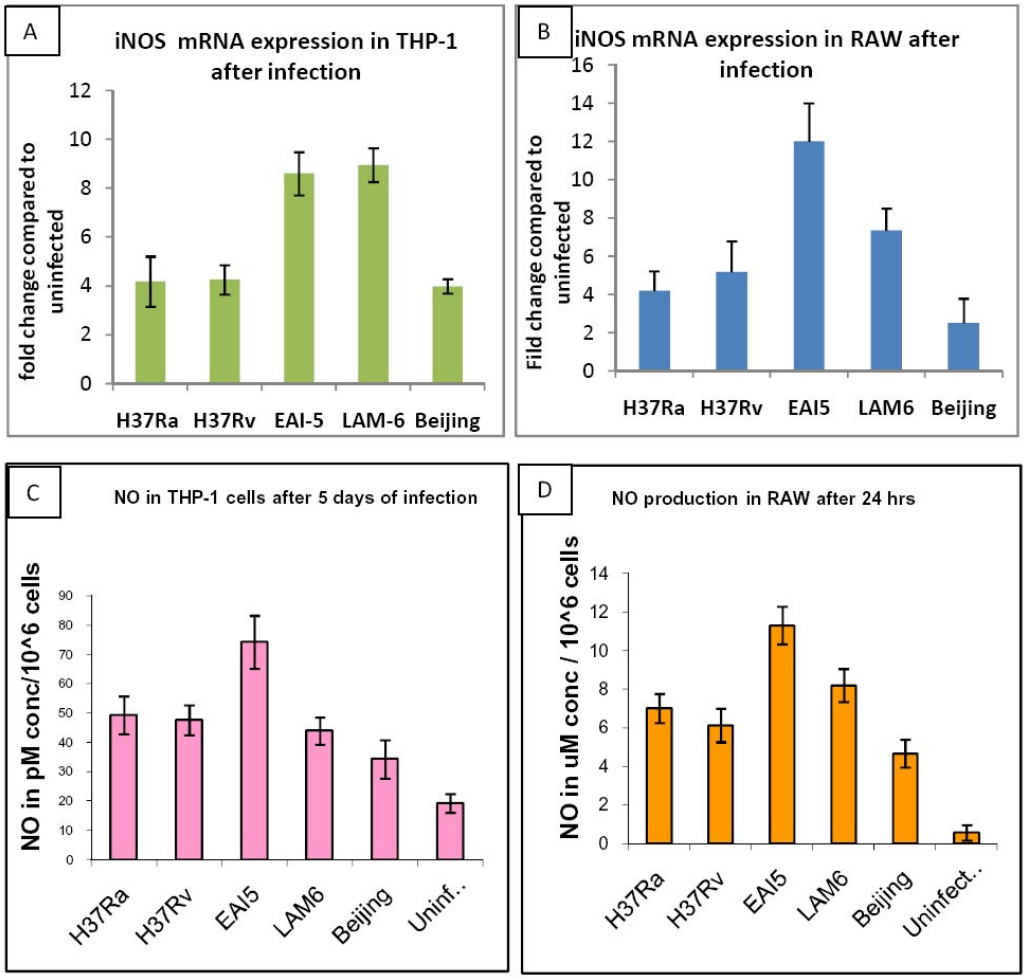


Figure 18 : RNI estimation in infected cells by different strains of MTB

(A, B) Real time PCR was carried out to estimate the mRNA expression for iNOS, 24 hrs after infection of THP-1 (A) and RAW (B) cells with *M. tuberculosis* H37Ra, H37Rv and three clinical isolates at MOI 10. The graph shows relative mRNA expression corrected for total mRNA using the housekeeping β-actin gene. Data represent the means ± standard deviation of three independent experiments.

(C, D) THP-1 and RAW cells were infected with H37Ra, H37Rv,EAI-5, LAM6 and Beijing strains of MTB at a MOI of 10 and after 120 hr and 24 hr respectively, 100 µl of cell free supernatant was removed from each culture well and was incubated with 100µl of Griess reagent. The graphs C & D represent the nitriteconcentrations in the supernatant of THP-1 and RAW cell lines respectively after infection with various strains. Data represent the means ± standard deviation of three independent experiments.

3.3.5. Cytokine profiles in THP-1 and RAW 264.7 cells after infection with different MTB strains

Differential gene expression for proinflammatory cytokines like TNF-α, IL-1β, IL-12 and anti-inflammatory cytokine IL-10, was assessed in infected THP-1 cells, as well as RAW cells by RT- PCR 24 hours after infection (Figure 19 and Figure 20 respectively). The mRNA expression of TNF-α, IL-12 and IL-1β was significantly ($P<0.05$) higher in THP-1 and RAW cells infected with EAI-5 than in cells infected with H37Rv, H37Ra and Beijing strains. However, the expression of these genes was comparable in LAM-6 and H37Rv infected cells. The infection with Beijing genotype showed lower expression levels for all cytokine genes mentioned above (Figure 19, 20). There was no significant difference in expression of IL-10 mRNA among the cells infected with different strains, except for Beijing which induced significantly lower amount of IL-10 mRNA compared to other strains ($P<0.05$). The most interesting observation about Beijing strain was that it induced lesser amount mRNA of both pro and anti-inflammatory cytokines in both the cell lines in contrast to cells infected with other strains.

The levels of different cytokines measured by ELISA corroborated with the mRNA expression pattern (Figure 21). TNF-α concentration in 48 hour post-infection supernatants of THP-1 cells were almost similar for H37Ra, H37Rv and LAM strains. However, Beijing genotype induced significantly lower and EAI induced significantly higher amount of TNF-α compared to H37Rv. Further, patterns for the secretion of other pro-inflammatory cytokines, IL-1β, IL-6 and IL-12, were similar to TNF-α, showing a highly significant correlation among them (TNF-α and IL-1β, R^2 =0.879, $P<0.05$; TNF-α and IL-6, R^2 = 0.799, $P<0.05$; IL-1β and IL-6, R^2 = 0.927, $P<0.05$). The induction of proinflammatory response by LAM-6 was comparable with laboratory strains. The concentrations of IL-10 produced in the

supernatants were less with no significant difference in 24 and 48 hours post-infection for all the strains.

3.3.6. Chemokine and TLR-2 mRNA expression in infected THP-1 cells

The mRNA expression patterns for the most important chemokines, IL-8 and MCP-1 were monitored in THP-1 cells infected with all the strains. The expression levels for EAI-5 infected cells were significantly higher compared to those for infection with other strains. Beijing strain induced lower levels of these chemokines (Figure 22). The TLR-2 expression was also assessed in infected THP-1 cells, but, there was no significant difference in the induction among the strains.

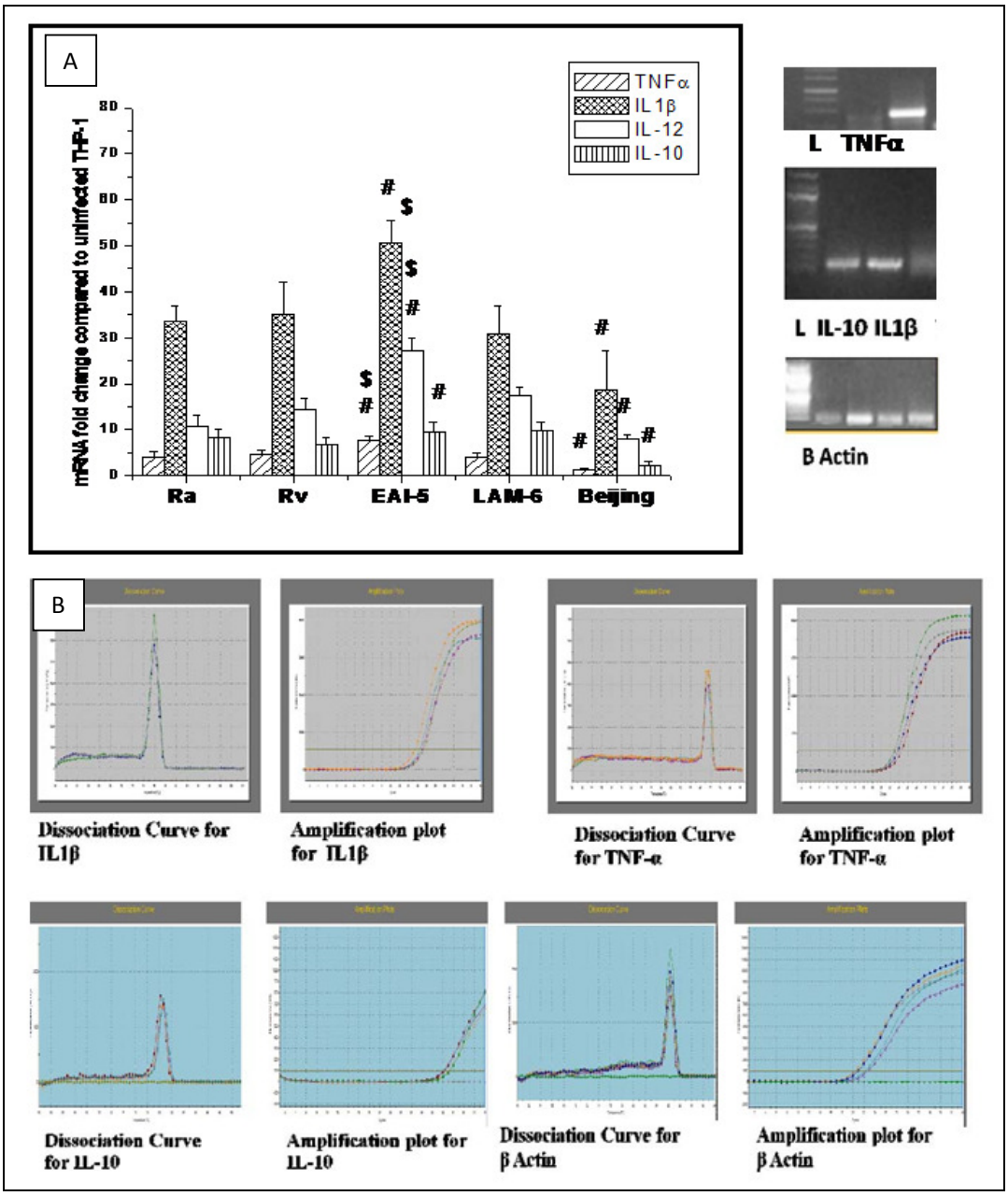


Figure 19: Cytokine induction in infected THP-1 cells (By Real time PCR)

(A) Real time PCR was carried out to estimate the mRNA expression for TNF-α, IL-1β, IL-12 and IL-10, 24 hrs after infection of THP-1 cells with *M. tuberculosis* H37Ra, H37Rv and three clinical isolates at MOI 10. The graph shows relative mRNA expression corrected for total mRNA using the housekeeping β-actin gene.Three independent experiments was carried out. Data represent the means ± standard deviation of one representative experiment. The symbols represent statistically significant difference with respect to specific strain, such as; #, compared to H37Rv and $, compared to Beijing strain.

(B) The respective dissociation curve and amplification plots for different cytokines are shown which indicate the specificity of the reaction.

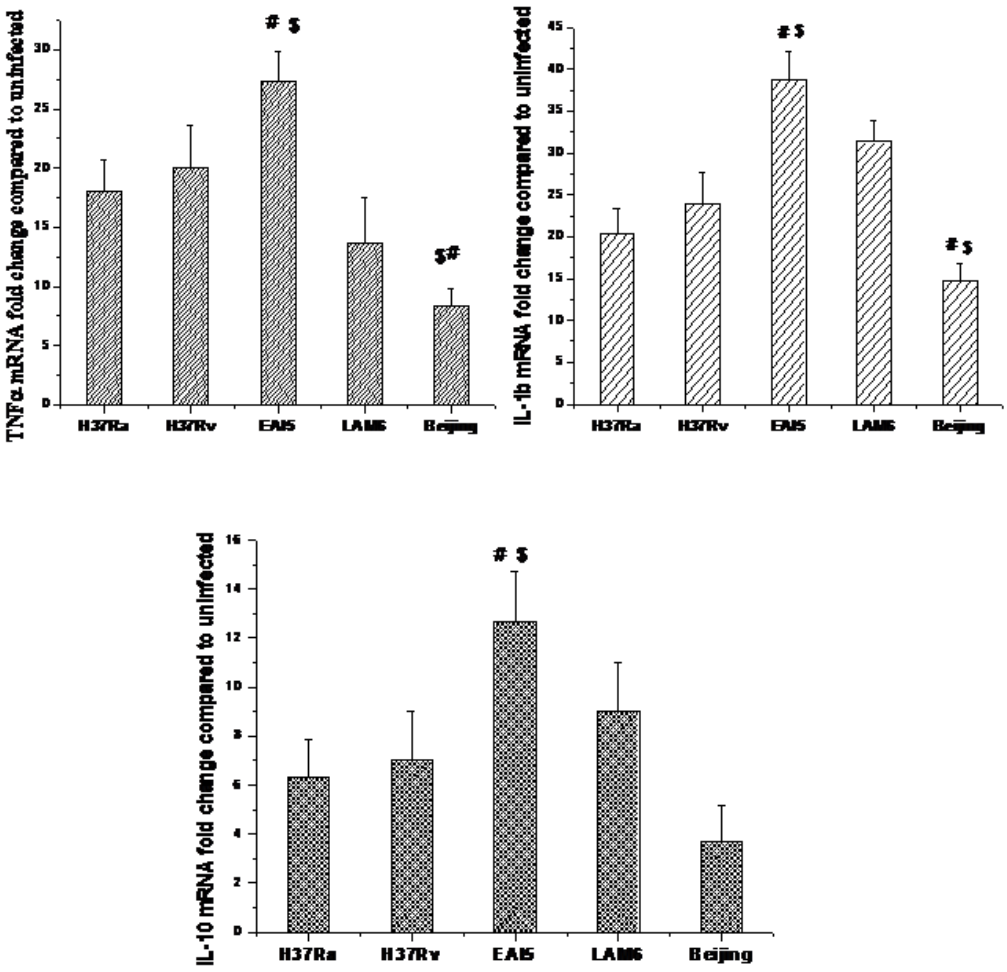


Figure 20: Cytokine induction in infected RAW 264.7 cells (By Real time PCR)

Real time PCR was carried out to estimate the mRNA expression for TNF-α, IL-1β and IL-10, 24 hrs after infection of RAW cells with *M. tuberculosis* H37Ra, H37Rv and three clinical isolates at MOI 10. The graph shows relative mRNA expression corrected for total mRNA using the housekeeping β-actin gene. Data represent the means ± standard deviation of three independent experiments. The symbol # and $ represent statistically significant difference when compared to infection by H37Rv and H37 Ra respectively.

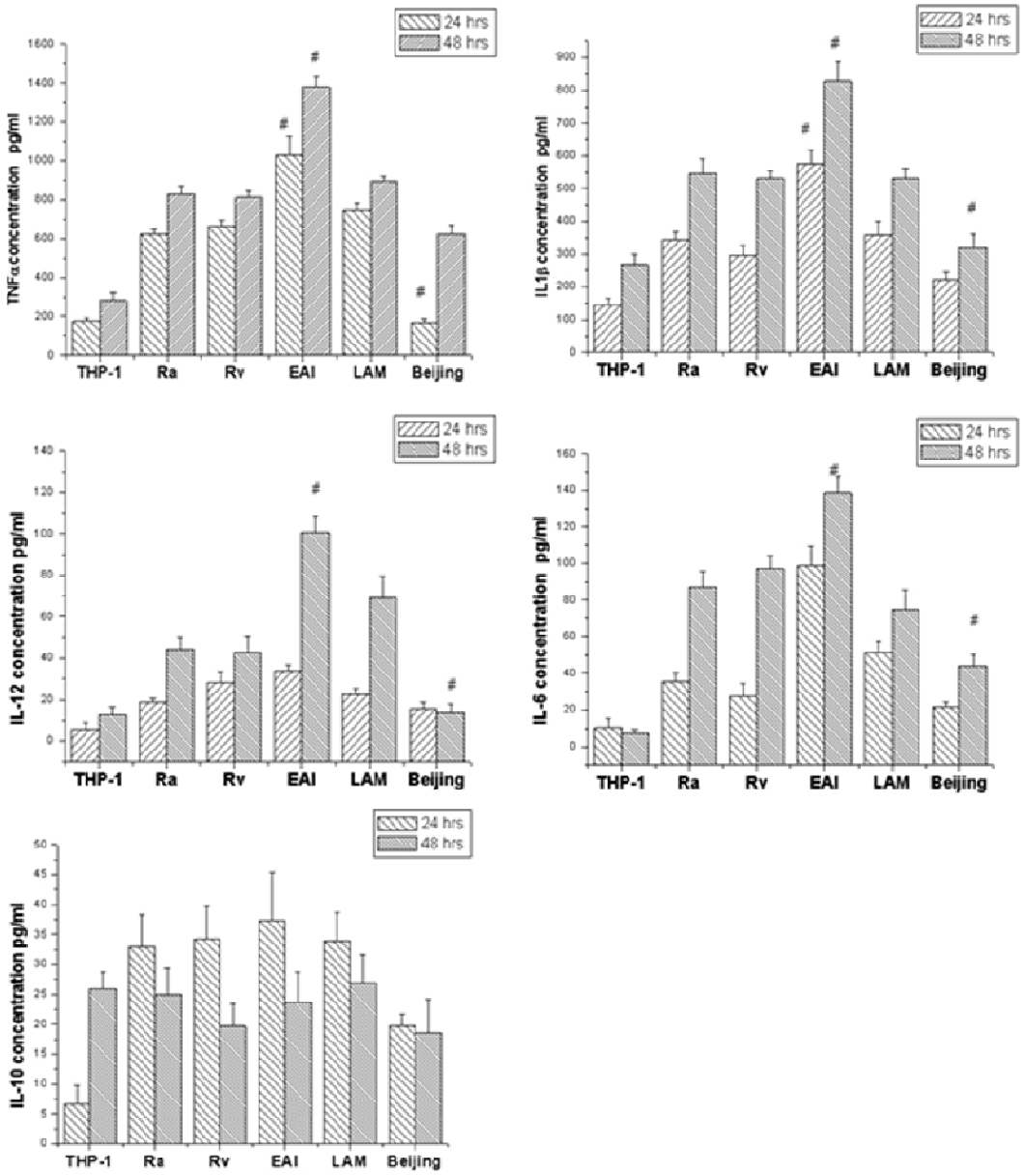


Figure 21: Cytokine induction in infected THP-1 cells (as estimated by ELISA)

The graphs describe the levels of TNF-α, IL-1β, IL-12, IL-6 and IL-10 as measured by ELISA, in the supernatants of THP-1 cells infected with different strains of MTB, 24 and 48 hrs after infection. Three such independent experiments were carried out and data represents the mean ± SD one of such experiments. The symbol #, represents statistically significant difference when compared toinfection by H37Rv.

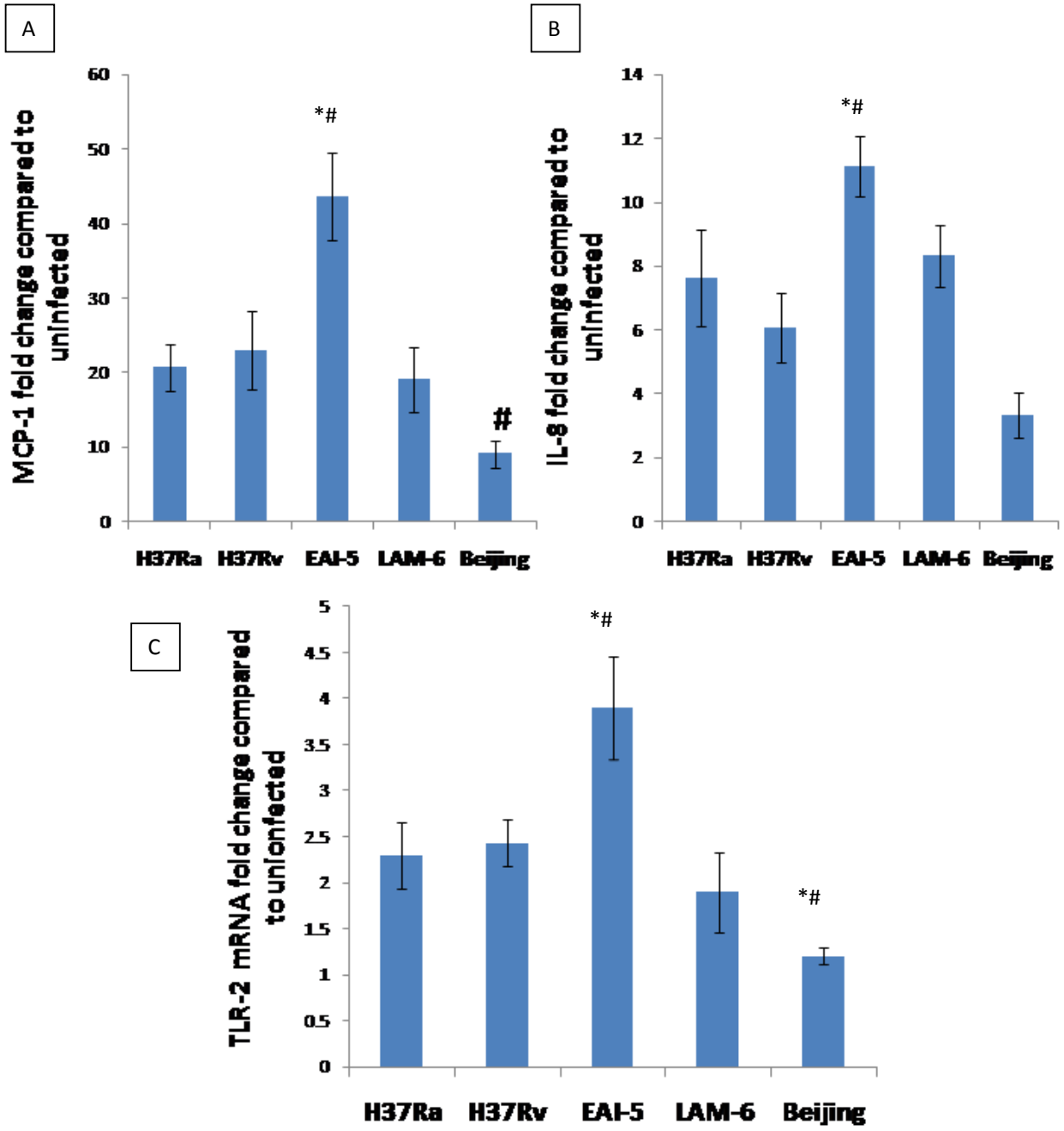


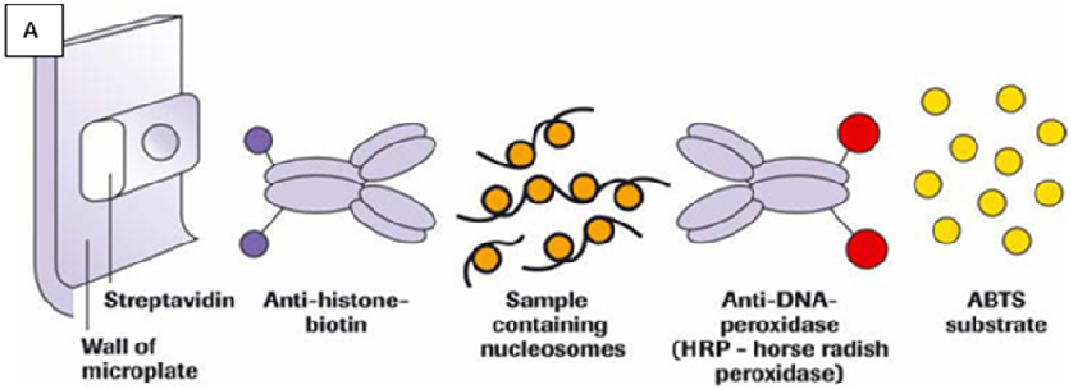
Figure 22 : Chemokine and TLR-2 induction in infected THP-1 cells

MCP-1, IL-8 and TLR-2 mRNA expression in THP-1 cells (A, B and C respectively) infected with different strains of M. tuberculosis (MTB) after 24 hr of infection. Expression was analyzed by Real time-PCR and the graphs show relative mRNA expression corrected for total mRNA using the housekeeping β-actin gene. Data are representative of three independent experiments. . The symbol # and * represent statistically significant difference when compared to infection by H37Rv and H37Ra respectively.

3.3.7. Apoptosis in infected cells

Apoptosis in host cells was confirmed by several different methods. Significant apoptosis was observed after 5 days of infection in THP-1 cells by apoptosis ELISA method (Figure 23). It estimates the fragmented DNA by anti-histone antibody. Strains of MTB induced apoptosis differentially according to their genotype. It was further confirmed by PI staining and annexin V staining. The pattern of apoptosis in THP-1 cells was also compared after five and six days of infection by PI staining (Figure 24) and by Annexin V labelling (Figure 25). Among the five strains H37Ra and EAI-5 induced significantly higher apoptosis compared to that in uninfected and Beijing infected cells. Further, the apoptotic response to LAM-6 was similar to that observed for H37Rv.

To understand the apoptotic pathway more clearly, the levels of *Bcl2*, *Bax* and *Caspase-3* genes in the infected THP-1 cells, were analyzed. It was observed those 24 hours post-infection, the levels of *bax* and *caspase-3* gene expression were higher in case of infection by EAI strain, compared to those for cells infected with Beijing and H37Rv. On the other hand, *bcl-2* gene expression was significantly less compared to that after infection with H37Ra, H37Rv and Beijing strains. Further, expression of Bcl2-associated X (*bax*), poly ADP-ribose polymerase (PARP) and Caspase-3 proteins was also confirmed by Western Blotting. Figure 26B depicts the relative expression of pro-apoptotic proteins, such as PARP, bax and caspase-3 in uninfected and infected THP-1 cells after 48 hrs of infection for all the five strains. The observed protein levels of degraded PARP, bax and caspase-3 also support the apoptotic events.



Scheme shows the principle of the cell death detection ELISA

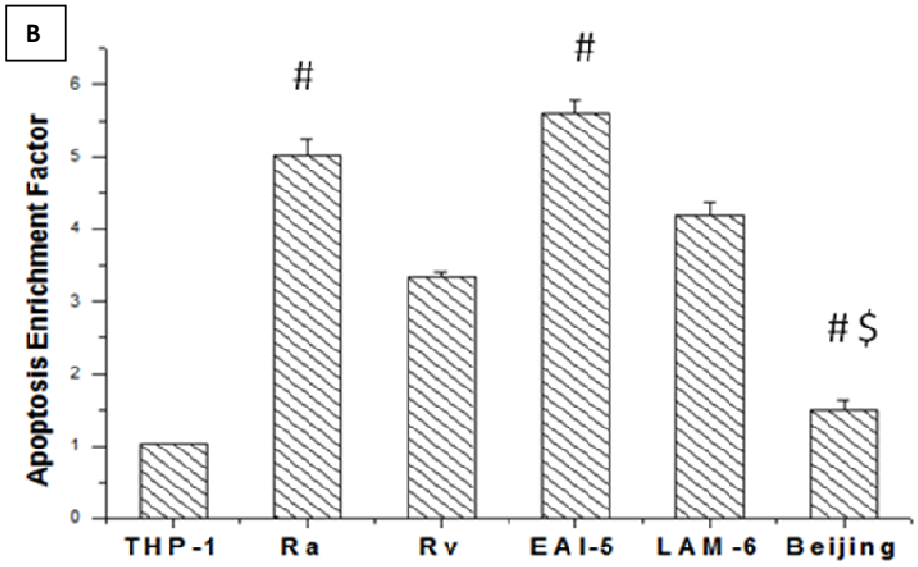


Figure 23: Apoptosis of infected THP-1 cells as estimated by ELISA

(A) The diagram shows the principle of cell death detection ELISA.

(B) Apoptosis was determined using the Cell Death Detection ELISA (Roche Applied Science) five days after infection. The bar diagram represents the extent of nucleosome fragmentation in infected THP-1 cells. Two such independent experiments were carried out and the graph represented as mean ± SD of one such experiment. The symbol # and $ represent statistically significant difference when compared to infection by H37Rv and H37Ra respectively.

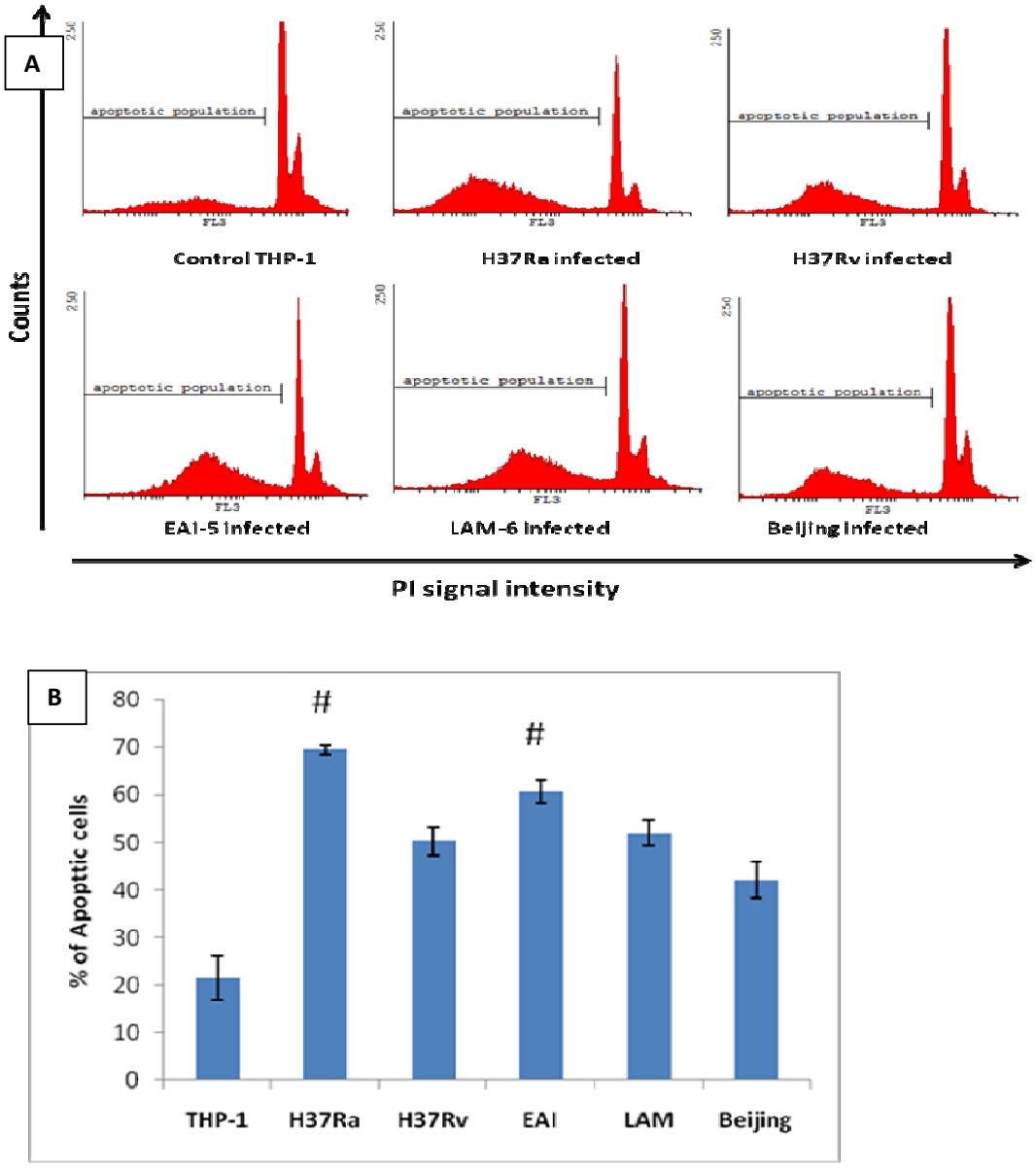


Figure 24 : Apoptosis of infected THP-1 cells as estimated by PI staining

(A) A representative flowcytometric analysis of propidium iodide (PI) labeled THP-1 cells infected with various MTB strains on 5th day of infection. The gated cell population represents cells showing apoptosis. Three such independent experiments were carried out.

(B) The bar diagram represents the percentage of apoptotic THP-1 cells. Three such independent experiments were carried out and the bars show mean ± SD values from one such experiment. The symbol, # represents statistically significant difference when compared to infection by H37Rv.

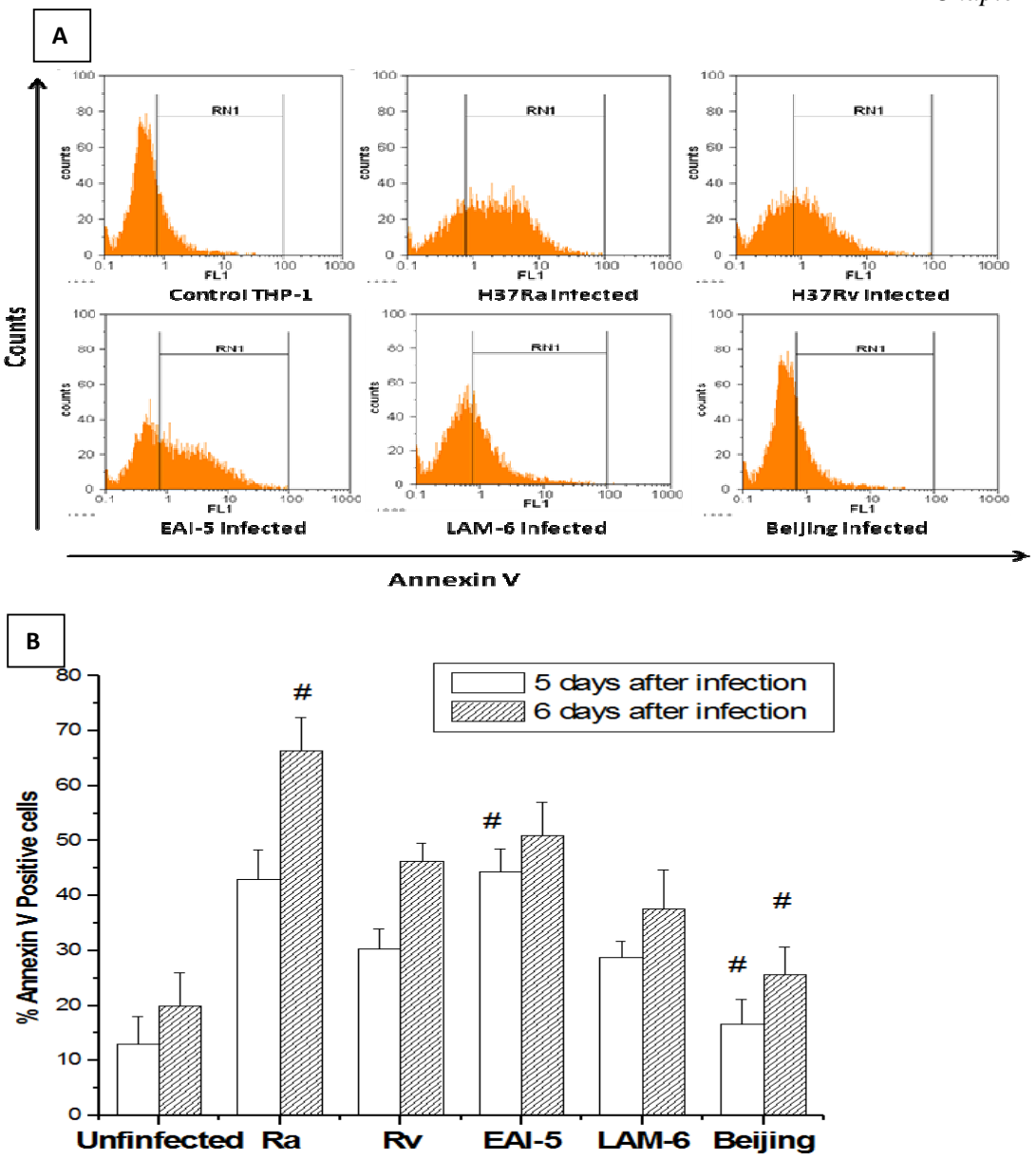


Figure 25 : The apoptosis of infected THP-1 cells as estimated by Annexin V-FITC

(A) A representative flowcytometric analysis of Annexin-V-FITC labeled THP-1 cells on 5th day of infection with various MTB strains. RN1 indicates the percentage of Annexin-V positive cells.

(B) The bar diagram represents the percentage of infected THP-1 cells stained with AnnexinV after 5 and 6 days of infection. Three such independent experiments were carried out and the graph represented as mean ± SD of one such experiment. The symbol, # represents statistically significant difference when compared to infection by H37Rv.

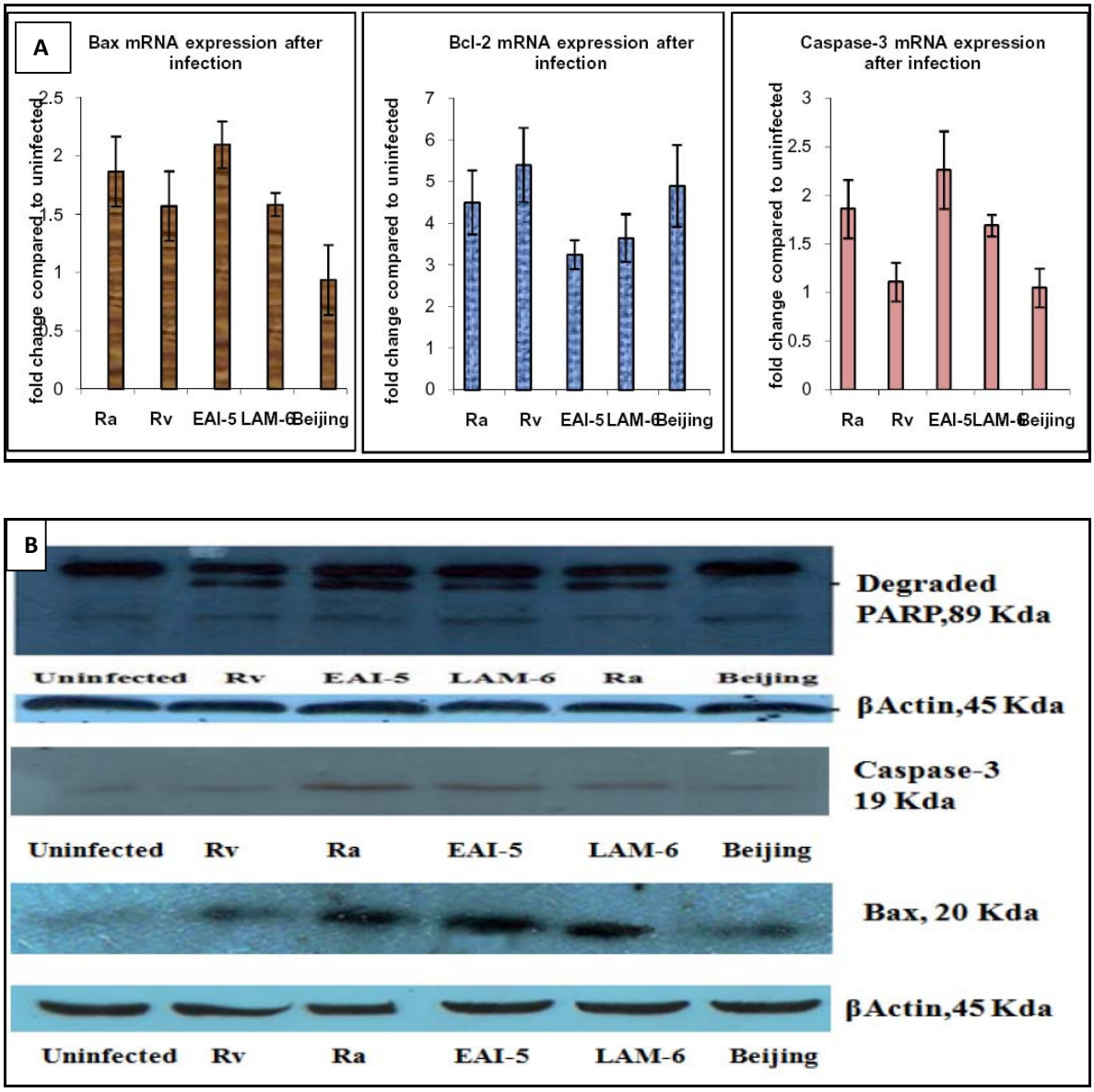


Figure 26 : Expression of apoptosis related genes in infected THP-1 cells

(A) *Bax, bcl-2* and *Caspase3* mRNA expression levels in THP-1 cells after 24 hours of infection with different strains of MTB, was measured by Real time RT PCR and fold change in gene expression relative to β actin was calculated by $2^{-\Delta\Delta CT}$ method. Mean fold change in gene expression is plotted on Y-axis and the X axis shows the strain names. Three such independent experiments were carried out and the data arerepresented as mean ± SD.
(B) Expression of degraded PARP, Caspase-3 and Bax and β-Actin was monitored in THP-1 cells after 48 h of infection withfive different MTB strains by Western Blotting. In this representative picture, the lanes were aligned from original picture.

3.4. Discussion:

Pathogenesis in tuberculosis is driven by many components of the host immune system, pathogen and environment (Comas & Gagneux, 2009). Hence, for understanding the disease, an integrative approach, considering many interactions between host and pathogen, is essential. Innate immune mechanisms are crucial to the outcome of infection. In the present study, innate responses were comparatively evaluated for three MTB clinical isolates and two laboratory strains in terms of phagocytosis, growth, induction of ROI/RNI, cytokines and apoptosis in infected host cells. The percentages of total THP-1 cells showing phagocytosis were not significantly different for laboratory strains and clinical isolates. However, infection with avirulent H37Ra resulted in comparatively higher accumulation of bacteria inside the host cells with higher number of infected cells containing more than 10 *Mycobacteria* per cell, compared to infection with all virulent strains which showed more infected cells in 1–5 *Mycobacteria* per cell group. This is in agreement with the report of Rajavelu et al, 2007. Other studies have compared the overall extent of phagocytosis for different MTB strains and have reported contradictory results. Torrelles et al (2010) demonstrated that clinical isolates from Beijing genotype exhibited lesser phagocytosis due to the presence of truncated mannose-capped lipoarabinomannan (ManLam) as compared to laboratory strains whereas, Sarkar et al, 2012 have demonstrated less uptake of laboratory strain H37Rv as compared to that of clinical isolates at the time of infection. Whether this difference in accumulation really has any role in virulence is not yet clear.

Intracellular bacillary load is considered to be an indicator of virulence of the MTB strains. Previous studies have suggested that virulent strains grow faster intracellularly and their survival depends on their adaptability inside the host (Silver et al, 1998; Zhang et al 1998; Park et al, 2006). Quantitation of the intracellular growth of MTB in human macrophages is technically demanding for several reasons. MTB can induce cell death during

the culture period which artificially reduces the number of CFU in macrophage lysates. Further, the extracellular growth of MTB and clumping of bacteria may complicate the interpretation of results. In the present study, two different techniques were used and compared. The radiorespirometry data obtained, correlated well with CFU counts (R^2=0.98, P<0.05), indicating that the former corresponded only to intracellular bacteria. The isolates displayed differential growth in THP-1 cells, which was apparently comparable or lower than that of H37Rv (Figure 15,16). Among the strains under study, H37Ra grew much faster extracellularly (Figure 14), but its intracellular growth was lowest compared to the virulent strains on day 5 of infection indicating the lack of ability to sustain inside the host, probably due to the intracellular killing by the host and induction of apoptosis. It is well established that mycobacteria have evolved strategies to block phagolysosomal fusion by different ways. It needs involvement of several different virulent factors of the pathogen. On the other hand, a number of factors of host macrophage, have been characterized so far which modulate the outcome of the entry of mycobacteria and the phagolysosomal fusion thereafter. The small GTPase LRG-47, membrane cholesterol and coronin-1 or TACO (tryptophan aspartate containing coat protein) are the most studied host molecules in this regard (MacMicking, 2005). Only viable Mycobacteria can recruit TACO in the phagosomal membrane and block phago-lysosomal fusion by activation of the Ca2+ dependent phosphatase calcineurin (Ferrari et al, 1999; Nguyen and Pieters, 2005; Jayachandran et al, 2007). Interestingly, it has also been observed that down regulation of TACO gene restricts mycobacterial intracellular survival in THP-1 cells (Anand & Kaul, 2005). In the current study, the role of TACO was not examined, but apoptosis of host cells was assessed.

Laboratory virulent strain, H37Rv grew faster intracellularly among all strains and presented itself as more virulent than clinical isolates which was in agreement with some other studies (Rajavelu et al, 2007). It was reported that the differences in apparent

intracellular growth rates (Figure 15, 16) presumably reflect differences in strain virulence (Park et al, 2006; Theus et al, 2006). Clinical isolates are supposed to be more virulent compared to laboratory strains (Comas & Gagneux, 2009). Possibly the strains under study might not have adapted to the intracellular environment compared to other prevalent strains; or, it was likely that five day observation of intracellular growth in THP-1(*in vitro* model) might not sufficiently indicate the virulent characteristic of the strains. Importantly, the variation in intracellular growth was observed after infection with different genotypes in spite of these isolates showing broadly comparable growth rates in extracellular cultures (Figure 14).

Induction of pro-inflammatory cytokines dictates containment of intracellular pathogen, granuloma formation and determines virulence, as well as prevalence of the strain (Silver et al, 1993). Persistent MTB strains induce much lower amount of pro-inflammatory cytokines in the host during infection (*ex vivo*) compared to non-prevalent isolates (Theus et al, 2005). A higher pro-inflammatory cytokine response was observed for the non-Beijing strains and significantly lower induction for Beijing strain, as reported earlier (Sarkar et al, 2012). Whether the suppression of innate immune response induced by Beijing strain is associated with the ability of this genotype to propagate faster, is still to be explored. However, it has been reported that Beijing strains produce a unique phenolic glycolipid that abolishes the host's ability to control the infection (Schlesinger, 1993). EAI-5 strain in the present study consistently elicited a stronger pro-inflammatory response like other ancient strains reported earlier (Sarkar et al, 2012). LAM-6 showed similar proinflammatory response compared to H37Rv and they both belong to lineage 4. Though the strains used in the study showed differences in their resistance to anti-TB drugs (Table 2, 3; Figure 9), all the clinical isolates showed patterns of immune response typical for their respective genotype with no deviations due to drug resistance. This observation strongly suggests that the innate

immune responses of the host varied with the lineage of the pathogen but not with its drug resistance.

It is acknowledged that ROS and RNI play an autoregulatory role in amplifying the synthesis of TNF-α and IL-1β. In addition, reactive oxygen species (ROS) can also act as signalling molecules (Yang et al, 2007). It was observed that mouse macrophage produced detectable levels of nitrite in culture supernatants after 24 hours of infection, whereas human macrophage hardly showed nitrite after 120 hours of infection. The probable reason may be the lack of tetrahydrobiopterin, a necessary cofactor for iNOS catalytic activity and a co-enzyme not constitutively synthesized by human monocytes or macrophages. Jagannath and co-workers (1998) demonstrated that the difficulty in detecting NO in human macrophages might, be due to the inability of the standard colorimetric assay to detect relatively low levels of NO in initial stages of infection. In the current study, results showed that each of the genotypes assessed had a differential ability to induce ROS and RNI in infected host cells. The Beijing genotype which is known to be highly virulent, induced the lowest proinflamatory cytokine, reactive nitrogen and reactive oxygen species in the host without affecting expression of immunosuppressive cytokines. These findings are consistent with previous reports with other members of the W/Beijing family of strains (Portevin et al, 2011).

The extent of apoptosis in THP-1 cells infected with Beijing strain was also significantly less than in cells infected with other strains, which indicated that selected Beijing isolate used in this study must have been virulent in nature. Manca et al (2004) showed that certain members of the highly pathogenic W/Beijing family of MTB strains produced lipids that fail to efficiently induce protective immune response. In the infected host this failure would ultimately result in delayed or impaired protective immunity, leading to more severe disease. In the present study, EAI5 ancient isolate induced higher

proinflammatory cytokines, ROS and RNI, which is corroborative as reactive species induce cytokines and cytokines induce apoptosis.

In the host defence against *Mycobacterium tuberculosis*, apoptosis promotes microbicidal activity in alveolar macrophages (Gan et al, 2008). Macrophage apoptosis may contribute to the innate immune system's response by containing and limiting the growth of tubercle bacilli (Kelly et al, 2008) by keeping the membrane barrier intact (Gan et al, 2008). Macrophage necrosis, on the other hand, releases viable tubercle bacilli from macrophages for the extracellular spread of infection (Gan et al, 2008). Macrophage necrosis causes cell lysis and death, leading to the inflammation that promotes tissue damage. This process is responsible for the recruitment of naive macrophages which ultimately become infected and promote MTB infection (Gan et al, 2008). The attenuated H37Ra strain induced macrophage apoptosis while the virulent H37Rv strain induced macrophage necrosis (Fratazzi et al, 1997; Gan et al, 2008). It was reported that infection of human alveolar macrophages by MTB was sufficient to induce classical apoptosis by extrinsic pathway mediated by TNF-α in an autocrine/paracrine manner and pro-inflammatory cytokines directly or indirectly modulated apoptotic response depending on the degree of virulence of the strain (Ciaramella et al, 2002). It was observed that addition of exogenous TNF- α enhanced apoptosis and, conversely, treatment with anti-TNF-α enhanced macrophage survival. On the other hand, treatment with anti-IL-10 diminished cell viability and Bcl-2 expression, while increasing caspase 1 activation, p53 expression, and the number of TNF-α producing cells. These findings are in agreement with those of Estaquier et al,(1996) showing that IL-10 was able to rescue the cells from apoptosis by down-regulating protease cascade rather than by inhibition of nitric oxide production. Furthermore, it has been reported that *Bcl-2* family genes are down-regulated in human mononuclear phagocytes after infection with *M. bovis* BCG (Klingler et al, 1997). The down-regulation of *Bcl-2* was accompanied up-regulation of Bax (Mogga et al, 2002), which

is known as a cause of apoptosis (Perskvist et al, 2002). In the present study too, a strain specific apoptotic response was observed, with EAI-5 inducing significantly higher rate of pro-inflammatory response and apoptosis compared to Beijing strain and H37Rv ($P<0.05$). This strain even induced higher amount of *bax* mRNA and protein while lowering *bcl-2* (mRNA), whereas, Beijing strain induced lower pro-inflammatory cytokines and apoptosis compared to all other strains. We observed a good correlation between all pro-inflammatory cytokine responses and apoptosis induced only by virulent strains after five and six days of infection. The correlation coefficients (R^2) for TNF-α, IL-1β, IL-6 and IL-12 with per cent apoptosis were 0.906, 0.864, 0.879 and 0.890 respectively ($P<0.05$) on 5^{th} day and 0.830, 0.669, 0.639 and 0.846 respectively ($P<0.05$) on 6^{th} day of infection . When H37Ra was included for the analysis, a good correlation at a significant level was not observed. It was evident that MTB strains trigger apoptosis mostly by induction of proinflammatory cytokines (Keane et al, 1997). However, as avirulent strains in present study showed higher apoptosis without inducing proportionately higher pro-inflammatory cytokines, possibility of involvement of other pathways along with proinflammatory cytokines do exist.

Till date only one report described the relationship between phagocytosis by and apoptosis of host cell after infection with MTB strains (Rajavelu & Das, 2007). According to that study, phagocytic index for '> 20 bacilli/ cell' has a positive correlation and for '1-10 bacilli /cell' has a negative correlation with apoptosis. In the present study, a positive correlation was observed between phagocytic index for '>10 bacilli/cell' and the per cent apoptosis after five (R^2 =0.708 , $P<0.05$) and six (R^2 = 0.608, $P<0.05$) days of infection and a negative correlation was seen between phagocytic index '1-5 bacilli/cell' and per cent apoptosis (R^2 =-0.6 and -0.606 $P<0.05$) for the same period. It was evident that pro-inflammatory cytokines and higher initial accumulation of bacilli inside the host cell were the two determining factors for host cell apoptosis. Furthermore, our results illustrate that more

virulent strains of MTB have more number of infected cells in 1-5 bacilli per cell category. On this basis it may be hypothesized that virulent strains once inside the host allow limited accumulation of bacilli by yet unknown mechanism which is beneficial to the pathogen. Taken together, it is apparent that diverse clinical isolates from different genetic background induce differential innate immune responses. The opposite responses illustrated by ancient and modern lineages, may reflect the differential virulence strategies employed to subvert the host immunity.

This study is the first report in which all the important parameters like bacterial extracellular, intracellular growth and their internalization in terms of phagocytic index were monitored, along with induction of cytokines, reactive oxygen-nitrogen species and apoptosis in host cells. It is also unique, as it involved the drug resistant strains from different lineages and for the first time illustrates that, the high and low inflammatory responses induced by ancient and modern lineages respectively, are not influenced by their drug resistant status. Moreover, significant correlation among the innate responses were-established for all these clinical isolates.

Chapter 4: *Ex vivo* infection of monocyte derived macrophage (MDM), monocyte derived dendritic cells (MDDC) and whole blood from healthy individuals, with different strains of *Mycobacterium tuberculosis* and assessment of interleukin responses.

4.1 Introduction

Alveolar macrophages are the primary cell types involved in the initial uptake of *M. tuberculosis*. This first encounter is usually followed by the phagocytosis by dendritic cells and monocyte-derived macrophages (Henderson et al, 1997; Thurnher et al, 1997). The host defence in tuberculosis involves both mononuclear phagocytes and lymphocytes involved in innate and acquired immune responses respectively (Andersen et al, 2000; Kaplan et al, 2003). Typically, mononuclear phagocytes in their innate response produce proinflammatory cytokines, e.g. tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-12 (IL-12) and anti-inflammatory cytokines, e.g. interleukin-10 (IL-10) and transforming growth factor-β (TGF-β). Similarly, after stimulation, T-lymphocytes in their acquired immune response produce Th1-type cytokines, e.g. interferon-γ (IFN-γ) and interleukin-2 (IL-2) or Th2-type cytokines, interleukin-4 (IL-4) and interleukin-10 (IL-10) (Lucey et al, 1996). The delicate balance between pro- and anti-inflammatory cytokines as well as Th-1 and Th-2 response is believed to influence the outcome of the infection (Rook et al, 1996).

In addition to macrophages, the dendritic cells (DC) are also known to play a critical role in the initiation of adaptive immune response (Banchereau et al, 1998) strengthening cellular immunity against mycobacterium infection (Henderson et al, 1997; Fortsch et al, 2000; Tascon et al, 2000, Demangel et al, 2000, Mohagheghpour et al 2000). After the onset of the inflammatory response, DCs are highly represented at the sites of MTB infection (Sertl et al, 1986; Holt et al, 1987; Van Haarst et al, 1994). Immature DCs present in the lung mucosa are specialized in antigen uptake and processing and after interacting with pathogens, they mature and migrate to lymphoid organs where they prime Th1 cells through the MHC

class II associated antigen presentation and secrete immunoregulatory cytokines such as IL-12 (Henderson et al, 1997; Banchereau et al, 1998).

It is well known that macrophages are the reservoir and also the main effectors for killing of *M.tuberculosis*. In the earlier chapter, *ex vivo* infection experiments with THP-1 cells, a human monocytic leukemia cell line, were described. However, cancerous cells have their own drawback of not responding accurately like the primary cells. Since the cell lines are genetically manipulated, it may alter their phenotype, native functions and their responsiveness to stimuli. Further, their serial passages can cause genotypic and phenotypic variations over an extended period and genetic drift may lead to heterogeneity in the cultures at a single time point. In view of this, and also with an aim to represent and understand the true interactions in the normal infection process in tuberculosis, the infection studies were carried out in primary cultures of MDM, MDDC and whole blood from several individuals. Cytokine production is the best reflection of the immune interactions taking place in humans during the infection. However, concentrations of circulating cytokines are often very low or undetectable and do not reflect local cytokine production (Barnes et al, 1993; Perenboom et al, 1996) and measurement of cytokines at the tissue level in humans has its own practical problems. Comparatively, studying ex-vivo cytokine production in isolated cells after infection is associated with less difficulties and more accurate results.

Hence, in the present study, monocyte derived macrophage (MDM) and monocyte derived dendritic cells (MDDC) were isolated and infected in *ex vivo* condition with different strains of MTB. Further, the whole blood assay was included as it has both practical and theoretical advantages of representing *in vivo* situation. To analyze the patterns of immune response to bacterial constituents, sonicates of different strains of MTB were also included for this study.

In the earlier part of the study, ancient strain, EAI-5 and modern strain Beijing showed extremely opposite responses. Since MDM and MDDC are not very abundant, only these two strains were selected along with laboratory strain H37Rv for monitoring the host immune responses induced by them.

4.2 Materials and Methods

4.2.1 Study subjects

Seven tuberculin skin test positive healthy subjects were included (5 men and 2 women; mean age 32 years) in this study. It is known that, MTB antigens induce the production of IFNγ in skin-test positive individuals but not in skin test negative controls. Hence, in the current study, Mantoux positive individual's blood samples were taken to compare the differential cytokine induction by live and dead bacteria. Blood samples were collected from three healthy individuals who had previous history of tuberculosis infection (2 pulmonary and 1 extra-pulmonary). All the subjects did not have any chronic illness and were not affected by any acute medical problem at the time of the study. Heparinized blood samples were drawn from each subject after obtaining informed consent and the approval from Medical Ethics Committee, BARC.

4.2.3. Mycobacterium tuberculosis strains

As described in chapter 2.2.3 section and Table 4.

4.2.4. *Mycobacteria*l growth, single cell suspension and whole cell sonicate

Single cell suspensions of bacteria were prepared as described in 3.2.1. Total sonicate of *M. tuberculosis* was prepared by suspending 10^8 CFU of dried bacilli per ml in ice-cold PBS and sonicating with a probe sonicator (Sonifier 450; Branson Ultrasonics Corp.,

Danbury, Conn.) at 40% maximum power output for 5 min. This *M. tuberculosis* sonicate represents a suspension of all of the antigens of the bacillus.

4.2.5. Ex-vivo infection of MDMs and MDDCs

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood by density gradient centrifugation using Histopaque-1077 (Sigma) according to the manufacturer's protocol. To isolate monocytes, PBMCs were plated in Nuncleon Suface 24-well plates (Nunc, Denmark) containing RPMI- 1640 (Sigma, Germany) with 10% heat-inactivated fetal bovine serum (FBS; Sigma, Germany), 2 mM L-glutamine and 100 units of gentamycin for 2 hours at 37^{o}C. Non-adherent cells were removed by washing with phosphate buffered saline (PBS) containing 3% FBS and adherent cells were incubated for 7 days at 37^{o} C, in 5% CO_2 to obtain MDMs. The purity of MDM was assessed microscopically as well as by flowcytometry labelling adherent cells with CD-14 primary antibody and FITC conjugated secondary antibody. Approximately 84% cells were CD-14+ or MDM (Figure 27).

MDDC were generated by culturing adherent monocytes in 24-well tissue cultures plates with 400U/ml GM-CSF and 400 U/ml IL-4 (BD) for 5 days in RPMI 1640 with supplements as mentioned above. The purity of MDDC was assessed by flowcytometry after labelling cells with CD1a primary antibody and FITC conjugated secondary antibody. Approximately 73% labelled cells were MDDC (Figure 27). FACS analysis for MDM and MDDC population was done only for one sample during protocol standardization and the same protocol was followed for rest of the samples.

Both MDM and MDDC were infected with single cell suspension of the two clinical isolates, EAI5 and Beijing, along with the vaccine strain BCG and H37Rv for 4 hours at a MOI of 5. The cells were washed to remove extracellular *Mycobacteria* and incubated for a further 20 h before RNA extraction.

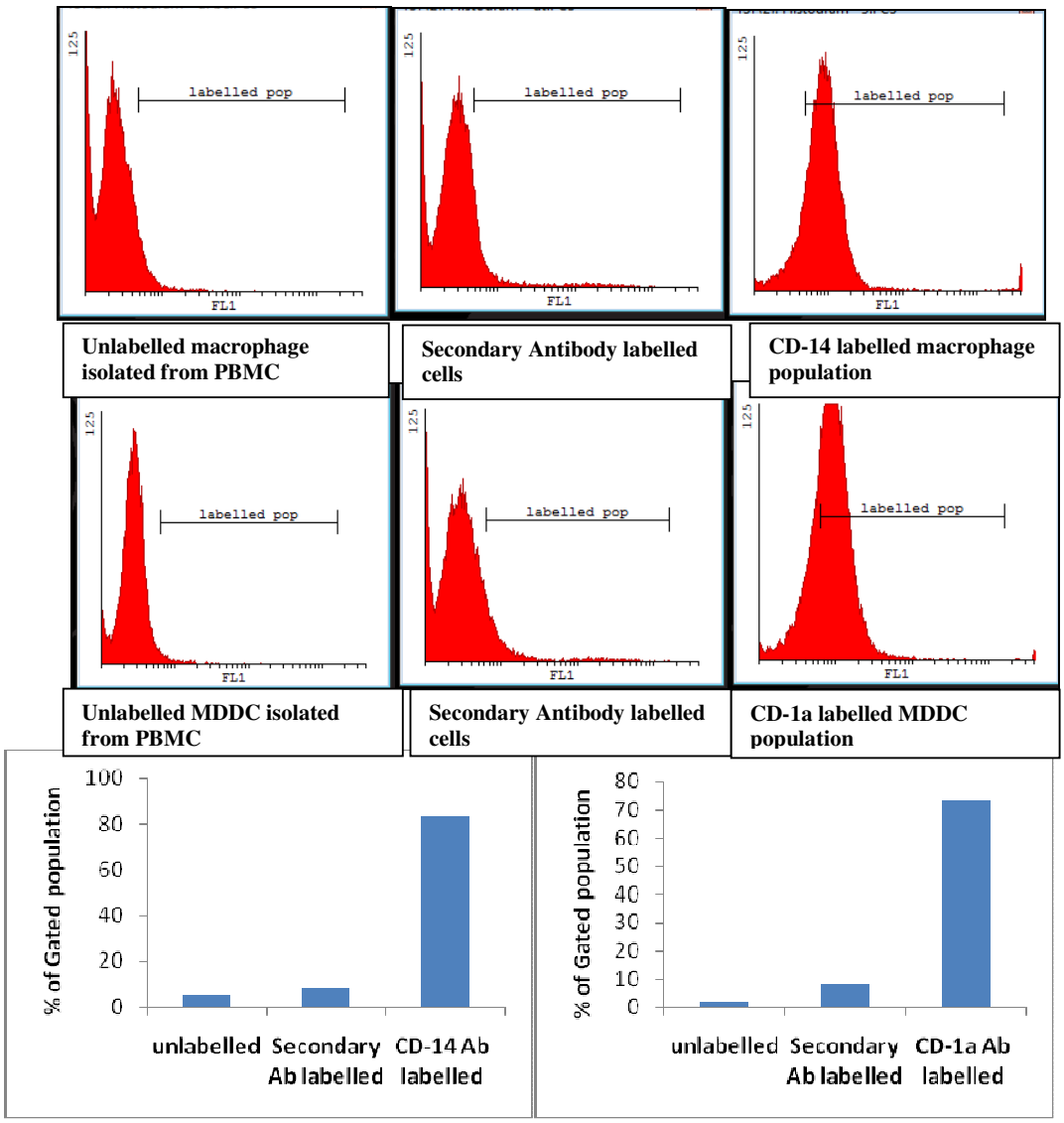


Figure 27: Phenotypic purityof MDM and MDDC isolated from PBMC by FACS analysis

PBMCs were plated in Nuncleon Suface 24-well plates (Nunc, Denmark) in complete medium for 2 hours at 37°C. Non-adherent cells were removed by washing with phosphate buffered saline (PBS) containing 3% FBS and adhered cells were incubated for 7 days at 37° C, 5% CO_2 to obtain MDMs. Adherent cells were labelled by anti-CD-14 primary antibody and FITC conjugated secondary antibody analyzed by flowcytometry. Approximately 84% cells were CD-14+. MDDC were generated by culturing adherent monocytes in 24-well tissue culture plates with 400U/ml GM-CSF and 400 U/ml IL-4 (BD) for 5 days in complete medium. The cells were labelled by CD1a primary antibody and FITC conjugated secondary antibody. Flowcytometric analysis revealed that 73% cells were labelled (MDDC).

4.2.6. RNA extraction, cDNA synthesis and RT-PCR

After 24 hr of infection, RNA was extracted from infected macrophage and DCs using RNA extraction kit (HIMEDIA) and it was digested with Dnase I (Roche). Thereafter, cDNA was synthesized from 1µg of RNA using cDNA synthesis kit (Fermentas Cat.No.#K1622). Quantitative RT-PCR was performed for TNF-α, IL-1β, IL-12, IL-10, MCP-1 ,IL-8 and β-actin (Table-F in Appendix-II) using SYBR Green master Mix (CAT # 600548, Stratagene) with the following amplification conditions: 95˚C for 10 min, for 40 cycles at 95˚C for 15s, 60˚C for 30s and 72˚C for 30s. Melting curve analysis was performed for confirming the specificity of PCR. Further, the Ct values for each gene amplification were normalized with respect to house-keeping gene, β-actin by $2^{-\Delta\Delta Ct}$ method and the expression levels are presented as fold induction in comparison to uninfected cells (Livak et al, 2004).

4.2.7. Whole blood assay with live bacteria and whole cell sonicates

Blood samples were collected in apyrogenic heparinized tubes (Vacutainer; Becton Dickinson, Mountain View, CA). The blood was processed immediately and diluted with 1 :1 RPMI 1640 supplemented with 2mM glutamine and 100 U/ml penicillin and was then distributed in 24 well tissue culture plates as 1 ml of diluted blood for each well. Either 10^5 CFU/ml live bacteria or *Mycobacteria*l whole cell sonicate from similar number of bacteria was added to each well. LPS (endotoxin from *E. coli* ; 026B6 Sigma) 1µg/ml was used as a positive control and only medium as a negative control. The plates were incubated at 37^0C with 5% CO_2 for 24 h. The contents of the wells were then collected and centrifuged at 900 g for 10 min. Supernatants were recovered and frozen at -70^0C until use.

4.2.8. Cytokine estimation by ELISA

Supernatants from infected cells were collected, after 24 hours, centrifuged and frozen at -70˚ C until used. Quantification of TNF-α, IL-12, IFN-γ and IL-10 was carried out using commercial ELISA kits (BD OptEIA). The procedures are briefly described insection 3.2.9. of Chapter 3.

4.2.9. Statistical analysis

Data analysis, correlation study, Mann-Whitney U tests and Kruskal-Wallis rank tests were performed using Sigmastat software version 3.5. Without assuming a pre-defined distribution of the response tested, non-parametric statistical analysis has been used all across the study.

4.3. Results

4.3.1 Mycobacterium tuberculosis isolates differ in their ability to induce cytokines in MDMs and MDDCs

To address the link between MTB genotype and host immune responses, two clinical isolates representative of the modern and ancient clades of human MTBC were selected from a well-characterized clinical strain collection from previous study, along with two laboratory strains (*M. tuberculosis* H37Rv and *M. bovis* BCG Pasteur). Monocyte derived macrophages (MDMs) and monocyte derived dendritic cells (MDDCs) were taken as host cells from seven different healthy individuals and infected *ex vivo* with MTB strains at a MOI of 5 for 4 hours. Using Acid Fast staining, it was observed that approximately 60% of cells had phagocytosed *Mycobacteria* at this time point. mRNA expression for the cytokines like TNF-α, IL-1β and IL-12 (proinflammatory), IL-10 (anti-inflammatory) and IL-8, MCP-1 (chemokines) was analyzed from 24 hr infected MDM and MDDC by SYBR Green Real time PCR and the levels are shown in Figure 28 and Figure 29. The relative hierarchy of low and high responses induced by modern and ancient lineages respectively was observed to be similar to one seen in previous study with THP-1. The ancient strain EAI-5 induced significantly higher pro-inflammatory cytokine and chemokine response compared to that

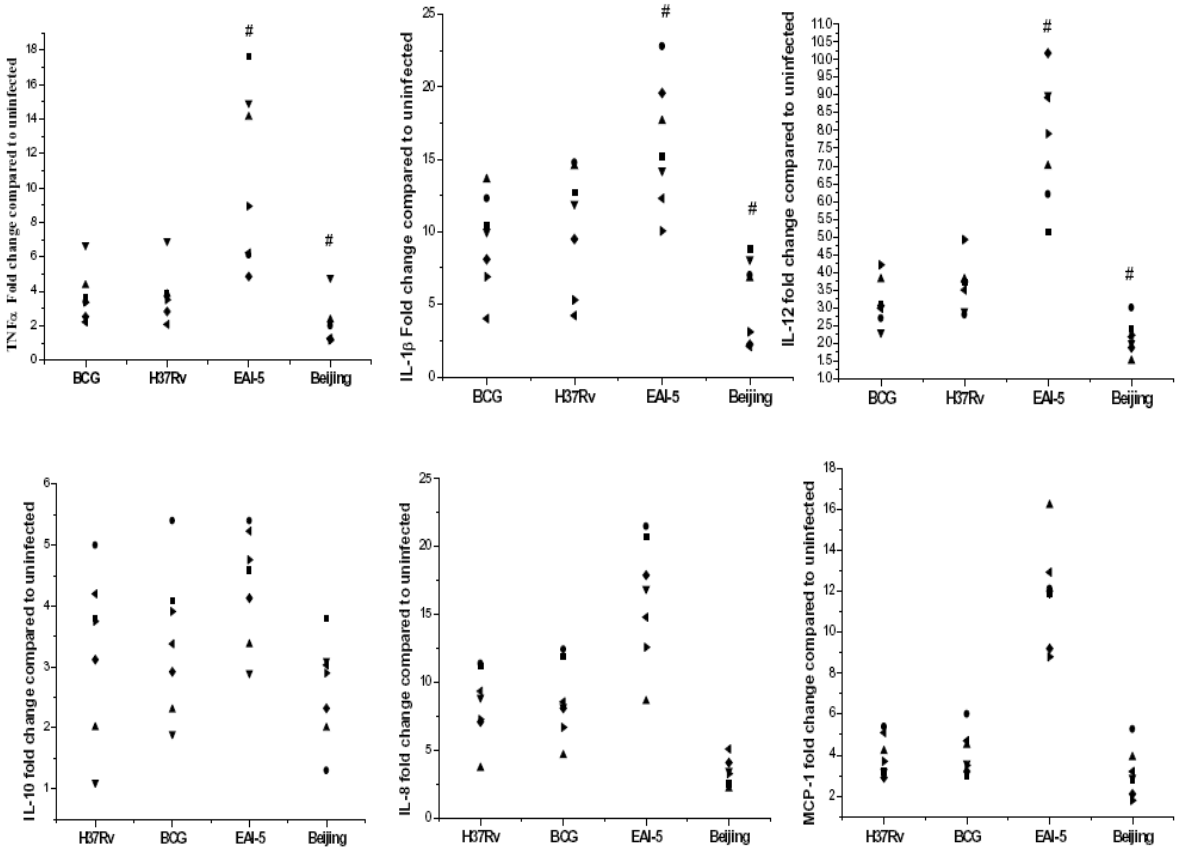


Figure 28: Cytokines and chemokines induced in infected MDMs by different strains of MTB.

Real time PCR was carried out to estimate the mRNA expression for TNF-α, IL-1β, IL-12, IL-10, MCP-1 and IL- 24 hrs after infection of. human monocyte-derived macrophages (MDM). Data from seven independent donors are shown in a scatter plot representation and have been clustered according to strain lineage. MDMs were infected with *M. tuberculosis* BCG, H37Rv and two clinical isolates (EAI and Beijing) at MOI 5 for four hours and after removing extracellular bacteria, the infected cells were kept for another 20 hours in complete medium. The total mRNA was extracted from infected MDMs at 24 hour of infection. The bar shows relative mRNA expression corrected for total mRNA using the housekeeping β-actin gene. Non-parametric Kruskal-Wallis test revealed a significant effect of different lineages in cytokine production (P,0.001). '#' sign indicates significant difference compared to infection by H37Rv where p is at least ≤0.05.

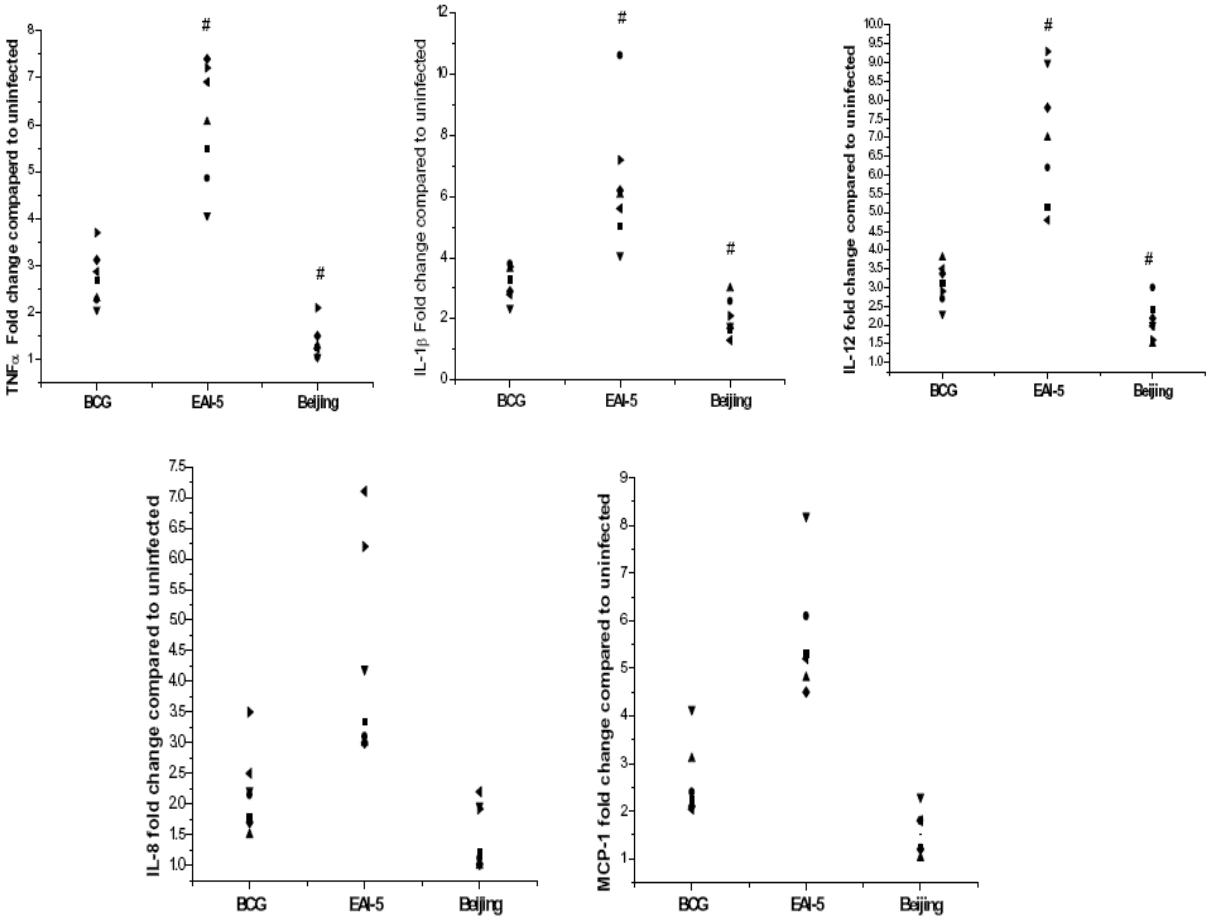


Figure 29: Cytokines and chemokines induced in infected MDDCs by different strains of MTB.

Real time PCR was carried out to estimate the mRNA expression for TNF-α, IL-1β, IL-12, MCP-1 and IL-8 24 hr after infection. Data for human monocyte-derived dendritic cells (MDDCs) from seven independent donors are shown in a scatter plot representation and are clustered according to strain lineage. MDDCs were infected with *M. tuberculosis* BCG, H37Rv and two clinical isolates (EAI and Beijing) at MOI 5 for four hours and after removing extracellular bacteria, the infected cells were kept for another 20 hours in complete medium. Then the total mRNA was extracted from infected MDDCs at 24 hour of infection. The bar shows relative mRNA expression corrected for total mRNA using the housekeeping β-actin gene. Non-parametric Kruskal-Wallis test revealed a significant effect of different lineages in cytokine production (P,0.001). '#' sign indicates significant difference compared to H37Rv where p is at least ≤0.05.

induced by laboratory strains and Beijing induced sometimes lower and sometimes similar cytokine levels compared to H37Rv. This pattern of cytokine secretion was maintained in all the seven donors, though there were fluctuations in the absolute levels of the same cytokine for different donors.

The patterns for the cytokine responses of MDM and MDDC following infection with different strains were similar in the present study. However, it was observed that mRNAs for pro-inflammatory cytokines, TNF-α, IL-1β and IL-12, were expressed at high levels preferentially by MTB-infected MDM, whereas MDDC produced low levels of these cytokines except for IL-12. On the other hand, IL-10 gene expression was not in the detectable range in infected DCs. In addition, similar to the earlier experiments in THP-1 cells, all the proinflammatory cytokines like TNF-α, IL-1β and IL-12 induced by a particular strain showed correlation among each other when analyzed across the panel of seven donors. The correlation coefficient between production of TNF-α and IL-1β was 0.890; TNF-α and IL-12 was 0.850; IL-1β and IL-12 was 0.849 (Spearman rank correlation coefficient when $P<0.001$) across the seven donors for all four strains. There were no significant difference in the expression of these cytokines induced by laboratory virulent strain H37Rv and vaccine strain BCG in the present study.

4.3.2. Cytokine response in whole blood infected with different clinical isolates of MTB

To evaluate how macrophages respond to infection with different MTB strains in the presence of lymphocytes, whole blood assay was done. It also reflects *in vivo* conditions (De Groote, et al., 1992). The concentrations of cytokines, monitored 24 hours post infection with ancient strain EAI, modern strain Beijing and reference strain H37Rv by ELISA are depicted in Figure 30. In this assay, lipopolysaccharide (LPS) was included as positive control stimulus for monocytes and only RPMI medium was kept as a negative control. There was a clear difference in the level of pro-inflammatory cytokines produced by a single host in

response to infection with different strains and the relative hierarchy of low and high responses was maintained as observed in MDM and MDDC infection. The concentration of TNFα induced by H37Rv and EAI-5 was 10-15 ng/ml and 19-26 ng/ml respectively in whole blood of different individuals; whereas uninfected cells produced 0.185-0.780 ng/ml. IFN-γ concentration also varied with strain type with EAI-5 and Beijing inducing significantly higher and lower levels respectively compared to H37Rv. No significant difference in the level of IL-10 was observed after infection by different strains.

Similar whole blood assay was also performed for three previously infected individuals and levels of TNF-α, IL-12 and IFN-γ were monitored after *ex vivo* co-culturing with live MTB. The responses were compared with healthy individuals who didn't have history of TB. There was significant difference in the levels of IFN-γ and IL-12 in these two groups when compared in whole blood supernatant 24 hours post-infection (Figure 31).

4.3.3. Cytokine response in whole blood stimulated by the sonicates of different clinical isolates of MTB

It was observed that cytokine expression pattern for whole blood, infected with live bacteria of different strains was similar to that observed with infected MDM and MDDC. To find out whether intact bacteria were required for the differential cytokine pattern of a given strain, cell sonicates were used for stimulating whole blood cells. Differential cytokine response was not seen when sonicates were used for stimulating the whole blood cells (Figure 32).

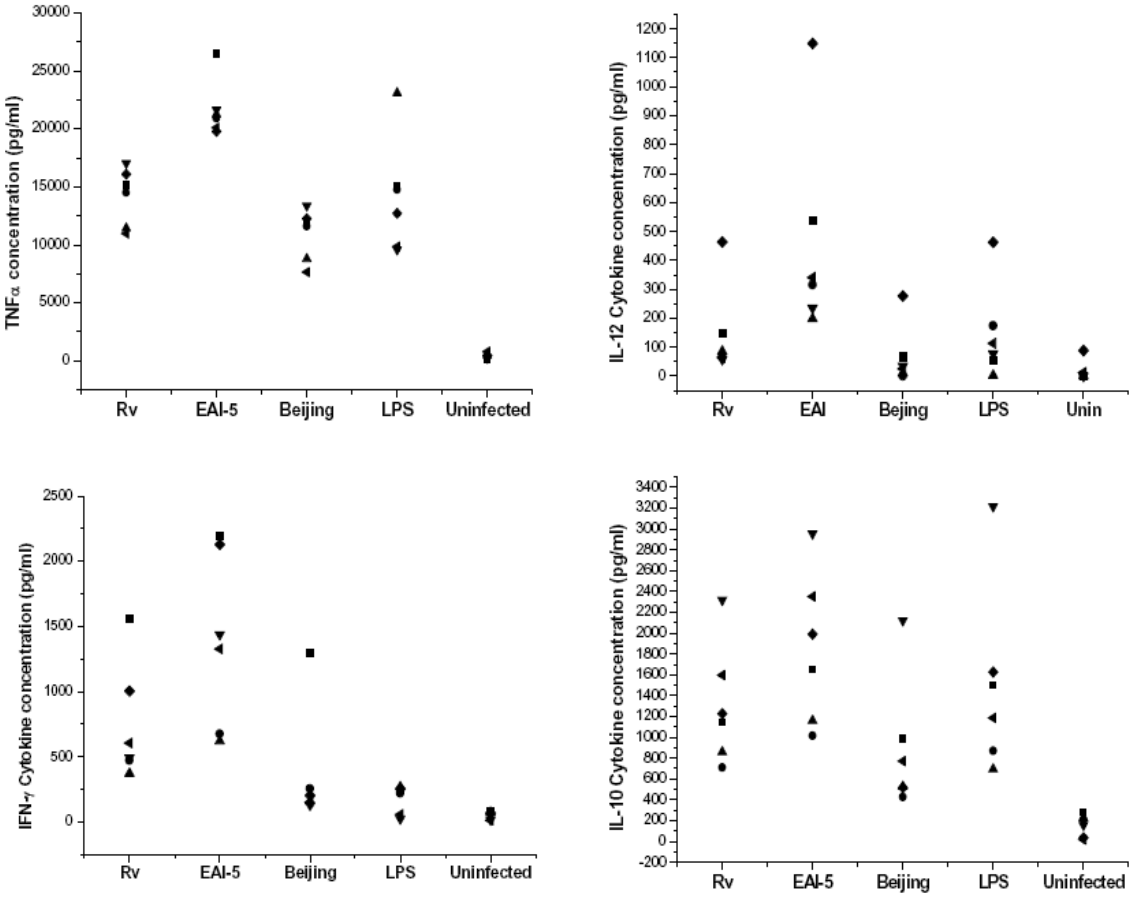


Figure 30: Induction of cytokines by live strains of MTB in whole blood assay

After ex-vivo infection by different strains of MTB the levels of TNF-α, IL-12, IFN-γ and IL-10in the supernatant were estimated by ELISA. Their levels are shown in scatter plot representation and according to strain lineage. Data from diluted whole blood from six healthy subjects after stimulation with 10^5 live MTB single cells along with LPS (positive control) after 24 hours are shown.

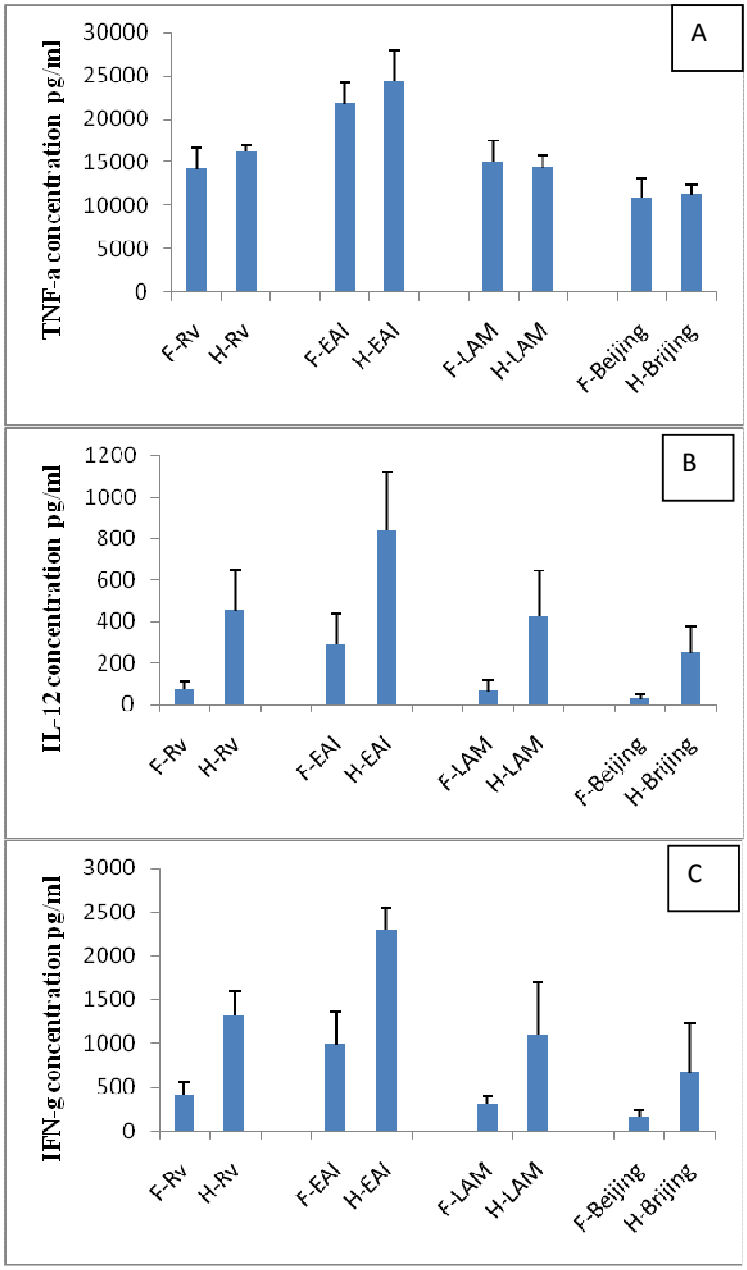


Figure 31: Cytokines induced by healthy sensitized persons and previously treated person after infection with strains of MTB in whole blood assay

Cytokines induced after ex-vivo infections were estimated by ELISA and averaged for each individual. Healthy individuals are referred to as 'F' and individuals with previous TB are referred to as H. The levels of TNF-α (A), IL-12 (B), and IFN-γ (C) in the supernatants of diluted whole blood after stimulation with 10^5/ml live MTB single cells from different genotypes after 24 hours are shown.

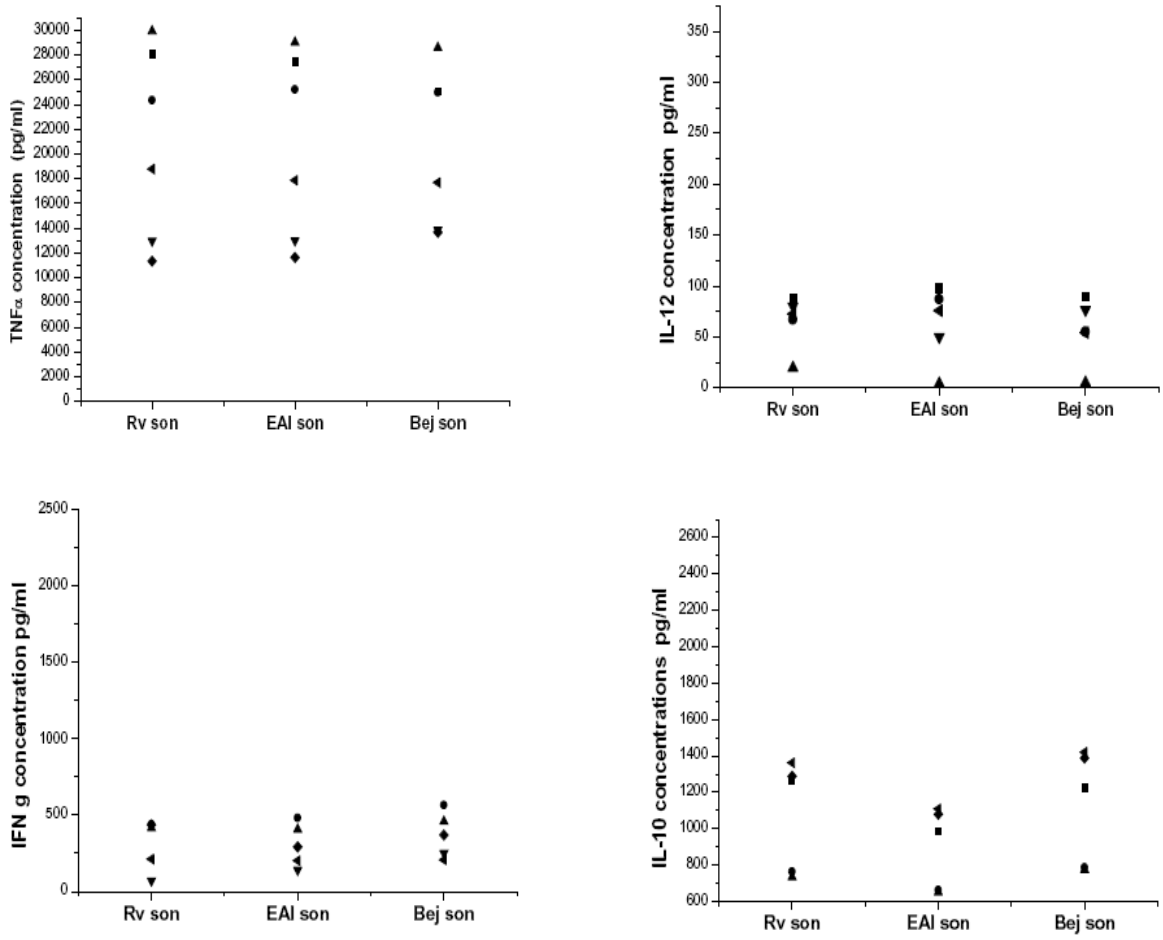


Figure 32: Cytokines induced in whole blood by whole cell sonicate of MTB

Cytokines induced following *ex vivo* stimulation of whole blood of six healthy individuals by sonicates of 10^5 MTB cells after 24 hours, were assessed by ELISA.

4.4. Discussion

The proinflammatory cytokines like TNF-α and IL-12 play essential role in host defence, as they dictate containment of intracellular pathogen and granuloma formation in TB (Senaldi et al, 1996; Klingler et al, 1997). In addition, the induction of proinflammatory cytokines by different MTB strains determines its virulence (Manca et al, 2001). TNF-α, produced by monocytes and macrophages in early infection plays a key role in protective immunity during TB infection. Further, IL-12 production is essential to induce a protective Th1 response (Cooper et al, 1997). Furthermore, chemokines like IL-8 and MCP-1 are important in TB and recruit inflammatory cells at the site of infection (Juffermans et al, 1999). As the interplay of the proinflammatory cytokines and chemokines is a hallmark of TB infection, the interleukins secreted by the host after infections with different genotypes can be considered as good markers for correlating MTB genotypes with the innate responses induced by them. In view of this, in the present study, the expression of different cytokines and chemokines was analyzed in MDM, MDDC and whole blood cells infected with different strains of MTB.

It has been reported that ancient lineage MTB strains induced higher proinflammatory cytokines in human macrophages and higher interferon-γ in peripheral blood T cells (Torres et al, 1998). In agreement with this report, in the present study, profound induction of proinflammatory cytokines (TNF-α, IL1b and IL-12) by ancient (EAI) was observed and modern (Beijing) lineages suppressed this response in THP-1 cells. This pattern of cytokine gene expression remained similar even in MDM, MDDC as well as whole blood infected with respective strains. In addition, there were clear differences in the levels of proinflammatory cytokines produced by a single donor in response to different strains and a significant correlation was observed among each of the proinflammatory cytokine pairs induced by a single strain across the multiple donors. IL-10, an anti-inflammatory cytokine

suppresses TNF-α and IL-12 expression. Differential expression of IL-10 induction was not observed in MDMs, on the other hand, IL-10 induction observed in MDDC, infected with different strains, was at a negligible level

It was observed in the earlier studies, that the response induced by MTB varies with the change in the microenvironment of the infection (Kaplan et al, 2003). Hence, whole blood assay was carried out to evaluate the innate response induced by different MTB strains which will have contributions from MDM and MDDC and lymphocytes. In one of the earlier studies the differential host response seen in isolated MDM, was not observed in peripheral blood mononuclear cells for different strains (Portevin et al, 2011). In contrast to this, some research groups showed the differential cytokine response to the MTB strains in whole PBMC (De Groote et al, 2003). The dilution of blood and incubation in tissue culture wells facilitates the use of much smaller volumes of blood and longer incubation periods, both of which may be advantageous for the detection of cytokines with a lower production rate (e.g. IL-10). Whole blood might therefore be a good substitute for purified populations like MDM and MDDC.

However, even with optimum standardization many cellular and humoral variables in the blood cannot be controlled. Plasma components such as cortisol (Petrovsky et al, 1994), lipids (Ulevitch et al, 1978) or soluble cytokine receptors (Dinarello, 1996) may influence results by enhancing or inhibiting cell stimulation and inactivating or capturing secreted cytokines. All these effects reflect *in vivo* conditions and are less prominent in diluted blood assay. The induction of proinflammatory responses across the isolates was compared and similar lineage specific response was observed. A typical pattern of a protective Th1 cytokine was observed for EAI strain in whole blood assay. These observations are in agreement with the results obtained in an *ex vivo* infection with MDM and MDDC of particular human subjects. Interestingly, the induction of IFN-γ and IL-12 was significantly higher in the blood

of previously diseased persons compared to others. These persons probably have higher percentage of sensitized T-cells which enable them to give higher response when co-cultured with live bacilli. This strain specific pattern of cytokine induction was absent when MTB cell sonicates were used instead of live bacteria. It is possible that different components of the sonicate induce contrasting pathways of regulation of cytokine secretion, so that the net concentration of induced cytokines was similar for sonicates of all the strains used.

The responses to different MTB strains were similar to THP-1 cells and primary cells isolated from PBMC. Several other factors like concentration of vitamin-D, glucose, mutation in cytokine genes are known to influence disease outcome *in vivo*. It is therefore, necessary to evaluate responses to the strains *in vivo* in an animal model.

Chapter 5: *In vivo* infection study in BALB/c mice using strains of *Mycobacterium tuberculosis* belonging to different genotypes

5.1. Introduction

After the first exposure to *Mycobacterium tuberculosis*, a series of immune responses are triggered that define the course of the infection. However, it is evident from the earlier part of the present study, that the host response is not uniform for different clinical isolates of MTB. Human macrophages, both from primary cultures and from transformed monocyte cell line, THP-1 serve as a model of infection for clinical isolates of MTB belonging to different genotypes. However, infection in cell lines and isolated primary cells has their own disadvantages as it doesn't truly represent the original infection condition. Therefore, the animal models for MTB infection studies are thought to be useful for further study of host–pathogen interaction with the selected strains.

Mice can be easily infected via several routes and the bacilli can grow easily in lungs and subsequently spread to liver and spleen (Gupta & Katoch, 2009). The resulting infection is well tolerated for more than one year in some strains of mice (Flynn et al, 2001). In addition, guinea pigs (Flynn et al, 2006), rabbits (Dannenberg et al, 1994), and non-human primates (Langermans et al, 2001) serve as additional mammalian hosts. *Mycobacteria*l infection is observed even in more distantly related ectotherms, including frogs and transparent zebra fish (Cosma et al, 2004; Cosma et al, 2006).

The mouse strains, BALB/c as well as C57BL/6 are well established for MTB infection studies. The mean survival time of these two strains are higher compared to DBA/2 and C3H/HeJ strains after infection (Forget A et al, 1981). Among the two mouse strains, it is observed that C57BL/6 is significantly resistant to MTB infection compared to BALB/c. C57BL/6 mice produce a strong Th1 response which is able to limit and resolve the infection whereas BALB/c mice, which are dominated by IL-4 production, develop progressive

disease. The difference between the two appears to be governed by multiple genes (Reiner et al, 1995), one of which may be related to the major histocompatibility complex (MHC) (Roberts et al, 1997). Therefore, BALB/c mice are widely used for the studies of disease susceptibility (Roch et al, 1990; Wakeham et al, 2000; Roque et al, 2007) and assessment of immunological parameters induced by the pathogen (Hernandez et al, 1996; Hernandez et al, 1997). The immune response to MTB in mouse has been shown to correlate with the human system, including the importance of CD4+ T cells (Muller et al, 1987; Caruso et al,1999; Scanga et al, 2000), interleukin-12 (Cooper et al, 1997) and tumour necrosis factor-α (Bean et al, 1999; Keane et al, 2001; Mohan et al, 2001). These cytokines activate macrophages and induce iNOS expression. The NO produced in this process is essential for mice to kill intracellular *Mycobacteria* (Cooper et al, 2000). This protective activity fails if there is a marked release of Th2 type cytokines (Powrie & Coffman, 1993; Lucey et al, 1996). The interplay of cytokines is depicted clearly in a BALB/c model of pulmonary tuberculosis (Wakeham et al, 2000). This model offers the following benefits: first the rate of bacterial multiplication in the lungs correlates with the extent of tissue damage and mortality. Second, the infection is controlled successfully as long as a strong T helper type 1 (Th1) cell response is sustained.

In experimental mouse TB models, the route of bacterial administration can influence the level or nature of immune responses generated. TB infection was mostly established by pulmonary or intravenous inoculation of MTB in mouse models. Higher extra-pulmonary load of MTB was observed in the intravenously (i.v.)-infected animals compared to the intra tracheally (i.t.)-infected animals. In addition, differences in immune response following i.t. infection versus i.v. infection may add to differences in MTB clearance from the various organs. North et al. (1995) described the difference in pathogenesis of TB in aerosol-infected mice versus intravenously-infected mice. Low dose aerosol infection is the most

physiologically natural method for infecting experimental animals with MTB. Aerosol infection was shown to allow more rapid lung pathology and higher rate of bacterial growth in mice than intravenous infection (North et al, 1995). Natural MTB infection through inhalation may also have an influence on the course of infection (Mc Kinney et al, 1998).

In aerosol model, mice are challenged with a low dose of aerosol of virulent MTB which can multiply in the lungs and spread to other organs, most notably spleen and the liver. The initial phase is dominated by high production of Th1 cell cytokines that, together with high levels of TNF-α and iNOS, temporarily control the infection. Granuloma develops in this phase. Three weeks after the infection, the expression of Th1 cell cytokines, TNFα and iNOS starts to decline. Gradually, pneumonic areas prevail over granulomas. In the current study, BALB/c mouse model of progressive pulmonary TB by aerosol infection was used, to examine the course of infection in terms of strain virulence (lung bacillary load, histopathology) and immune responses (cytokine expression determined by RT-PCR) induced by different MTB strains selected from previous study.

5.2 Materials and Methods

5.2.1 *Mycobacteria*l growth and single cell suspension

As described in section 3. 2.1. in chapter 3.

5.2.2 Murine model of progressive pulmonary tuberculosis

The experimental model of pulmonary tuberculosis has been described in detail previously (Hemandez-Pando et al 1996, Hemandez-Pando et al, 1997, Hemandez-Pando et al, 2001). Eight week-old, male BALB/c mice, free of common viral pathogens were purchased from National Institute of Nutrition (Hyderabad). Mice were exposed to the aerosol with the final bacterial count of; 10^6 bacilli/ml. for 15 min using a Lovelace nebulizer (In-Tox Products, Albuquerque, NM). This implants approximately 200 organisms into the lungs of each mouse as confirmed by plating lung homogenates next day after infection. Groups of 17 mice were

infected by *M. bovis* BCG and each strain of *M tuberculosis*, such as H37Rv, EAI-5, LAM-6 and Beijing. Another group of 15 mice was left uninfected, undisturbed and were sacrificed at specific time points with other infected mice which served as a negative control. Of the 17 mice from five infected groups, 2 mice were sacrificed to confirm the infection by plating their respective lung homogenate and five mice from each groups were killed by cervical dislocation at 15, 30 and 60 days after infection. Lungs were used for CFU analysis, RNA extraction and histopathological study. The protocol was approved by the animal ethic committee of International Centre for Genetic Engineering and Biotechnology (ICGEB).

5.2.3.Colony-forming unit (CFU) counts from infected lung

Lungs of 5 mice from each experimental group were harvested 15, 30 and 60 days after infection, for colony-forming unit determination. The right or left lungs were homogenized with a Polytron homogenizer (Kinematica, Luzern, Switzerland) in sterile tubes containing 2 ml of isotonic saline. Three different dilutions of each homogenate were spread onto Bacto Middlebrook 7H11 agar plates enriched with oleic acid, albumin, catalase and dextrose (Difco Laboratories, Detroit, MI, USA) in duplicate. The numbers of colonies were counted 21 days post-infection.

5.2.4. RT-PCR analysis of cytokines and iNOS in lung homogenates

RNA from mouse lung was isolated with RNA extraction Kit (HIMEDIA, India) according to the manufacturer's instructions. Briefly, lung homogenate was prepared in lysis buffer and subsequently passed through RNA extraction column. DNaseI digestion was performed on the extraction column itself and RNA was isolated in DEPC treated water. Thereafter, cDNA was synthesized using 1µg of RNA by cDNA synthesis kit (Cat.No.#K1622, Fermentas Life Science). Quantitative real-time RT-PCR was performed with TNF-α, IL-12, IL-10, IFN-γ, iNOS and β-actin primers (shown in Table-G in Appendix-II) using SYBR Green master Mix (CAT # 600548, Stratagene, La Jolla, Ca, USA) with the

following amplification conditions: 95˚C for 10 min, for 40 cycles at 95˚C for 15s, 60˚C for 30s and 72˚C for 30s. Melting curve analysis was performed for confirming the specificity of PCR. Further, the Ct values for each gene amplification were normalized with respect to house-keeping gene, β-actin by $2^{-\Delta\Delta Ct}$ method (Livak et al, 2004) and the expression levels were presented as fold induction in comparison to uninfected mice.

5.2.5. Restimulation Assay

Spleens from infected and uninfected mice were removed aseptically and single cell suspensions were prepared by pressing the spleen through a sterile mesh. Erythrocytes were removed by incubation in red cell lysis solution (1.5 M NH_4Cl, 100 mM NaHCO3, 10 mM disodium EDTA) (Sigma), pH 7.2 for 12 min, followed by centrifugation and resuspension in RPMI 1640 medium (Sigma) supplemented with 10% foetal bovine serum (FBS) (Hyclone Laboratories), 200 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were plated at a concentration of 2×10^5 cells/ ml in a 24 well plate and were stimulated with either PPD (10μg/ml) or ConA (5μg/ml) for each group of mice. After 48 hours, the supernatants were collected, centrifuged (2000 rpm for 5 minutes) and stored at -70^{o}C and later used for estimation of IFN-γ by ELISA (described in section 3.2.9.).

5.2.6. Histopathology

Immediately after dissection the lungs were immersed in 10% formalin in PBS. After 24 hours, the specimens were embedded in paraffin. Five micrometre thick haematoxilin and eosin stained transverse sections were examined by light microscopy. Coded sections were viewed and scored. For each time point, the lungs of three animals were examined for histopathological parameters such as perivascular infiltration and granuloma formation.

5.2.7 Statistical Analysis

Statistical analysis of lung CFU, histopathology features and cytokine expression was performed using student's t-test and $P<0.05$ was considered as statistically significant.

5.3. Results

5.3.1. Bacterial load in the lungs

Successful infection by all the strains was confirmed by the mean number of CFUs obtained 24 hr post infection in the lungs of mice which was about 200 for all the clinical isolates. Figure 33 shows CFU in lungs of mice infected with strains belonging to each genotypic family. After 15 days of infection, the MTB loads in the lungs of the mice were similar for all the strains. However, mice infected with five different strains of *M tuberculosis* showed a significantly different lung bacillary load at 30 days post-infection. Beijing and LAM strain showed significantly higher bacillary load compared to H37Rv, BCG and EAI strains ($P<0.05$). CFU in the lungs of mice infected with BCG and EAI did not differ statistically from each other or from counts obtained with H37Rv. Interestingly, this increase in the load of MTB in the lungs of infected mice was transient, and by 60 days of infection the bacterial count decreased significantly ($p<0.05$) in the mice infected with Beijing, LAM and H37Rv.

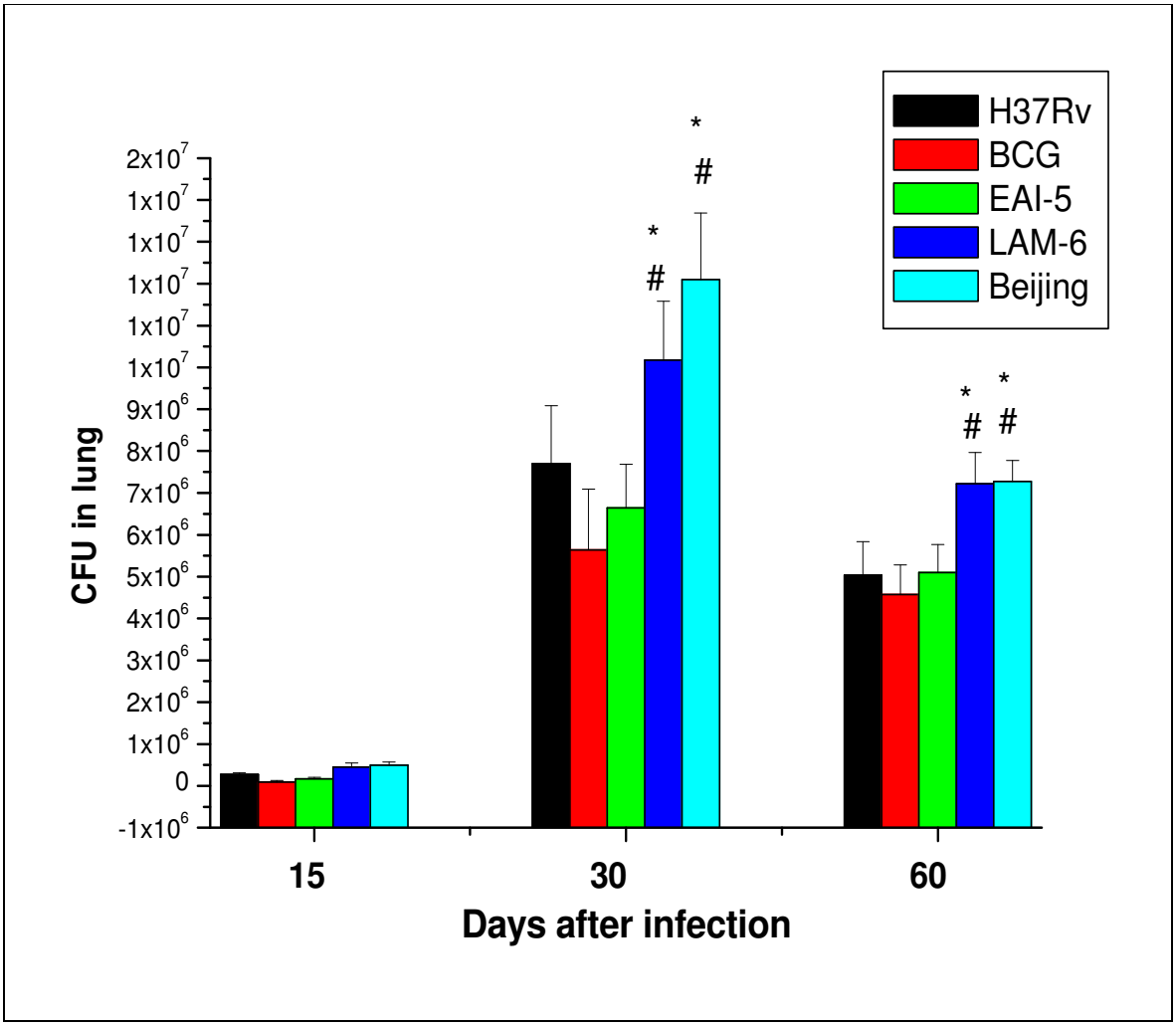


Figure 33: Bacterial load in the lungs
BALB/c mice were infected with aerosolized *M tuberculosis* strains of BCG, H37Rv, EAI-5, LAM and Beijing and after 15, 30 and 60 days of infection, the lung CFU was determined. The data are presented as mean CFU counts ± SD (5 mice/group/time point). The symbol # and * represent statistically significant difference when compared to infection at a similar time point by H37Rv and BCG respectively.

5.3.2. Cytokines and iNOS gene expression in lung homogenates

Gene expression for proinflammatory cytokines like TNF-α, IL-12 and anti-inflammatory cytokine IL-10, was assessed in infected mouse lungs by Real Time PCR 15, 30 and 60 days after infection (Figure 34 and 35). In view of the fact that serum cytokines do not represent the true infection situation, assessment of local mRNA expression is considered to be the best way to measure strain dependent immune response. The mRNA expression of TNF-α, IL-12, iNOS (Figure 36) and IFN-γ (Figure 34) was significantly ($P<0.05$) higher in mouse lungs infected with EAI-5 and BCG than in those infected with LAM and Beijing strains at 15 days after infection. However, the expression of these genes was comparable in Beijing, LAM-6 and H37Rv infected mice at this time point. H37Rv infected mice showed higher TNF-α compared to Beijing and LAM at 30 days after infection. The lower levels of TNF-α and IFN-γ detected in the lungs of mice infected with Beijing indicated less Th1 immune activation in mice infected with this strain. In addition, IL-10 mRNA levels were also lower in Beijing-infected mice. There was no significant difference in expression of IL-10 mRNA in the lungs infected with different strains, except for LAM which induced significantly higher amount of IL-10 at 30 days after infection ($P<0.05$). The most exciting observation about different strains was that the cytokine induction pattern at 15 days post infection was similar to the one observed in *in vitro* infection studies with THP-1 cells.

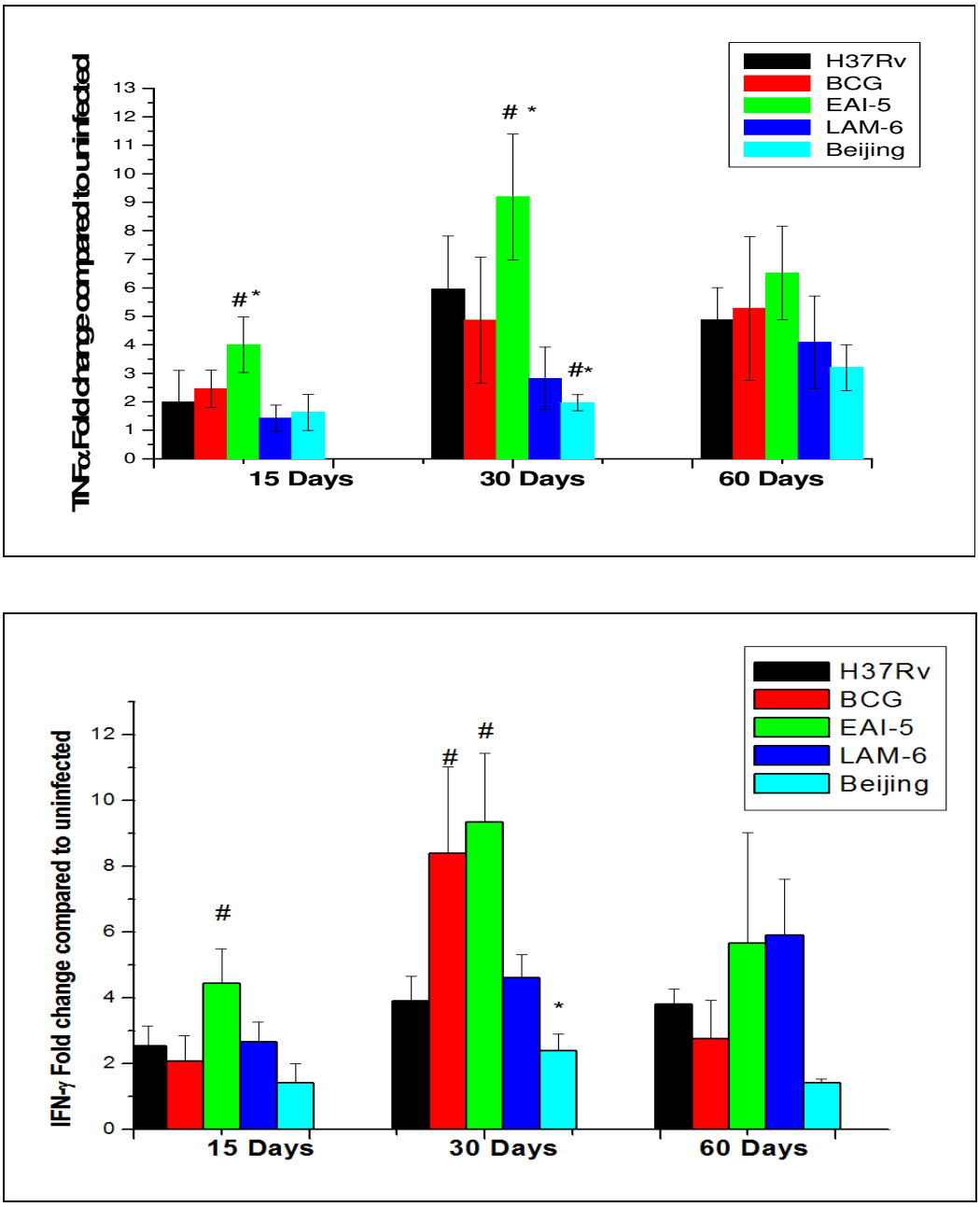


Figure 34: Assessment of TNF-α and IFN-γ in infected mouse lung (By Real time PCR)

Real time PCR was carried out to estimate the mRNA expression for TNF-α and IFN-γ at indicated time point, after infection of BALB/c mice with *MTB* H37Ra, H37Rv and three clinical isolates. The bars show relative mRNA expression corrected for total mRNA using the housekeeping β-actin gene. Data represent the means ± standard deviation of the samples. The symbol # and * represent statistically significant difference when compared to infection at a similar time point by H37Rv and BCG respectively.

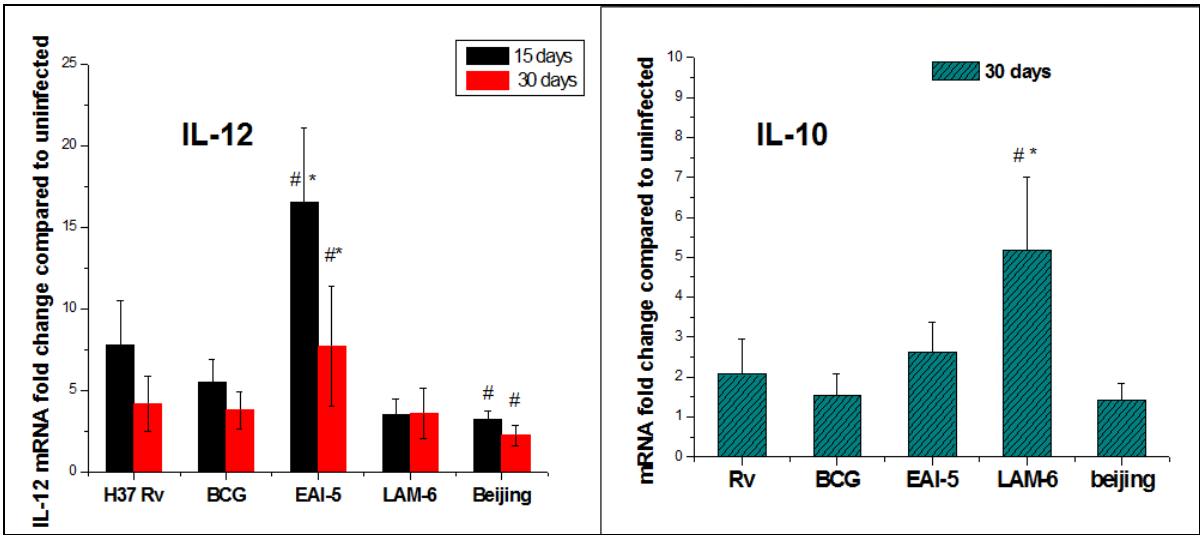


Figure 35: Assessment of IL-12 and IL-10 in infected mouse lung (By Real time PCR)

Real time PCR was carried out to estimate the mRNA expression for IL-12 and IL-10 at indicated time point, after infection in BALB/c mice with *MTB* H37Ra, H37Rv and three clinical isolates. The bars show relative mRNA expression corrected for total mRNA using the housekeeping β-actin gene. Data represent the mean ± standard deviation of the samples. The symbol # and * represent statistically significant difference when compared to infection at a similar time point by H37Rv and BCG respectively.

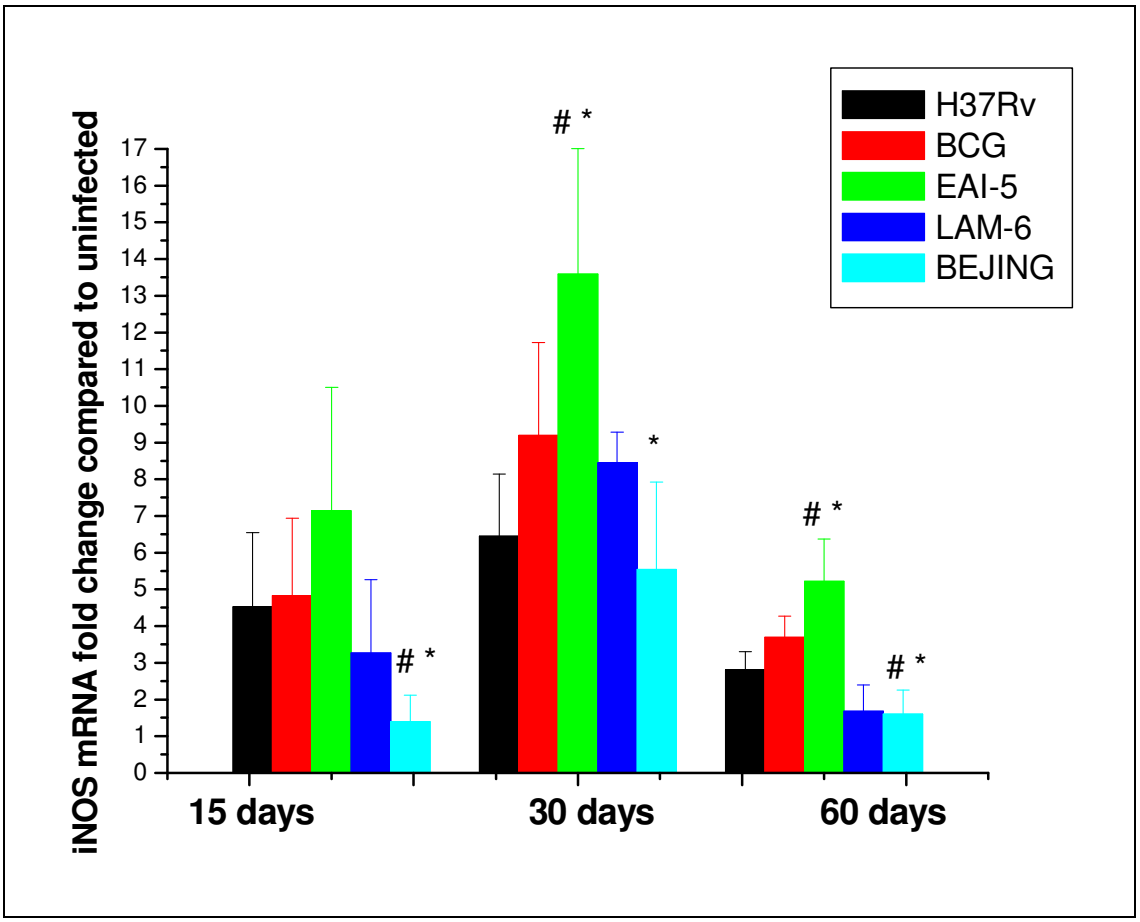


Figure 36: Estimation of iNOS mRNA induction in infected mouse lung (By Real time PCR)

Real time PCR was carried out to estimate the mRNA expression for iNOS in BALB/c mice at 15 days, 30 days and 60 days post infection with MTB H37Ra, H37Rv and three clinical isolates. The bars show relative mRNA expression corrected for total mRNA using the housekeeping β-actin gene. Data represent the mean ± standard deviation of the samples**.** The symbol # and * represent statistically significant difference when compared to infection at a similar time point by H37Rv and BCG respectively.

5.3.3 Splenocyte restimulation and IFN-γ production in vitro

To further characterize the role of acquired immunity in the immune response against different genotypes of *MTB*, the state of T cell activation in the splenocytes of infected mice were investigated. At 60 days post infection, IFN-γ production in response to PPD was significantly lower in spleen cells from mice infected with Beijing strain than in spleen cells from mice infected with BCG and EAI-5 (Figure 37). IFN-γ production by splenocytes isolated from mice infected with different clinical isolates in response to Con A were similar. ConA served as positive control and unstimulated cells served as negative control. IFN-γ concentration was very low for all unstimulated splenocytes.

5.3.4 Histopathology

Histopathological parameters, such as lymphocyte infiltration in the lung were observed with the increased severity in the course of infection. Large differences in the time-dependent induction of lung pathology were noticed among mice infected with different strains (Figure 38). At 15 day post infection, a minimal infiltrates was detected in lungs of mice infected with LAM strain. Most of the strains, however, were able to induce reasonable lung damage within 30 days after infection. Beijing and LAM strains caused modest perivascular infiltration at 30 days after infection. It was significantly larger than that produced by H37Rv.

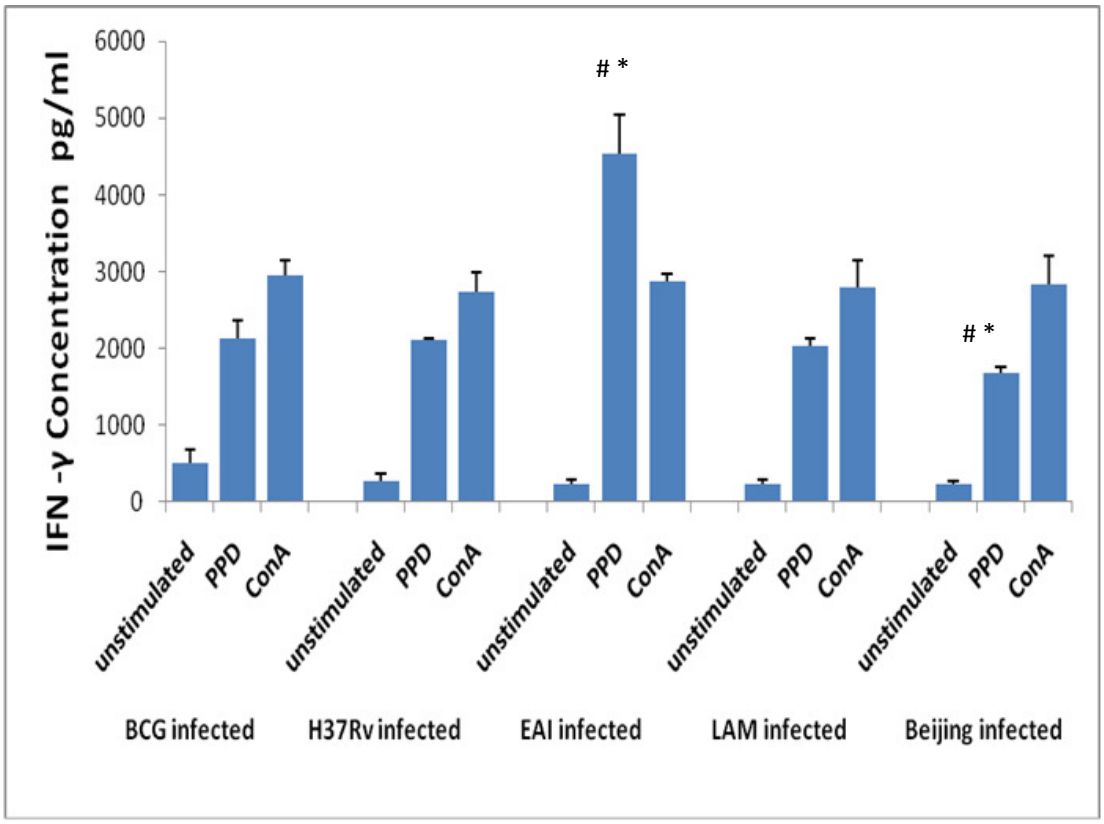


Figure 37: Estimation of IFN-γ by stimulated splenocytes isolated from 60 days post infected mice

Splenocytes were isolated from mice 60 days post-infection with different strains of MTB (as indicated) and stimulated with PPD (10 µg/ml) and Con A (5µg/ml) for 48 hours. IFN-γ levels were estimated in cell free supernatants of unstimulated, PPD and Con A stimulated cells by ELISA. The data are presented as mean IFN-γ concentration ± SD (3 mice/group/time point). The symbol # and * represent statistically significant difference when compared to infection at a similar time point by H37Rv and BCG respectively.

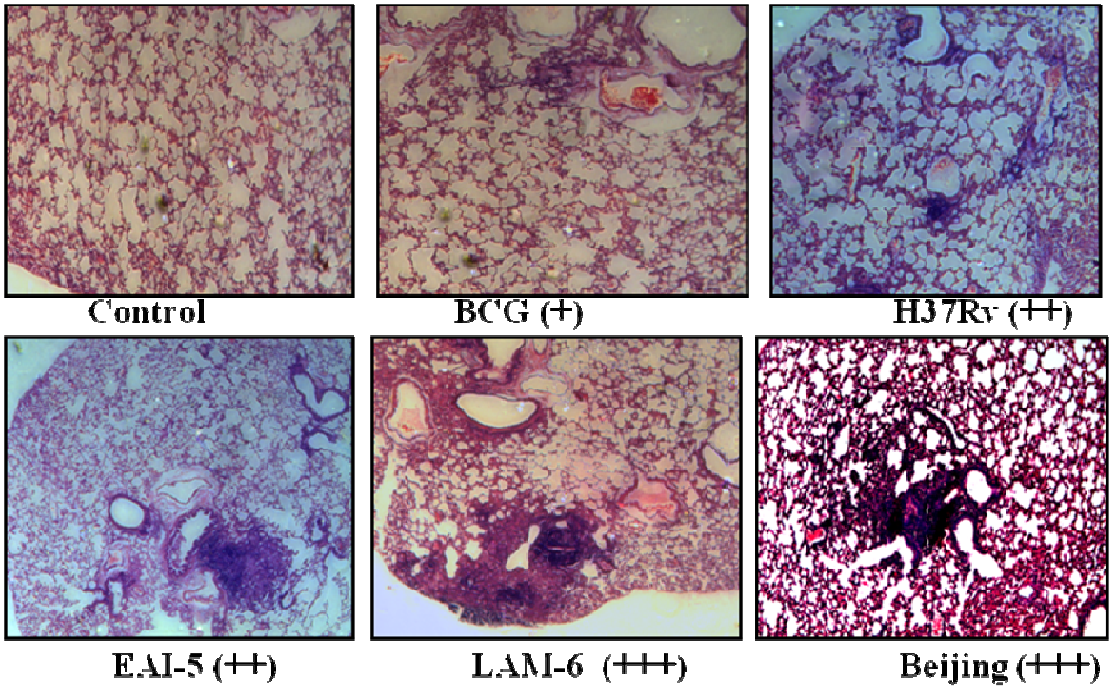


Figure 38: Lung histopathology in MTB infected BALB/c mice
Sections of the lungs were stained with H&E stain. Representative lung sections for each *M. tuberculosis* infected group at day 30 post-infection are displayed. Lung sections were scored for lymphocytic infiltrates, (0) = normal lung; a score of + = very little infiltrates ; ++ = moderate lymphocytic infiltrate and a score of +++ = extensive lymphocytic infiltrate. Representative photographs are shown.

5.4. Discussion

Clinical isolates of MTB from different genetic background have already shown their ability to induce differential immune response in different hosts in *ex vivo* infection models. When, the same strains were studied in BALB/c mouse model, dissimilar patterns of immune response were observed yet again.

It has been established that both in mice and humans the control of Mycobacterial infections depends mainly on macrophage activation, through the Th1 type cytokines. Th1 type cytokines provoke inflammation which may lead to the development of tissue pathology by granuloma formation and necrosis. Eventually, the containment of the infection depends on intracellular killing of the *Mycobacteria* or at least suppression of bacterial growth leading to a state of latency. The precise mechanisms involved in containment of MTB during *in vivo* infection are only partly known. In mice this depends on reactive oxygen and nitrogen intermediates whereas these do not seem to be involved in the effecter mechanism in humans (Chan e al, 1992; MacMicking et al, 1997). It was found that in human, granulysin in combination with perforin was an important mycobactericide (Stenger et al, 1998). This protective activity fails if there is a marked release of Th2 type cytokines (Seah et al, 2000; Wangoo et al, 2001), thus the Th1/Th2 balance is thought to determine the outcome of the encounter with the pathogen. This interplay of cytokines is clearly depicted in a BALB/c model of pulmonary tuberculosis following aerosol infection. In the present study, the initial phase of infection (15 to 30 days) was dominated by high production of Th1 cell cytokines, in coexistence with high levels of TNF-α and iNOS in EAI-5, BCG and H37Rv infected mice, which temporarily controlled the infection. Their lung bacillary load was also significantly lower than of Beijing and LAM strains. Further, 60 days post infection, probably the predominance of Th2 cytokines helped comparatively decrease the TNF-α and iNOS levels in EAI-5, BCG and H37Rv infected mice (Figures 33 and 35).

The reduced expression of IFN-γ and iNOS was seen in case of infection by Beijing genotype, which suggests suppression of immune activation during the early phase of the infection. The minuscule expression of IFN-γ induced by Beijing strain, failed to stimulate the arrest of bacillary multiplication in the lungs, resulting in massive tissue damage compared to that inflicted by other strains. In contrast with the Beijing strain, EAI-5 elicited prompt and conspicuous inflammation. The high and sustained TNF-α and iNOS expression might be the reason for the arrest of progression of the bacterial growth in case of EAI-5, which was reflected by limited tissue damage and lower lung bacillary load. This early and very efficient control of the infection by activated macrophages was also accompanied by high IFN-γ expression in the mice 30 days post infection with EAI-5 strain.

The initial host-pathogen interactions by strains of MTB differentially induced host adaptive response. EAI-5 strain which induced higher Th1 response in early infection showed more bacillary clearance and higher number of primed T cells in the splenocytes of infected mice. In presence of PPD, these activated T-cells produced significantly higher IFN-γ compared to that by spleen cells infected by other strains. The IFN-γ levels were even higher compared to those induced in response to BCG, a vaccine strain, known to induce stronger adaptive response. T-cell activation observed for Beijing and LAM strains, was comparable with that for H37Rv, although both the strains grew to a much higher bacillary load in the infected lung, than H37Rv. This observation suggests that lung bacillary load and severity of infection might have little role in induction of adaptive immune responses whereas the strain genotype might play a key role in this regard.

Different genotypes of MTB were also divergent in their virulence, as illustrated by bacillary burden and lung histopathology at different time points after infection. Strains such as Beijing and LAM that caused the highest levels of infiltration or granuloma formation, also showed the highest bacillary burden. Earlier studies with the Beijing strains in an animal

model, had also demonstrated similar results (Lopez et al, 2003; Dormans et al, 2004). It was reported in several studies that Beijing, Lineage-2 strains grow rapidly in animal models compared to H37Rv and other clinical isolates (Li Q et al, 2002; Tsenova et al, 2005) which is in agreement with the present results. It was also confirmed in earlier studies that Beijing (clinical isolate and NH898, an outbreak strain from Texas) triggered less Th1 response and caused severe mortality (Lopez 2003; Manca et al, 2005). Relatively, very few studies have so far been published on the animals infected with Lineage-4 strains other than laboratory strains H37Rv. The strains of LAM lineage are also known for causing cavitary disease (Lazzarini et al, 2008) and the acquisition of drug resistance (Ignatova et al, 2006). Similar to the results observed with present LAM strain, CDC1551 (an outbreak LAM strain from Tennessee) and RD^{Rio} (LAM strains from Rio de Janeiro, Brazil) also showed higher lung bacillary load in mice, compared to laboratory strain after 3-4 weeks of infection (Valway et al, 1998; Lazzarini et al, 2007). Ancient strains from Lineage-1 are known to be less virulent and easy to treat. In addition, recurrence is rarely observed in infection with this strain type compared to that with Beijing (Lan et al, 2003). It is evident from current study, that the ancient strain induces higher Th1 response initially which probably responsible for the lesser bacterial burden in the lung.

Comparing both *in vivo* and *ex vivo* infections for the MTB strains, it is clear that a genotype of MTB induced a particular immune pattern in the host, which is comparable with the early infection (15 days) in mice and *ex vivo* infection in THP-1 cells. The results obtained for the cytokines induction, iNOS and intracellular bacterial growth were similar for all the strains in these two infection models. Beijing showed lowest bacterial growth in THP-1; but, showed much higher growth in mouse model, which clearly indicated that *ex vivo* intracellular growth, might not be an important parameter to determine virulence.

In conclusion, using a well-studied model of progressive pulmonary tuberculosis, it was demonstrated that genetically different MTB strains elicit diverse immune responses and it is an attribute of particular genotype. This divergence led to differences in the induced pathology and lung bacillary burden. In particular, the emerging Beijing genotype and LAM were the most virulent among the genotypes studied, as they induced less adaptive response and high lung bacillary burden.

Summary:

The stable infection of host macrophages by *Mycobacterium tuberculosis* involves and depends on the subversion of the diverse microbicidal responses mounted by the host cells. This is primarily achieved through targeted perturbation of the host cellular machinery by bacterial effecter molecules. Broadly, the innate and adaptive immune responses triggered by mycobacterium create a cascade of events that work to limit and contain the growth of infection. Strains from different genotype have been shown to subvert the host response in different ways and regulate multiple events at a time. Hence, a coherent knowledge of such parameters might shed light on actual TB pathogenesis. The present study was aimed to characterize different clinical isolates from Mumbai and to analyze how mycobacterium strains might modulate several responses of macrophage according to their genotype.

Three different clinical isolates of MTB from three different lineages were selected for the study and were used for *ex vivo* as well as *in vivo* infection experiments. The strains were further characterized on the basis of TbD-1, MIRU-VNTR and their drug susceptibility. The *ex vivo* infection study was carried out in THP-1 and RAW264.7 cells, as well as in primary cells MDMs and MDDCs, isolated from blood of healthy individuals. Several different interactions between host and pathogen such as phagocytosis, intracellular growth of *Mycobacteria*, induction of proinflammatory cytokines and chemokines, reactive oxygen/nitrogen species and apoptosis in THP-1 cells, were monitored. Induction of proinflammatory cytokines followed a particular pattern for a single clinical isolate, in *ex vivo* experiments and the pattern was unchanged in different host cells. It was observed that the ancient EAI-5 strain from lineage-1 induced significantly higher proinflammatory response in host compared to modern clinical isolates, as marked in earlier studies. On the other hand, modern Beijing strain from lineage-2 consistently induced low levels of proinflammatory cytokines and IL-10 according to its known lineage characteristic. Further, LAM-6 and

H37Rv both belonging to lineage 4, showed similar proinflammatory responses. Overall, it was noticed that there exist a lineage specific cytokine induction throughout *ex vivo* and *in vivo* infections as reported in previous studies and interestingly these patterns were not influenced by the drug resistant status of the strains. A strain specific apoptotic response was observed only for virulent strains with a significant correlation with induction of pro-inflammatory cytokines. Further, virulent strains accumulated less in number of bacteria (less than 5) compared to virulent strain H37Ra inside the host during their initial interaction or phagocytosis and it showed a relation with the induction of apoptosis of the host. In the present study, a positive correlation was observed between phagocytic index for '>10 bacilli/cell' and the percent apoptosis after five and six days of infection. It was evident that pro-inflammatory cytokines and initial higher accumulation of bacilli inside the host cell were the two determining factors for host cell apoptosis.

Similar interplay of different immune interactions as observed in *ex vivo* studies was also clearly observed in the BALB/c model of TB following aerosol infection. In the present study, the initial phase of infection (15 & 30 days) was dominated by high production of Th1 type cytokines, in coexistence with high levels of TNF-α and iNOS for EAI-5, BCG and H37Rv infected mice which showed simultaneously lower bacillary load in lungs. However, clinical isolates belonging to LAM and Beijing genotype showed the opposite picture of higher bacillary load in lungs. The early Th1 response observed in lungs, induced by different strains also caused dissimilar adaptive immune responses, which was confirmed by splenocyte restimulation assay using PPD. Thus, EAI-5 strain which induced higher Th1 response in early infection showed more bacillary clearance and higher number of primed T cells in the restimulated splenocytes whereas Beijing and LAM strains induced T-cell activation comparable to H37Rv. It is evident from current study, that the strain exhibiting higher proinflammatory responses *in vitro* (EAI-5), induced higher Th1 type of response

which was protective in nature. It is also reflected in the bacillary load in the lungs of mice infected with the same strain. Similarly, the Beijing strain which showed suppression of immune responses showed higher lung bacillary load in infected mice.

So far, this kind of study was conducted with laboratory strains and with very few clinical isolates of MTB. The present study was conducted with clinical strains isolated from TB patients from Mumbai, India and hence is the first of its kind. Additionally, this is also the first report where all the important parameters of immune interactions like intracellular growth, phagocytic index, induction of cytokine and apoptosis were monitored in hosts infected with highly drug resistant, well characterized MTB lineages in a single study. It illustrated lineage-specific patterns of innate immune responses induced by the strains of different genotypes in *ex vivo* and *in vivo* conditions. This might have consequences in TB patients infected with different lineages of MTB. The study has generated a strong base for future in-depth research in the field of TB pathogenesis. The detailed study of the underlying mechanisms behind these differences might pave way to the new treatment regimen.

Appendix I

Composition of buffers and reagents:

A. Solutions for DNA extraction by CTAB method:

TE buffer :100 mM Tris-Cl (pH 8.0) and10 mM EDTA (pH 8.0)

CTAB/NaCl solution: 4.1 gm NaCl was dissolved in 80 ml distilled water. While stirring 10 gm CTAB was added and heated at 65°C. The volume was adjusted to 100 ml with distilled water and stored at room temperature (RT).

B. Solutions for Spoligotyping:

20X SSPE : 0.2 M $Na_2HPO_4.2H_2O$; 3.6 M NaCl; 20 mM EDTA
The pH was adjusted to 7.4 and solution was autoclaved and stored at room temperature.

20X SSC : 3 M NaCl; 0.3 M Na-citrate.
The pH was adjusted to 7.0 with NaOH and solution was autoclaved and stored at room temperature.

C. Concentrations used first line and second line Drugs in LJ slant
Dihydrostreptomycin : 4 µg/ml
Isoniazid : 0. 2 µg/ml
Rifampicin : 40 µg/ml
Ethambutol : 2 µg/ml
Pyrazinamide: 100 µg/ml
Kanamycin : 30 µg/ml
Ethionamide : 40 µg/ml
Ofloxacin : 2 µg/ml
Amikacin : 40 µg/ml
Capriomycin : 40 µg/ml
P-amino Salycyclic acid: 1 µg/ml
Rifabutin: 20µg/ml
Cycloserine: 40 µg/ml

D. Solutions for PAGE and Western Blotting

Lysis buffer for cytoplasmic extract : 20mM HEPES, 10mMNaCl, 1.5nM $MgCl_2$, 0.2mM EDTA, 0.5% (v/v) Triton X-100, 0.5 mM DTT, 1mM sodium orthovanadate, 1mM NaF and a cocktail of protease inhibitors.

2X SDS-PAGE gel loading Buffer : 100mM Tris-HCL, pH 6.8, 4%(w/v) SDS, 20% (v/v) glycerol, 200mM β-mercaptoethanol, 0.2% (w/v) bromophenol blue

Transfer buffer: 25mM Tris base, 250 mM Glycine, 0.01% SDS (Sodium Dodecyl Sulphate) (w/v) and 20% (v/v) Methanol

1X PBS (phosphate-buffered saline): NaCl, 80.0 g; Na_2HPO_4, 11.6 g; KH_2PO_4 , 2.0 g; KCl, 2.0g H_2O to 10 liter; Adjust pH to 7.2 - 7.4

Blocking Buffer: 5 % (w/v) Bovine Serum Albumin (BSA) in 1XPBS-T. Antibody dilutions were prepared either in 1 X PBS-T or 5% (w/v) BSA.

Wash Buffer: 0.1% (v/v) Tween-2- in 1 X PBS.

E. Solutions for Microscopy

Fixative Solution: 3% (w/v) Para formaldehyde in 1 X PBS

Quenching Solution: 50 mM ammonium chloride in 1XPBS

Wash Buffer: 0.05% (v/v) Triton X-100- in 1 X PBS.

F. Solutions for Flowcytometry

Fixative solution: 0.1% (w/v) Para formaldehyde in 1 X PBS

Wash Buffer: 0.1% (w/v) BSA in 1 X PBS containing 0.01% (w/v) Azide

Appendix II

Table A: PCR reaction mixture for spoligotyping

A 50 µl polymerase chain reaction mixture was set-up as follows:

Reagents	Volume (µl)	Final reaction conc.
Buffer 10 X	5	1 X
dNTPs	1	0.2mM of each
MgCl2 25mM	4	2 mM
DRa- GGTTTTGGGTCTGACGAC	4	20 pmoles
DRb-CCCGAGAGGGGACGGAAAC	4	20 pmoles
Taq polymerase	1	1 u
Template DNA	10 µl	10 ng
Mineral oil	40 µl	
H2O		11.75

Table B: PCR reaction mixture for MIRU-VNTR typing

A 25 µl polymerase chain reaction mixture was set-up as follows :

Reagents	Final reaction conc	Volume (µl)
Buffer 10 X	1x	2.5
dNTPs 10mM	0.2mM	0.5
MgCl2 50mM	2.5mM	1.5
Primers (listed separately)	0.4mM	1+1=2
DMSO	5%	1.25
Enzyme	2-3 units	0.5
Template DNA 4ng/ul	4 ng/ ul	5
H_2O		11.75

Table C: Primers of MIRU-VNTR analysis

MIRU locus	PCR primer sequence (5'-3')
MIRU-2F	TGGACTTGCAGCAATGGACCAACT
MIRU-2R	TACTCGGACGCCGGCTCAAAAT
MIRU-4F	GTCAAACAGGTCACAACGAGAGGAA
MIRU-4R	CCTCCACAATCAACACACTGGTCAT
MIRU-10F	GTTCTTGACCAACTGCAGTCGTCC
MIRU-10R	GCCACCTTGGTGATCAGCTACCT
MIRU-16F	TCGGTGATCGGGTCCAGTCCAAGTA
MIRU-16R	CCCGTCGTGCAGCCCTGGTAC
MIRU-20F	TCGGAGAGATGCCCTTCGAGTTAG
MIRU-20R	GGAGACCGCGACCAGGTACTTGTA
MIRU-23F	CTGTCGATGGCCGCAACAAAACG
MIRU-23R	AGCTCAACGGGTTCGCCCTTTTGTC
MIRU-24F	CGACCAAGATGTGCAGGAATACAT
MIRU-24R	GGGCGAGTTGAGCTCACAGAA
MIRU-26F	TAGGTCTACCGTCGAAATCTGTGAC
MIRU-26R	CATAGGCGACCAGGCGAATAG
MIRU-27F	TCGAAAGCCTCTGCGTGCCAGTAA
MIRU-27R	GCGATGTGAGCGTGCCACTCAA
MIRU-31F	ACTGATTGGCTTCATACGGCTTTA
MIRU-31R	GTGCCGACGTGGTCTTCAT
MIRU-39F	CGCATCGACAAACTGGAGCCAAAC
MIRU-39R	CGGAAACGTCTACGCCCCACACAT
MIRU-40F	GGGTTGCTGGATGACAACGTGT
MIRU-40R	GGGTGATCTCGGCGAAATCAGATA

Table D: PCR reaction mixture for TbD-1, RD1 and seven different genes selected for mutation analysis:

The PCR condition was 40 cycles of denaturation at 94°C for 30 s, annealing (at respective temperature) for 30 s and extension at 72°C for 1 min.

A 25 µl polymerase chain reaction mixture was set-up as follows :

Reagents	Final reaction conc	Volume (µl)
Buffer 10 X	1x	2.5
dNTPs 10mM	0.2mM	0.5
MgCl2 50mM	2.5mM	1.5
Primers (listed separately)	0.4mM	1+1=2
Taq polymerase	1u	0.5
Template DNA 4ng/ul	4ng/ ul	5
H2O		13

Table E: Primers for TbD-1, RD1 and mutation analysis:

Primers	5' to 3'	Annealing Temp ^{0}C
flaTBD1F	CTA CCT CAT CTT CCG GTC CA	59
flaTBD1R	CAT AGA TCC CGG ACA TGG TG	
TBD1intS.F	CGT TCA ACC CCA AAC AGGTA	59
TBD1intS.R	AAT CGA ACT CGT GGA ACA CC	
RD1F	GTC AGC CAA GTC AGG CTA CC	60
RD1R	CAA CGT TGT GGT TGT TGA GG	
RD1FF	GAA ACA GTC CCC AGC AGG T	60
RD1FR	TTC AAC GGG TTA CTG CGA AT	
rpoB F	TGG TCC GCT TGC ACG AGG GTC AGA-	70
rpoB R	CTC AGG GGT TTC GATCGGGCACAT	
AhpC F	ACT GCT TTG CCG CCA CC	57
AhpC F	CFGA TGA GAG CGC TGA GCT G	
InhA F	CGCTGCCCAGAAAGGGA	70
InhA R	CCGGGTTTCCTCCGGT	
KatG F	CAG ATG GGC TIG GGC TGG AAG AGC	57
KatG R	-GAC GAC GCC GCC GCC CA	
16S F	GGA A TI CAG AGT TGG A TC MTG GYT CAG	70
16S R	CGG GAT CCC TIT A CG CCC ART RAW TCCG	
rps F	CGA CCC GCA GCG TCG TGG TG	70
rps R	GCT GCG TGC CTG TTTGCG GTT CTT	
emb F	TGG ACG G*GC* GGG GCT CAA T	70
emb R	GGC AGG CGC ATC CAC AGA CT	

Table F: Primers for cytokines, chemokines and other genes

Primers	Sequence	Annealing temperature (^{0}C)
TNF-αF	GAGTGACAAGCCTGTAGCCCATGTTGTAGC	60
TNF-αR	GCAATGATCCCAAAGTAGCCTGCCCAGAC	
IL-1βF	CCTGTCCTGCGTGTTGAAAGA	60
IL-1βR	GGGAACTGGGCAGACTCAAA	
IL-10F	GCTGGAGGACTTTAAGGGTTACCT	60
IL-10R	CTTGATGTCTGGGTCTTGGTTCT	
IL-8F	ATGACTTCCAAGCTGGCCGTG	65
IL-8F	TTATGAATTCTCAGCCCTCTTCAAAAACTTCTC	
TLR-2F	GGCCAGCAAATTACCTGTGTG	60
TLR-2R	AGGCGGACATCCTGAACCT	
iNOSF	TGGAATTCACTCAGCTGTGC	55
iNOSR	GATGTTGTAGCGCTGGACG	
IL-12F	GCTGGAGGACTTTAAGGGTTACCT	60
IL-12R	CTTGATGTCTGGGTCTTGGTTCT	
MCP-1F	ATGACTTCCAAGCTGGCCGTG	65
MCP-1R	TTATGAATTCTCAGCCCTCTTCAAAAACTTCTC;	
TLR-4F	GGCCAGCAAATTACCTGTGTG	60
TLR-4R	AGGCGGACATCCTGAACCT	
Bcl2F	TGGAATTCACTCAGCTGTGC	60
Bcl2R	GATGTTGTAGCGCTGGACG	
BaxF	GGAATTCGCGGTGATGGACGGGTCCGG	60
BaxR	GGAATTCTCAGCCCATCTTCTTCCAGA	
Caspase3F	TTCAGAGGGGATCGTTGTAGAAGTC	60
Caspase3R	CAAGCTTGTCGGCATACTGTTTCAG	
B ActinF	GTGGGCCGCTCTAGGCACCA	60
B ActinR	CGGTTGGCCTTAGGGTTCAGGGGGG	

Table G: Primers for mouse cytokines and other genes

Primers	Sequence	Annealing temperature (^{0}C)
TNF-αF	GGCAGGTCTACTTTGGAGTCATTGC	60
TNF-αR	ACATTCGAGGCTCCAGTGAATTCGG	
IFN-γF	TGAACGCTACACACT GCA TCT TGG	60
IFN-γR	CGACTC CTTTTCCGCTTCCTGAG	
IL-10F	CGGGAAGACAATAACTG	60
IL-10R	CATTTCCGATAAGGCTTGG	
iNOSF	CCCTTCCGAAGTTTCTGGCAGCAGC3	60
iNOSR	GGCTGTCAGAGCCTCGTGGCTTTGG	
IL-12F	GCTGGAGGACTTTAAGGGTTACCT	60
IL-12R	CTTGATGTCTGGGTCTTGGTTCT	
β ActinF	TGG AAT CCT GTG GCA TCC ATG AAA C	60
β ActinR	TAA AAC GCA GCT CAG TAA CAG TCC G	

References

Abadi FJ, Carter PE, Cash P, Pennington TH (1996) Rifampicin resistance in *Neisseria meningitidis* due to alterations in membrane permeability. Antimicrobial agents and chemotherapy 40: 646-651.

Achtman M (2008) Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. Annu Rev Microbiol 62: 53-70.

Aderem A, Underhill DM (1999) Mechanisms of phagocytosis in macrophages. Annual review of immunology 17: 593-623.

Agarwal R, Malhotra P, Awasthi A, Kakkar N, Gupta D (2005) Tuberculous dilated cardiomyopathy: an under-recognized entity? BMC infectious diseases 5: 29.

Akahoshi M, Nakashima H, Miyake K, Inoue Y, Shimizu S, et al. (2003) Influence of interleukin-12 receptor β 1 polymorphisms on tuberculosis. Human genetics 112: 237-243.

Allix-Barguec C, Fauville-Dufaux M, Supply P (2008) Three-year population-based evaluation of standardized *Mycobacteria*l interspersed repetitive-unit-variable-number tandem-repeat typing of *Mycobacterium tuberculosis*. Journal of clinical microbiology 46: 1398-1406.

Anand, P. K. and D. Kaul (2005). "Downregulation of TACO gene transcription restricts mycobacterial entry/survival within human macrophages." FEMS microbiology letters 250(1): 137-144.

Andersen P, Munk ME, Pollock JM, Doherty TM (2000) Specific immune-based diagnosis of tuberculosis. The Lancet 356: 1099-1104.

Asiimwe BB, Ghebremichael S, Kallenius G, Koivula T, Joloba ML (2008) *Mycobacterium tuberculosis* spoligotypes and drug susceptibility pattern of isolates from tuberculosis patients in peri-urban Kampala, Uganda. BMC infectious diseases 8: 101.

Astarie-Dequeker C, N Diaye E-N, Le Cabec V, Rittig MG, Prandi J, et al. (1999) The mannose receptor mediates uptake of pathogenic and nonpathogenic *Mycobacteria* and bypasses bactericidal responses in human macrophages. Infection and immunity 67: 469-477.

Awomoyi AA, Charurat M, Marchant A, Miller EN, Blackwell JM, et al. (2005) Polymorphism in IL1B: IL1B 511 association with tuberculosis and decreased lipopolysaccharide-induced IL-1β in IFN-γ primed ex-vivo whole blood assay. Journal of endotoxin research 11: 281-286.

Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392: 245-252.

Barnes PF, Lu S, Abrams JS, Wang E, Yamamura M, et al. (1993) Cytokine production at the site of disease in human tuberculosis. Infection and immunity 61: 3482-3489.

Barreiro LB, Neyrolles O, Babb CL, Tailleux L, Quach H, et al. (2006) Promoter variation in the DC-SIGN encoding gene CD209 is associated with tuberculosis. PLoS medicine 3: e20.

Bean AGD, Roach DR, Briscoe H, France MP, Korner H, et al. (1999) Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol *Mycobacterium tuberculosis* infection, which is not compensated for by lymphotoxin. The Journal of Immunology 162: 3504-3511.

Beharka AA, Gaynor CD, Kang BK, Voelker DR, McCormack FX, et al. (2002) Pulmonary surfactant protein A up-regulates activity of the mannose receptor, a pattern recognition receptor expressed on human macrophages. The Journal of Immunology 169: 3565-3573.

Bekker LG, Maartens G, Steyn L, Kaplan G (1998) Selective increase in plasma tumour necrosis factor-α and concomitant clinical deterioration after initiating therapy in patients with severe tuberculosis. Journal of Infectious Diseases 178: 580-584.

Bekker L-G, Freeman S, Murray PJ, Ryffel B, Kaplan G (2001) TNF-alpha controls intracellular *Mycobacteria*l growth by both inducible nitric oxide synthase-dependent and inducible nitric oxide synthase-independent pathways. The Journal of Immunology 166: 6728-6734.

Belisle JT, Brennan PJ (1989) Chemical basis of rough and smooth variation in *Mycobacteria*. Journal of bacteriology 171: 3465-3470.

Bellamy R (2003) Susceptibility to *Mycobacteria*l infections: the importance of host genetics. Genes and immunity 4: 4-11.

Bellamy R, Ruwende C, Corrah T, McAdam K, Whittle HC, et al. (1998) Assessment of the interleukin 1 gene cluster and other candidate gene polymorphisms in host susceptibility to tuberculosis. Tubercle and Lung Disease 79: 83-89.

Bercovier H, Kafri O, Sela S (1986) *Mycobacteria* possess a surprisingly small number of ribosomal RNA genes in relation to the size of their genome. Biochemical and biophysical research communications 136: 1136-1141.

Berg S, Kaur D, Jackson M, Brennan PJ (2007) The glycosyltransferases of *Mycobacterium tuberculosis*roles in the synthesis of arabinogalactan, lipoarabinomannan, and other glycoconjugates. Glycobiology 17: 35R-56R.

Bermudez LE, Goodman J (1996) *Mycobacterium tuberculosis* invades and replicates within type II alveolar cells. Infection and immunity 64: 1400-1406.

Berrington WR, Hawn TR (2007) *Mycobacterium tuberculosis*, macrophages, and the innate immune response: does common variation matter? Immunological reviews 219: 167-186.

Bhowruth V, Alderwick L, Brown A, Bhatt A, Besra G (2008) Tuberculosis: a balanced diet of lipids and carbohydrates. Biochemical Society Transactions 36: 555.

Bifani PJ, Mathema B, Kurepina NE, Kreiswirth BN (2002) Global dissemination of the *Mycobacterium tuberculosis* W-Beijing family strains. Trends Microbiol 10: 45-52.

Bodnar KA, Serbina NV, Flynn JL (2001) Fate of *Mycobacterium tuberculosis* within murine dendritic cells. Infection and immunity 69: 800-809.

Boeuf P, Vigan-Womas I, Jublot D, Loizon S, Barale JC, et al. (2005) CyProQuant-PCR: a real time RT-PCR technique for profiling human cytokines, based on external RNA standards, readily automatable for clinical use. BMC Immunol 6: 5.

Boom WH (1996) The role of T-cell subsets in *Mycobacterium tuberculosis* infection. Infectious agents and disease 5: 73.

Bosio CM, Gardner D, Elkins KL (2000) Infection of B cell-deficient mice with CDC 1551, a clinical isolate of *Mycobacterium tuberculosis*: delay in dissemination and development of lung pathology. The Journal of Immunology 164: 6417.

Brandt L, Elhay M, Rosenkrands I, Lindblad EB, Andersen P (2000) ESAT-6 Subunit Vaccination against*Mycobacterium tuberculosis*. Infection and immunity 68: 791-795.

Brightbill HD, Libraty DH, Krutzik SR, Yang R-B, Belisle JT, et al. (1999) Host defence mechanisms triggered by microbial lipoproteins through toll-like receptors. Science 285: 732-736.

Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser C, et al. (2002) A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proceedings of the national academy of Sciences 99: 3684.

Brosch R, Philipp WJ, Stavropoulos E, Colston MJ, Cole ST, et al. (1999) Genomic Analysis Reveals Variation between *Mycobacterium tuberculosis* H37Rv and the AttenuatedM. tuberculosis H37Ra Strain. Infection and immunity 67: 5768-5774.

Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, et al. (2006) *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC microbiology 6: 23.

Brudey K, Gutierrez MC, Vincent Vr, Parsons LM, Salfinger M, et al. (2004) *Mycobacterium africanum* genotyping using novel spacer oligonucleotides in the direct repeat locus. Journal of clinical microbiology 42: 5053-5057.

Burman WJ, Bliven EE, Cowan L, Bozeman L, Nahid P, et al. (2009) Relapse associated with active disease caused by Beijing strain of *Mycobacterium tuberculosis*. Emerging Infectious Diseases 15: 1061.

Canetti G, Fox W, Khomenko Aa, Mahler HT, Menon NK, et al. (1969) Advances in techniques of testing *Mycobacteria*l drug sensitivity, and the use of sensitivity tests in tuberculosis control programmes. Bulletin of the World Health Organization 41: 21.

Canetti G, Froman S, Grosset Ja, Hauduroy P, Langerovo M, et al. (1963) *Mycobacteria*: laboratory methods for testing drug sensitivity and resistance. Bulletin of the World Health Organization 29: 565.

Caruso AM, Serbina N, Klein E, Triebold K, Bloom BR, et al. (1999) Mice deficient in CD4 T cells have only transiently diminished levels of IFN-γ yet succumb to tuberculosis. The Journal of Immunology 162: 5407-5416.

Casanova J-L, Abel L (2002) Genetic dissection of immunity to *Mycobacteria*: the human model. Annual review of immunology 20: 581-620.

Caws M, Thwaites G, Dunstan S, Hawn TR, Lan NTN, et al. (2008) The influence of host and bacterial genotype on the development of disseminated disease with *Mycobacterium tuberculosis*. PLoS pathogens 4: e1000034.

Caws M, Thwaites G, Stepniewska K, Lan NTN, Duyen NTH, et al. (2006) Beijing genotype of *Mycobacterium tuberculosis* is significantly associated with human immunodeficiency virus infection and multidrug resistance in cases of tuberculous meningitis. Journal of clinical microbiology 44: 3934-3939.

Cervino ACL, Lakiss S, Sow O, Bellamy R, Beyers N, et al. (2002) Fine mapping of a putative tuberculosis-susceptibility locus on chromosome 15q1113 in African families. Human molecular genetics 11: 1599-1603.

Chacón Salinas R, Serafin□Lopez J, Ramoso□Payán R, Méndeze□Aragón P, Hernandez□Pando R, et al. (2005) Differential pattern of cytokine expression by macrophages infected *in vitro* with different *Mycobacterium tuberculosis* genotypes. Clinical & Experimental Immunology 140: 443-449.

Chan J, Fan XD, Hunter SW, Brennan PJ, Bloom BR (1991) Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages. Infection and immunity 59: 1755-1761.

Chan J, Xing Y, Magliozzo RS, Bloom BR (1992) Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages. The Journal of experimental medicine 175: 1111-1122.

Chensue SW, Warmington KS, Ruth JH, Sanghi PS, Lincoln P, et al. (1996) Role of monocyte chemoattractant protein-1 (MCP-1) in Th1 (*Mycobacteria*l) and Th2 (schistosomal) antigen-induced granuloma formation: relationship to local inflammation, Th cell expression, and IL-12 production. The Journal of Immunology 157: 4602-4608.

Chihota V, Apers L, Mungofa S, Kasongo W, Nyoni IM, et al. (2007) Predominance of a single genotype of *Mycobacterium tuberculosis* in regions of Southern Africa. The International Journal of Tuberculosis and Lung Disease 11: 311-318.

Cho S, Mehra V, Thoma-Uszynski S, Stenger S, Serbina N, et al. (2000) Antimicrobial activity of MHC class I-restricted CD8+ T cells in human tuberculosis. Proceedings of the National Academy of Sciences 97: 12210-12215.

Chua J, Vergne I, Master S, Deretic V (2004) A tale of two lipids:*Mycobacterium tuberculosis* phagosome maturation arrest. Current opinion in microbiology 7: 71-77.

Ciaramella A, Cavone A, Santucci MB, Amicosante M, Martino A, et al. (2002) Proinflammatory cytokines in the course of *Mycobacterium tuberculosis*–induced apoptosis in monocytes/macrophages. Journal of Infectious Diseases 186: 1277.

Co Do, Hogan LH, Kim S-I, Sandor M (2004) *Mycobacteria*l granulomas: keys to a long-lasting host pathogen relationship. Clinical Immunology 113: 130-136.

Cole S, Brosch R, Parkhill J, Garnier T, Churcher C, et al. (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393: 537-544.

Comas I, Gagneux S (2009) The past and future of tuberculosis research. PLoS pathogens 5: e1000600.

Comas I, Homolka S, Niemann S, Gagneux S (2009) Genotyping of genetically monomorphic bacteria: DNA sequencing in *Mycobacterium tuberculosis* highlights the limitations of current methodologies. PloS one 4: e7815.

Comstock GW (1978) Tuberculosis in twins: a re-analysis of the Prop hit survey. The American review of respiratory disease 117: 621-624.

Condos R, Rom WN, Liu YM, Schluger NW (1998) Local immune responses correlate with presentation and outcome in tuberculosis. American journal of respiratory and critical care medicine 157: 729-735.

Connolly SF, Kusner DJ (2007) The regulation of dendritic cell function by calcium-signalling and its inhibition by microbial pathogens. Immunologic research 39: 115-127.

Converse PJ, Dannenberg AM, Estep JE, Sugisaki K, Abe Y, et al. (1996) Cavitary tuberculosis produced in rabbits by aerosolized virulent tubercle bacilli. Infection and immunity 64: 4776-4787.

Cooke GS, Campbell SJ, Sillah J, Gustafson P, Bah B, et al. (2006) Polymorphism within the interferon-γ/receptor complex is associated with pulmonary tuberculosis. American journal of respiratory and critical care medicine 174: 339-343.

Cooper AM, Magram J, Ferrante J, Orme IM (1997) Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*. The Journal of experimental medicine 186: 39-45.

Cooper AM, Segal BH, Frank AA, Holland SM, Orme IM (2000) Transient loss of resistance to pulmonary tuberculosis in p47 phox-/- mice. Infection and immunity 68: 1231-1234.

Cosma CL, Humbert O, Ramakrishnan L (2004) Superinfecting *Mycobacteria* home to established tuberculous granulomas. Nature immunology 5: 828-835.

Cosma CL, Swaim LE, Volkman H, Ramakrishnan L, Davis J (2006) Zebrafish and frog models of Mycobacterium marinum infection. Current Protocols in Microbiology: 10B. 12.11-10B. 12.33.

Cowley S, Ko M, Pick N, Chow R, Downing KJ, et al. (2004) The *Mycobacterium tuberculosis* protein serine/threonine kinase PknG is linked to cellular glutamate/glutamine levels and is important for growth *in vivo*. Molecular microbiology 52: 1691-1702.

Cox RA, Katoch VM (1986) Evidence for genetic divergence in ribosomal RNA genes in *Mycobacteria*. FEBS letters 195: 194-198.

Crowley L, Crowley LV (2010) An introduction to human disease: Pathology and pathophysiology correlations: Jones & Bartlett Publishers.

Daffe M, McNeil M, Brennan PJ (1991) Novel type-specific lipooligosaccharides from *Mycobacterium tuberculosis*. Biochemistry 30: 378-388.

Dahl KE, Shiratsuchi H, Hamilton BD, Ellner JJ, Toossi Z (1996) Selective induction of transforming growth factor beta in human monocytes by lipoarabinomannan of *Mycobacterium tuberculosis*. Infection and immunity 64: 399-405.

Daley P, Thomas S, Pai M (2007) Nucleic acid amplification tests for the diagnosis of tuberculous lymphadenitis: a systematic review [Review Article]. The International Journal of Tuberculosis and Lung Disease 11: 1166-1176.

Dannenberg Jr AM (1994) Roles of cytotoxic delayed-type hypersensitivity and macrophage-activating cell-mediated immunity in the pathogenesis of tuberculosis. Immunobiology 191: 461-473.

Davidson PT, Le HQ (1992) Drug treatment of tuberculosis1992. Drugs 43: 651-673.

De Groote D, Zangerle P-Fo, Gevaert Y, Fassotte M-F, Beguin Y, et al. (1992) Direct stimulation of cytokines (IL-1B , TNF-A, IL-6, IL-2, IFN-γ and GM-CSF) in whole blood. I. Comparison with isolated PBMC stimulation. Cytokine 4: 239-248.

De Jong BC, Antonio M, Awine T, Ogungbemi K, De Jong YP, et al. (2009) Use of spoligotyping and large sequence polymorphisms to study the population structure of the *Mycobacterium tuberculosis* complex in a cohort study of consecutive smear-positive tuberculosis cases in The Gambia. Journal of clinical microbiology 47: 994-1001.

De Viedma DoGa, Infantes MdSDa, Lasala Ft, Chaves F, Alcala L, et al. (2002) New real-time PCR able to detect in a single tube multiple rifampin resistance mutations and high-level isoniazid resistance mutations in *Mycobacterium tuberculosis*. Journal of clinical microbiology 40: 988-995.

Demangel C, Britton WJ (2000) Interaction of dendritic cells with *Mycobacteria*: where the action starts. Immunology and cell biology 78: 318-324.

Deretic V, Singh S, Master S, Harris J, Roberts E, et al. (2006) *Mycobacterium tuberculosis* inhibition of phagolysosome biogenesis and autophagy as a host defence mechanism. Cellular microbiology 8: 719-727.

Desjardins M, Houde M, Gagnon E (2005) Phagocytosis: the convoluted way from nutrition to adaptive immunity. Immunological reviews 207: 158-165.

Dinarello CA (1996) Biologic basis for interleukin-1 in disease. Blood 87: 2095-2147.

Ding AH, Nathan CF, Stuehr DJ (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. The Journal of Immunology 141: 2407-2412.

Domenech P, Reed MB (2009) Rapid and spontaneous loss of phthiocerol dimycocerosate (PDIM) from *Mycobacterium tuberculosis* grown in vitro: implications for virulence studies. Microbiology 155: 3532.

Dormans J, Burger M, Aguilar D, Hernandez-Pando R, Kremer K, et al. (2004) Correlation of virulence, lung pathology, bacterial load and delayed type hypersensitivity responses after infection with different *Mycobacterium tuberculosis* genotypes in a BALB/c mouse model. Clin Exp Immunol 137: 460-468.

Downing JF, Pasula R, Wright JR, Twigg HL, Martin WJ (1995) Surfactant protein A promotes attachment of *Mycobacterium tuberculosis* to alveolar macrophages during

infection with human immunodeficiency virus. Proceedings of the National Academy of Sciences 92: 4848-4852.

Drobniewski FA, Wilson SM (1998) The rapid diagnosis of isoniazid and rifampicin resistance in *Mycobacterium tuberculosis*a molecular story. Journal of medical microbiology 47: 189-196.

Duan L, Gan H, Arm J, Remold HG (2001) Cytosolic phospholipase A2 participates with TNF-A in the induction of apoptosis of human macrophages infected with *Mycobacterium tuberculosis* H37Ra. The Journal of Immunology 166: 7469.

Dye C, Scheele S, Dolin P, Pathania V, Raviglione MC (1999) Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. JAMA 282: 677-686.

El Baghdadi J, Orlova M, Alter A, Ranque B, Chentoufi M, et al. (2006) An autosomal dominant major gene confers predisposition to pulmonary tuberculosis in adults. The Journal of experimental medicine 203: 1679-1684.

Epstein FH, Blobe GC, Schiemann WP, Lodish HF (2000) Role of transforming growth factor B in human disease. New England Journal of Medicine 342: 1350-1358.

Ernst JD (1998) Macrophage receptors for *Mycobacterium tuberculosis*. Infection and immunity 66: 1277-1281.

Estaquier J, Tanaka M, Suda T, Nagata S, Golstein P, et al. (1996) Fas-mediated apoptosis of CD4+ and CD8+ T cells from human immunodeficiency virus-infected persons: differential *in vitro* preventive effect of cytokines and protease antagonists. Blood 87: 4959-4966.

Ezekowitz RA, Sastry K, Bailly P, Warner A (1990) Molecular characterization of the human macrophage mannose receptor: demonstration of multiple carbohydrate recognition-like domains and phagocytosis of yeasts in Cos-1 cells. The Journal of experimental medicine 172: 1785-1794.

Fang Z, Morrison N, Watt B, Doig C, Forbes KJ (1998) IS6110 transposition and evolutionary scenario of the direct repeat locus in a group of closely related *Mycobacterium tuberculosis* strains. Journal of bacteriology 180: 2102-2109.

Farhat M, Greenaway C, Pai M, Menzies D (2006) False-positive tuberculin skin tests: what is the absolute effect of BCG and non-tuberculous *Mycobacteria*?[Review Article]. The International Journal of Tuberculosis and Lung Disease 10: 1192-1204.

Feng CG, Kaviratne M, Rothfuchs AG, Cheever A, Hieny S, et al. (2006) NK cell-derived IFN-γ differentially regulates innate resistance and neutrophil response in T cell-deficient hosts infected with *Mycobacterium tuberculosis*. The Journal of Immunology 177: 7086-7093.

Ferguson, J. S., J. L. Martin, et al. (2006). "Surfactant protein D increases fusion of Mycobacterium tuberculosis-containing phagosomes with lysosomes in human macrophages." Infection and immunity 74(12): 7005-7009.

Ferguson, J. S., D. R. Voelker, et al. (1999). "Surfactant protein D binds to Mycobacterium tuberculosis Bacilli and Lipoarabinomannan via carbohydrate-lectin interactions resulting in reduced phagocytosis of the bacteria by macrophages1." The Journal of Immunology 163(1): 312-321.

Ferrari, G., Naito, M., Langen, H., and Pieters, J. (1999). A coat protein on phagosomes involved in the intracellular survival of mycobacteria. Cell 97, 435–447.

Filliol I, Driscoll JR, Van Soolingen D, Kreiswirth BN, Kremer K, et al. (2002) Global distribution of *Mycobacterium tuberculosis* spoligotypes. Emerging Infectious Diseases 8: 1347.

Filliol I, Driscoll JR, van Soolingen D, Kreiswirth BN, Kremer K, et al. (2003) Snapshot of moving and expanding clones of *Mycobacterium tuberculosis* and their global distribution assessed by spoligotyping in an international study. Journal of clinical microbiology 41: 1963-1970.

Filliol I, Sola C, Rastogi N (2000) Detection of a previously unamplified spacer within the DR locus of *Mycobacterium tuberculosis*: epidemiological implications. Journal of clinical microbiology 38: 1231-1234.

Fink SL, Cookson BT (2005) Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. Infection and immunity 73: 1907-1916.

Flesch IE, Hess JH, Huang S, Aguet M, Rothe J, et al. (1995) Early interleukin 12 production by macrophages in response to *Mycobacteria*l infection depends on interferon gamma and tumour necrosis factor alpha. The Journal of experimental medicine 181: 1615-1621.

Flynn JL (2006) Lessons from experimental *Mycobacterium tuberculosis* infections. Microbes and Infection 8: 1179-1188.

Flynn JL, Chan J (2001) Immunology of tuberculosis. Annual review of immunology 19: 93-129.

Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, et al. (1993) An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. The Journal of experimental medicine 178: 2249-2254.

Forget A, Skamene E, Gros P, Miailhe A-C, Turcotte R (1981) Differences in response among inbred mouse strains to infection with small doses of Mycobacterium bovis BCG. Infection and immunity 32: 42-47.

Fortsch D, Rollinghoff M, Stenger S (2000) IL-10 converts human dendritic cells into macrophage-like cells with increased antibacterial activity against virulent *Mycobacterium tuberculosis*. The Journal of Immunology 165: 978-987.

Fratazzi C, Arbeit RD, Carini C, Remold HG (1997) Programmed cell death of *Mycobacterium avium* serovar 4-infected human macrophages prevents the *Mycobacteria* from spreading and induces *Mycobacteria*l growth inhibition by freshly added, uninfected macrophages. The Journal of Immunology 158: 4320-4327.

Freidag BL, Melton GB, Collins F, Klinman DM, Cheever A, et al. (2000) CpG oligodeoxynucleotides and interleukin-12 improve the efficacy of Mycobacterium bovis BCG vaccination in mice challenged with M. tuberculosis. Infection and immunity 68: 2948-2953.

Friedland JS, Hartley JC, Hartley CGC, Shattock RJ, Griffin GE (1995) Inhibition of *ex vivo* proinflammatory cytokine secretion in fatal *Mycobacterium tuberculosis* infection. Clinical & Experimental Immunology 100: 233-238.

Friedman CR, Quinn GC, Kreiswirth BN, Perlman DC, Salomon N, et al. (1997) Widespread dissemination of a drug-susceptible strain of *Mycobacterium tuberculosis*. Journal of Infectious Diseases 176: 478-484.

Frothingham R, Meeker-O'Connell WA (1998) Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. Microbiology 144: 1189-1196.

Gagneux S (2009) Fitness cost of drug resistance in *Mycobacterium tuberculosis*. Clinical Microbiology and Infection 15: 66-68.

Gagneux S, DeRiemer K, Van T, Kato-Maeda M, de Jong BC, et al. (2006) Variable host-pathogen compatibility in *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A 103: 2869-2873.

Gagneux S, Small PM (2007) Global phylogeography of *Mycobacterium tuberculosis* and implications for tuberculosis product development. The Lancet infectious diseases 7: 328-337.

Gan H, Lee J, Ren F, Chen M, Kornfeld H, et al. (2008) *Mycobacterium tuberculosis* blocks crosslinking of annexin-1 and apoptotic envelope formation on infected macrophages to maintain virulence. Nature immunology 9: 1189-1197.

Ganatra RD, Buddemeyer EU, Deodhar MN, Narkar AA, Powar DM, et al. (1980) Modifications in biphasic liquid-scintillation vial system for radiometry. The Journal of Nuclear Medicine 21: 480-483.

Gangadharam PRJ, Perumal VK, Crawford JT, Bates JH (1988) Association of Plasmids and Virulence of Mycobacterium avium Complex1-3. Am Rev Respir Dis 137: 212-214.

Garcia de Viedma D (2003) Rapid detection of resistance in *Mycobacterium tuberculosis*: a review discussing molecular approaches. Clinical Microbiology and Infection 9: 349-359.

Gaynor CD, McCormack FX, Voelker DR, McGowan SE, Schlesinger LS (1995) Pulmonary surfactant protein A mediates enhanced phagocytosis of *Mycobacterium tuberculosis* by a direct interaction with human macrophages. The Journal of Immunology 155: 5343-5351.

Geluk A, van Meijgaarden KE, Franken KLMC, Drijfhout JW, D Souza S, et al. (2000) Identification of major epitopes of *Mycobacterium tuberculosis* AG85B that are recognized by HLA-A* 0201-restricted CD8+ T cells in HLA-transgenic mice and humans. The Journal of Immunology 165: 6463-6471.

Gercken J, Pryjma J, Ernst M, Flad H-D (1994) Defective antigen presentation by *Mycobacterium tuberculosis*-infected monocytes. Infection and immunity 62: 3472-3478.

Gerosa F, Nisii C, Righetti S, Micciolo R, Marchesini M, et al. (1999) CD4+ T Cell Clones Producing both Interferon-γ and Interleukin-10 Predominate in Bronchoalveolar Lavages of Active Pulmonary Tuberculosis Patients. Clinical Immunology 92: 224-234.

Gibson AL, Huard RC, van Pittius NCG, Lazzarini LCO, Driscoll J, et al. (2008) Application of sensitive and specific molecular methods to uncover global dissemination of the major RDRio sublineage of the Latin American-Mediterranean *Mycobacterium tuberculosis* spoligotype family. Journal of clinical microbiology 46: 1259-1267.

Glynn JR, Whiteley J, Bifani PJ, Kremer K, van Soolingen D (2002) Worldwide occurrence of Beijing/W strains of *Mycobacterium tuberculosis*: a systematic review. Emerg Infect Dis 8: 843-849.

Goble M, Iseman MD, Madsen LA, Waite D, Ackerson L, et al. (1993) Treatment of 171 patients with pulmonary tuberculosis resistant to isoniazid and rifampicin. New England Journal of Medicine 328: 527-532.

Golesi F, Brignatz J, Bellenfant M, Raoult D, Drancourt M (2012) *Mycobacterium tuberculosis* Beijing outbreak in a school in Marseille, France, 2012. Euro surveillance: bulletin europaen sur les maladies transmissibles. European communicable disease bulletin 18.

Gonzalez-y-Merchand JA, Colston MJ, Cox RA (1996) The rRNA operons of Mycobacterium smegmatis and *Mycobacterium tuberculosis*: comparison of promoter elements and of neighbouring upstream genes. Microbiology 142: 667-674.

Gordon SV, Brosch R, Billault A, Garnier T, Eiglmeier K, et al. (1999) Identification of variable regions in the genomes of tubercle bacilli using bacterial artificial chromosome arrays. Molecular microbiology 32: 643-655.

Greenwood CMT, Fujiwara TM, Boothroyd LJ, Miller MA, Frappier D, et al. (2000) Linkage of Tuberculosis to Chromosome 2q35 Loci, Including*NRAMP1*,in a Large Aboriginal Canadian Family. The American Journal of Human Genetics 67: 405-416.

Groenen P, Bunschoten AE, Soolingen Dv, Errtbden JDAv (1993) Nature of DNA polymorphism in the direct repeat cluster of *Mycobacterium tuberculosis*; application for strain differentiation by a novel typing method. Molecular microbiology 10: 1057-1065.

Guinn, K. M., M. J. Hickey, et al. (2004). "Individual RD1 region genes are required for export of ESAT-6/CFP-10 and for virulence of Mycobacterium tuberculosis." Molecular microbiology 51(2): 359-370.

Gupta UD, Katoch VM (2009) Animal models of tuberculosis for vaccine development. Indian J Med Res 129: 11-18.

Gutierrez MC, Ahmed N, Willery E, Narayanan S, Hasnain SE, et al. (2006) Predominance of ancestral lineages of *Mycobacterium tuberculosis* in India. Emerging Infectious Diseases 12: 1367.

Hahn M-Y, Raman S, Anaya M, Husson RN (2005) The *Mycobacterium tuberculosis* extracytoplasmic-function sigma factor SigL regulates polyketide synthases and secreted or membrane proteins and is required for virulence. Journal of bacteriology 187: 7062-7071.

Hancock JM (1995) The contribution of slippage-like processes to genome evolution. Journal of Molecular Evolution 41: 1038-1047.

Hao M, Maxfield FR (2000) Characterization of rapid membrane internalization and recycling. Journal of Biological Chemistry 275: 15279-15286.

Harada M, Taniguchi, M.et al. (2003). "The regulatory role of V alpha14 NKT cells in innate and acquired immune response." Annual review of immunology 21(1): 483-513.

Harris ES, McIntyre TM, Prescott SM, Zimmerman GA (2000) The leukocyte integrins. Journal of Biological Chemistry 275: 23409-23412.

Heep M, BrandstA¤tter B, Rieger U, Lehn N, Richter E, et al. (2001) Frequency of *rpoB* mutations inside and outside the cluster I region in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates. Journal of clinical microbiology 39: 107-110.

Henderson RA, Watkins SC, Flynn JL (1997) Activation of human dendritic cells following infection with *Mycobacterium tuberculosis*. The Journal of Immunology 159: 635-643.

Hermans PW, Van Soolingen D, Bik EM, De Haas PE, Dale JW, et al. (1991) Insertion element IS987 from Mycobacterium bovis BCG is located in a hot-spot integration region for insertion elements in *Mycobacterium tuberculosis* complex strains. Infection and immunity 59: 2695-2705.

Hernandez-Pando R, Orozcoe H, Sampieri A, Pavon L, Velasquillo C, et al. (1996) Correlation between the kinetics of Th1, Th2 cells and pathology in a murine model of experimental pulmonary tuberculosis. Immunology 89: 26.

Hernandez-Pando R, Pavon L, Arriaga K, Orozco H, Madrid-Marina V, et al. (1997) Pathogenesis of tuberculosis in mice exposed to low and high doses of an environmental *Mycobacteria*l saprophyte before infection. Infection and immunity 65: 3317-3327.

Hernandez-Pando R, Schon T, Orozco EH, Serafin J, Estrada-Garcia I (2001) Expression of inducible nitric oxide synthase and nitrotyrosineduring the evolution of experimental pulmonary tuberculosis. Experimental and Toxicologic Pathology 53: 257-265.

Herrmann J-L, Lagrange P-H (2005) Dendritic cells and *Mycobacterium tuberculosis*: which is the Trojan horse? Pathologie Biologie 53: 35-40.

Hershberg R, Lipatov M, Small PM, Sheffer H, Niemann S, et al. (2008) High functional diversity in *Mycobacterium tuberculosis* driven by genetic drift and human demography. PLoS biology 6: e311.

Hetherington SV, Watson AS, Patrick CC (1995) Sequence and analysis of the rpoB gene of Mycobacterium smegmatis. Antimicrobial agents and chemotherapy 39: 2164-2166.

Hoal-van Helden EG, Stanton LA, Warren R, Richardson M, van Helden PD (2001) Diversity of *in vitro* cytokine responses by human macrophages to infection by *Mycobacterium tuberculosis* strains. Cell Biol Int 25: 83-90.

Holland SM, Dorman SE, Kwon A, Pitha-Rowe IF, Frucht DM, et al. (1998) Abnormal regulation of interferon-γ, interleukin-12, and tumour necrosis factor-α in human interferon-γ receptor 1 deficiency. Journal of Infectious Diseases 178: 1095-1104.

Holt PG, Schon-Hegrad MA (1987) Localization of T cells, macrophages and dendritic cells in rat respiratory tract tissue: implications for immune function studies. Immunology 62: 349.

Holtmeier W, Kabelitz D (2005) γδ T cells link innate and adaptive immune responses.

Hou JM, D'Lima NG, Rigel NW, Gibbons HS, McCann JR, et al. (2008) ATPase activity of *Mycobacterium tuberculosis* SecA1 and SecA2 proteins and its importance for SecA2 function in macrophages. Journal of bacteriology 190: 4880-4887.

Howard AD, Zwilling BS (1999) Reactivation of tuberculosis is associated with a shift from type 1 to type 2 cytokines. Clinical and experimental immunology 115: 428-434.

Hsieh S-M, Hung C-C, Chen M-Y, Sheng W-H, Chang S-C (1999) Dynamics of plasma cytokine levels in patients with advanced HIV infection and active tuberculosis: implications for early recognition of patients with poor response to anti-tuberculosis treatment. Aids 13: 935-941.

Huebner RE, Schein MF, Bass Jr JB (1993) The tuberculin skin test. Clinical infectious diseases: 968-975.

Hull J, Thomson A, Kwiatkowski D (2000) Association of respiratory syncytial virus bronchiolitis with the interleukin 8 gene region in UK families. Thorax 55: 1023-1027.

Ignatova A, Dubiley S, Stepanshina V, Shemyakin I (2006) Predominance of multi-drug-resistant LAM and Beijing family strains among *Mycobacterium tuberculosis* isolates recovered from prison inmates in Tula Region, Russia. Journal of medical microbiology 55: 1413.

Jacob JT, Mehta AK, Leonard MK (2009) Acute forms of tuberculosis in adults. The American journal of medicine 122: 12-17.

Jagannath C, Actor JK, Hunter RL (1998) Induction of Nitric Oxide in Human Monocytes and Monocyte Cell Lines by *Mycobacterium tuberculosis*. Nitric Oxide 2: 174-186.

Jamieson SE, Miller EN, Black GF, Peacock CS, Cordell HJ, et al. (2004) Evidence for a cluster of genes on chromosome 17q11q21 controlling susceptibility to tuberculosis and leprosy in Brazilians. Genes and immunity 5: 46-57.

Janeway CA. Approaching the asymptote? Evolution and revolution in immunology; 1989.Cold Spring Harbor Laboratory Press. pp. 1-13.

Janis EM, Kaufmann SH, Schwartz RH, Pardoll DM (1989) Activation of gamma delta T cells in the primary immune response to *Mycobacterium tuberculosis*. Science 244: 713-716.

Jansen R, van Embden JDA, Gaastra W, Schouls LM (2002) Identification of a novel family of sequence repeats among prokaryotes. Omics: a journal of integrative biology 6: 23-33.

Jayachandran Rajesh, Varadharajan Sundaramurthy, Benoit Combaluzier, Philipp Mueller et al (2007) Survival of Mycobacteria in Macrophages Is Mediated by Coronin 1-Dependent Activation of Calcineurin. Cell 130, 37–50

Juffermans NP, Florquin S, Camoglio L, Verbon A, Kolk AH, et al. (2000) Interleukin-1 signalling is essential for host defense during murine pulmonary tuberculosis. Journal of Infectious Diseases 182: 902-908.

Juffermans NP, Verbon A, van Deventer SJH, van Deutekom H, Belisle JT, et al. (1999) Elevated chemokine concentrations in sera of human immunodeficiency virus (HIV)-seropositive and HIV-seronegative patients with tuberculosis: a possible role for *Mycobacteria*l lipoarabinomannan. Infection and immunity 67: 4295-4297.

Jung Y-J, LaCourse R, Ryan L, North RJ (2002) Virulent but not avirulent *Mycobacterium tuberculosis* can evade the growth inhibitory action of a T helper 1dependent, nitric oxide synthase 2independent defense in mice. The Journal of experimental medicine 196: 991-998.

Kamath AT, Feng CG, Macdonald M, Briscoe H, Britton WJ (1999) Differential protective efficacy of DNA vaccines expressing secreted proteins of *Mycobacterium tuberculosis*. Infection and immunity 67: 1702-1707.

Kamerbeek J, Schouls L, Kolk A, Van Agterveld M, Van Soolingen D, et al. (1997) Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. Journal of clinical microbiology 35: 907-914.

Kaplan G, Post FA, Moreira AL, Wainwright H, Kreiswirth BN, et al. (2003) *Mycobacterium tuberculosis* growth at the cavity surface: a microenvironment with failed immunity. Infection and immunity 71: 7099-7108.

Kapsenberg ML (2003) Dendritic-cell control of pathogen-driven T-cell polarization. Nature Reviews Immunology 3: 984-993.

Kaufmann SHE (2001) How can immunology contribute to the control of tuberculosis? Nature Reviews Immunology 1: 20-30.

Kaufmann SHE, Schaible UE (2003) A Dangerous Liaison between Two Major Killers *Mycobacterium tuberculosis* and HIV Target Dendritic Cells through DC-SIGN. The Journal of experimental medicine 197: 1-5.

Keane J, Balcewicz-Sablinska MK, Remold HG, Chupp GL, Meek BB, et al. (1997) Infection by *Mycobacterium tuberculosis* promotes human alveolar macrophage apoptosis. Infection and immunity 65: 298-304.

Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, et al. (2001) Tuberculosis associated with infliximab, a tumour necrosis factor Aneutralizing agent. New England Journal of Medicine 345: 1098-1104.

Keane J, Remold HG, Kornfeld H (2000) Virulent *Mycobacterium tuberculosis* strains evade apoptosis of infected alveolar macrophages. The Journal of Immunology 164: 2016.

Kelly, D. M., A. M. C. ten Bokum, et al. (2008). "Bystander macrophage apoptosis after Mycobacterium tuberculosis H37Ra infection." Infection and immunity 76(1): 351-360.

Kent PT, Kubica GP (1985) Public health mycobacteriology: a guide for the level III laboratory: US Department of Health and Human Services, Public Health Service, Centers for Disease Control.

Khader SA, Pearl JE, Sakamoto K, Gilmartin L, Bell GK, et al. (2005) IL-23 compensates for the absence of IL-12p70 and is essential for the IL-17 response during tuberculosis but is dispensable for protection and antigen-specific IFN-γ responses if IL-12p70 is available. The Journal of Immunology 175: 788-795.

Khan, M. R. and M. E. Rahman (2000). "Essence of pediatrics." Anwar Khan Ltd, Dhaka.

Khoo K-H, Dell A, Morris HR, Brennan PJ, Chatterjee D (1995) Inositol phosphate capping of the nonreducing termini of lipoarabinomannan from rapidly growing strains of Mycobacterium. Journal of Biological Chemistry 270: 12380-12389.

Kindler V, Sappino A-P, Grau GE, Piguet P-Fo, Vassalli P (1989) The inducing role of tumour necrosis factor in the development of bactericidal granulomas during BCG infection. Cell 56: 731-740.

Klingler K, Tchou-Wong K-M, Brandli O, Aston C, Kim R, et al. (1997) Effects of *Mycobacteria* on regulation of apoptosis in mononuclear phagocytes. Infection and immunity 65: 5272-5278.

Koksalan OK (2006) Use of *Mycobacteria*l interspersed repetitive unit locus 26 for rapid identification of Beijing genotype *Mycobacterium tuberculosis* strains. Journal of clinical microbiology 44: 1612-1613.

Kong Y, Cave MD, Yang D, Zhang L, Marrs CF, et al. (2005) Distribution of insertion-and deletion-associated genetic polymorphisms among four *Mycobacterium tuberculosis* phospholipase C genes and associations with extrathoracic tuberculosis: a population-based study. Journal of clinical microbiology 43: 6048-6053.

Kong Y, Cave MD, Zhang L, Foxman B, Marrs CF, et al. (2007) Association between *Mycobacterium tuberculosis* Beijing/W lineage strain infection and extrathoracic tuberculosis: insights from epidemiologic and clinical characterization of the three principal genetic groups of M. tuberculosis clinical isolates. Journal of clinical microbiology 45: 409-414.

Kremer K, Arnold C, Cataldi A, GutiArrez MC, Haas WH, et al. (2005) Discriminatory power and reproducibility of novel DNA typing methods for *Mycobacterium tuberculosis* complex strains. Journal of clinical microbiology 43: 5628-5638.

Kremer L, Estaquier Jrm, Brandt E, Ameisen JC, Locht C (1997) Mycobacterium bovis Bacillus Calmette Guerin infection prevents apoptosis of resting human monocytes. European journal of immunology 27: 2450-2456.

Kulkarni S, Singh P, Memon A, Nataraj G, Kanade S, et al. (2012) An in-house multiplex PCR test for the detection of *Mycobacterium tuberculosis*, its validation & comparison with a single target TB-PCR kit. The Indian journal of medical research 135: 788.

Kulkarni S, Sola C, Filliol I, Rastogi N, Kadival G (2005) Spoligotyping of *Mycobacterium tuberculosis* isolates from patients with pulmonary tuberculosis in Mumbai, India. Research in microbiology 156: 588-596.

Kumar, A., J. C. Toledo, et al. (2007). "Mycobacterium tuberculosis DosS is a redox sensor and DosT is a hypoxia sensor." Proceedings of the National Academy of Sciences 104(28): 11568-11573.

Ladel CH, Szalay G, Riedel D, Kaufmann SH (1997) Interleukin-12 secretion by *Mycobacterium tuberculosis*-infected macrophages. Infection and immunity 65: 1936-1938.

Lambrecht RS, Carriere JF, Collins MT (1988) A model for analyzing growth kinetics of a slowly growing Mycobacterium sp. Applied and environmental microbiology 54: 910-916.

Lan NTN, Lien HTK, Tung LB, Borgdorff MW, Kremer K, et al. (2003) *Mycobacterium tuberculosis* Beijing genotype and risk for treatment failure and relapse, Vietnam. Emerging Infectious Diseases 9: 1633.

Langermans JAM, Andersen P, van Soolingen D, Vervenne RAW, Frost PA, et al. (2001) Divergent effect of bacillus CalmetteGuerin (BCG) vaccination on *Mycobacterium tuberculosis* infection in highly related macaque species: Implications for primate

models in tuberculosis vaccine research. Proceedings of the National Academy of Sciences 98: 11497-11502.

Lari N, Rindi L, Cristofani R, Rastogi N, Tortoli E, et al. (2009) Association of *Mycobacterium tuberculosis* complex isolates of *Bovis* and Central Asian (CAS) genotypic lineages with extrapulmonary disease. Clinical Microbiology and Infection 15: 538-543.

Lasunskaia E, Ribeiro S, Manicheva O, Gomes LL, Suffys PN, et al. (2010) Emerging multidrug resistant *Mycobacterium tuberculosis* strains of the Beijing genotype circulating in Russia express a pattern of biological properties associated with enhanced virulence. Microbes and Infection 12: 467-475.

Law K, Weiden M, Harkin T, Tchou-Wong K, Chi C, et al. (1996) Increased release of interleukin-1 beta, interleukin-6, and tumour necrosis factor-alpha by bronchoalveolar cells lavaged from involved sites in pulmonary tuberculosis. American journal of respiratory and critical care medicine 153: 799-804.

Lazzarini LCO, Huard RC, Boechat NL, Gomes HM, Oelemann MC, et al. (2007) Discovery of a novel *Mycobacterium tuberculosis* lineage that is a major cause of tuberculosis in Rio de Janeiro, Brazil. Journal of clinical microbiology 45: 3891-3902.

Lazzarini LCO, Spindola SM, Bang H, Gibson AL, Weisenberg S, et al. (2008) RDRio *Mycobacterium tuberculosis* infection is associated with a higher frequency of cavitary pulmonary disease. Journal of clinical microbiology 46: 2175-2183.

Legrand E, Filliol I, Sola C, Rastogi N (2001) Use of Spoligotyping To Study the Evolution of the Direct Repeat Locus by IS6110 Transposition in*Mycobacterium tuberculosis*. Journal of clinical microbiology 39: 1595-1599.

Li Q, Whalen CC, Albert JM, Larkin R, Zukowski L, et al. (2002) Differences in rate and variability of intracellular growth of a panel of *Mycobacterium tuberculosis* clinical isolates within a human monocyte model. Infection and immunity 70: 6489-6493.

Lienhardt C, Fielding K, Sillah JS, Bah B, Gustafson P, et al. (2005) Investigation of the risk factors for tuberculosis: a casecontrol study in three countries in West Africa. International Journal of Epidemiology 34: 914-923.

Lin Y, Gong J, Zhang M, Xue W, Barnes PF (1998) Production of monocyte chemoattractant protein 1 in tuberculosis patients. Infection and immunity 66: 2319-2322.

Lin Y, Zhang M, Hofman FM, Gong J, Barnes PF (1996) Absence of a prominent Th2 cytokine response in human tuberculosis. Infection and immunity 64: 1351-1356.

Ling DI, Flores LL, Riley LW, Pai M (2008) Commercial nucleic-acid amplification tests for diagnosis of pulmonary tuberculosis in respiratory specimens: meta-analysis and meta-regression. PloS one 3: e1536.

Ling LU, Tan KB, Lin H, Chiu GNC (2011) The role of reactive oxygen species and autophagy in safingol-induced cell death. Cell death & disease 2: e129.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-[Delta][Delta] CT method. Methods 25: 402-408.

Lopez B, Aguilar D, Orozco H, Burger M, Espitia C, et al. (2003) A marked difference in pathogenesis and immune response induced by different *Mycobacterium tuberculosis* genotypes. Clin Exp Immunol 133: 30-37.

Lucey DR, Clerici M, Shearer GM (1996) Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. Clinical Microbiology Reviews 9: 532-562.

Lukacs NW, Addison CL, Gauldie J, Graham F, Simpson K, et al. (1997) Transgene-induced production of IL-4 alters the development and collagen expression of T helper cell 1-type pulmonary granulomas. The Journal of Immunology 158: 4478-4484.

Lurie, M. B. (1928). "The fate of human and bovine tubercle bacilli in various organs of the rabbit." The Journal of experimental medicine 48(2): 155-182.

Ma X, Reich RA, Wright JA, Tooker HR, Teeter LD, et al. (2003) Association between interleukin-8 gene alleles and human susceptibility to tuberculosis disease. Journal of Infectious Diseases 188: 349-355.

MacMicking, J.D. (2005). Immune control of phagosomal bacteria by p47 GTPases. Curr. Opin. Microbiol. 8, 74–82.

MacMicking J, Xie Q-w, Nathan C (1997) Nitric oxide and macrophage function. Annual review of immunology 15: 323-350.

MacMicking JD, Taylor GA, McKinney JD (2003) Immune control of tuberculosis by IFN-γ-inducible LRG-47. Science 302: 654-659.

Manca C, Reed MB, Freeman S, Mathema B, Kreiswirth B, et al. (2004) Differential monocyte activation underlies strain-specific *Mycobacterium tuberculosis* pathogenesis. Infection and immunity 72: 5511-5514.

Manca C, Tsenova L, Barry CE, Bergtold A, Freeman S, et al. (1999) *Mycobacterium tuberculosis* CDC1551 induces a more vigorous host response *in vivo* and in vitro, but is not more virulent than other clinical isolates. The Journal of Immunology 162: 6740.

Manca C, Tsenova L, Freeman S, Barczak AK, Tovey M, et al. (2005) Hypervirulent M. tuberculosis W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway. Journal of interferon & cytokine research 25: 694-701.

Marino S, Pawar S, Fuller CL, Reinhart TA, Flynn JL, et al. (2004) Dendritic cell trafficking and antigen presentation in the human immune response to *Mycobacterium tuberculosis*. The Journal of Immunology 173: 494-506.

Marquina-Castillo B, Garcia-Garcia L, Ponce-de-Leon A, Jimenez-Corona ME, Bobadilla-Del Valle M, et al. (2009) Virulence, immunopathology and transmissibility of selected strains of *Mycobacterium tuberculosis* in a murine model. Immunology 128: 123-133.

Marttila HJ, Soini H, Eerola E, Vyshnevskaya E, Vyshnevskiy BI, et al. (1998) A Ser315Thr substitution in KatG is predominant in genetically heterogeneous multidrug-resistant *Mycobacterium tuberculosis* isolates originating from the St. Petersburg area in Russia. Antimicrobial agents and chemotherapy 42: 2443-2445.

Mazars E, Lesjean S, Banuls A-L, Gilbert Ml, Vincent Vr, et al. (2001) High-resolution minisatellite-based typing as a portable approach to global analysis of *Mycobacterium tuberculosis* molecular epidemiology. Proceedings of the National Academy of Sciences 98: 1901-1906.

Mazzaccaro RJ, Gedde M, Jensen ER, Van Santen HM, Ploegh HL, et al. (1996) Major histocompatibility class I presentation of soluble antigen facilitated by *Mycobacterium tuberculosis* infection. Proceedings of the National Academy of Sciences 93: 11786-11791.

Mc Kinney JD, Jacobs Jr WR, Bloom BR (1998) 3 Persisting problems in tuberculosis. Biomedical Research Reports 1: 51-146.

McShane H, Pathan AA, Sander CR, Goonetilleke NP, Fletcher HA, et al. (2005) Boosting BCG with MVA85A: the first candidate subunit vaccine for tuberculosis in clinical trials. Tuberculosis 85: 47-52.

Means TK, Wang S, Lien E, Yoshimura A, Golenbock DT, et al. (1999) Human toll-like receptors mediate cellular activation by *Mycobacterium tuberculosis*. The Journal of Immunology 163: 3920-3927.

Mege J-L, Meghari S, Honstettre Al, Capo C, Raoult D (2006) The two faces of interleukin 10 in human infectious diseases. The Lancet infectious diseases 6: 557-569.

Merle CSC, Cunha SS, Rodrigues LC (2010) BCG vaccination and leprosy protection: review of current evidence and status of BCG in leprosy control. Expert review of vaccines 9: 209-222.

Meyer CG, Scarisbrick G, Niemann S, Browne ENL, Chinbuah MA, et al. (2008) Pulmonary tuberculosis: Virulence of *Mycobacterium africanum* and relevance in HIV co-infection. Tuberculosis 88: 482-489.

Middelkoop K, Bekker L-G, Mathema B, Shashkina E, Kurepina N, et al. (2009) Molecular epidemiology of *Mycobacterium tuberculosis* in a South African community with high HIV prevalence. Journal of Infectious Diseases 200: 1207-1211.

Mischenko VV, Kapina MA, Eruslanov EB, Kondratieva EV, Lyadova IV, et al. (2004) *Mycobacteria*l dissemination and cellular responses after 1-lobe restricted tuberculosis infection of genetically susceptible and resistant mice. Journal of Infectious Diseases 190: 2137-2145.

Mogga SJ, Mustafa T, Sviland L, Nilsen R (2002) Increased Bcl?2 and Reduced Bax Expression in Infected Macrophages in Slowly Progressive Primary Murine *Mycobacterium tuberculosis* Infection. Scandinavian journal of immunology 56: 383-391.

Mohagheghpour N, van Vollenhoven A, Goodman J, Bermudez LE (2000) Interaction of *Mycobacterium avium* with human monocyte-derived dendritic cells. Infection and immunity 68: 5824-5829.

Mohan VP, Scanga CA, Yu K, Scott HM, Tanaka KE, et al. (2001) Effects of tumour necrosis factor alpha on host immune response in chronic persistent tuberculosis: possible role for limiting pathology. Infection and immunity 69: 1847-1855.

Mokrousov I, Ly HM, Otten T, Lan NN, Vyshnevskyi B, et al. (2005) Origin and primary dispersal of the *Mycobacterium tuberculosis* Beijing genotype: clues from human phylogeography. Genome research 15: 1357-1364.

Morgan MA, Horstmeier CD, DeYoung DR, Roberts GD (1983) Comparison of a radiometric method (BACTEC) and conventional culture media for recovery of *Mycobacteria* from smear-negative specimens. Journal of clinical microbiology 18: 384-388.

Muller I, Cobbold SP, Waldmann H, Kaufmann SH (1987) Impaired resistance to *Mycobacterium tuberculosis* infection after selective *in vivo* depletion of L3T4+ and Lyt-2+ T cells. Infection and immunity 55: 2037-2041.

Munsiff SS, Nivin B, Sacajiu G, Mathema B, Bifani P, et al. (2003) Persistence of a highly resistant strain of tuberculosis in New York City during 19901999. Journal of Infectious Diseases 188: 356-363.

Murray PJ, Wang L, Onufryk C, Tepper RI, Young RA (1997) T cell-derived IL-10 antagonizes macrophage function in *Mycobacteria*l infection. The Journal of Immunology 158: 315-321.

Nardell EA (1993) Fans, filters, or rays? Pros and cons of the current environmental tuberculosis control technologies. Infection control and hospital epidemiology 14: 681-685.

Neill MA, Klebanoff SJ (1988) The effect of phenolic glycolipid-1 from Mycobacterium leprae on the antimicrobial activity of human macrophages. The Journal of experimental medicine 167: 30-42.

Ngai P, McCormick S, Small C, Zhang X, Zganiacz A, et al. (2007) Gamma interferon responses of CD4 and CD8 T-cell subsets are quantitatively different and independent

of each other during pulmonary Mycobacterium bovis BCG infection. Infection and immunity 75: 2244-2252.
Nguyen, L., and Pieters, J. (2005). The Trojan horse: survival tactics of pathogenic mycobacteria in macrophages. Trends Cell Biol. 15, 269–276.
Nicas M, Nazaroff WW, Hubbard A (2005) Toward understanding the risk of secondary airborne infection: emission of respirable pathogens. Journal of occupational and environmental hygiene 2: 143-154.
Nicholson S, da G Bonecini-Almeida M, e Silva JRL, Nathan C, Xie QW, et al. (1996) Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. The Journal of experimental medicine 183: 2293-2302.
Nicol MP, Sola C, February B, Rastogi N, Steyn L, et al. (2005) Distribution of strain families of *Mycobacterium tuberculosis* causing pulmonary and extrapulmonary disease in hospitalized children in Cape Town, South Africa. Journal of clinical microbiology 43: 5779-5781.
Nicol MP, Wilkinson RJ (2008) The clinical consequences of strain diversity in *Mycobacterium tuberculosis*. Transactions of the Royal Society of Tropical Medicine and Hygiene 102: 955-965.
Nigou Jrm, Zelle-Rieser C, Gilleron M, Thurnher M, Puzo G (2001) Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor. The Journal of Immunology 166: 7477-7485.
Niobe-Eyangoh SN, Kuaban C, Sorlin P, Thonnon J, Vincent V, et al. (2004) Molecular characteristics of strains of the cameroon family, the major group of *Mycobacterium tuberculosis* in a country with a high prevalence of tuberculosis. Journal of clinical microbiology 42: 5029-5035.
North RJ (1995) *Mycobacterium tuberculosis* is strikingly more virulent for mice when given via the respiratory than via the intravenous route. Journal of Infectious Diseases 172: 1550-1553.
North RJ (1998) Mice incapable of making IL-4 or IL-10 display normal resistance to infection with *Mycobacterium tuberculosis*. Clinical & Experimental Immunology 113: 55-58.
Noss EH, Pai RK, Sellati TJ, Radolf JD, Belisle J, et al. (2001) Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of *Mycobacterium tuberculosis*. The Journal of Immunology 167: 910-918.
Nozaki Y, Hasegawa Y, Ichiyama S, Nakashima I, Shimokata K (1997) Mechanism of nitric oxide-dependent killing of Mycobacterium bovis BCG in human alveolar macrophages. Infection and immunity 65: 3644-3647.
Oddo M, Renno T, Attinger A, Bakker T, MacDonald HR, et al. (1998) Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular *Mycobacterium tuberculosis*. The Journal of Immunology 160: 5448-5454.
Oelemann MC, Diel R, Vatin V, Haas W, RAsch-Gerdes S, et al. (2007) Assessment of an optimized *Mycobacteria*l interspersed repetitive-unit-variable-number tandem-repeat typing system combined with spoligotyping for population-based molecular epidemiology studies of tuberculosis. Journal of clinical microbiology 45: 691-697.
Orme IM, Andersen P, Boom WH (1993) T cell response to *Mycobacterium tuberculosis*. Journal of Infectious Diseases 167: 1481-1497.
Pabst MJ, Gross JM, Brozna JP, Goren MB (1988) Inhibition of macrophage priming by sulfatide from *Mycobacterium tuberculosis*. The Journal of Immunology 140: 634-640.

Pai M, Zwerling A, Menzies D (2008) Systematic review: T-cellbased assays for the diagnosis of latent tuberculosis infection: an update. Annals of internal medicine 149: 177-184.

Palomino JC (2005) Nonconventional and new methods in the diagnosis of tuberculosis: feasibility and applicability in the field. European Respiratory Journal 26: 339-350.

Pancholi P, Mirza A, Bhardwaj N, Steinman RM (1993) Sequestration from immune CD4+ T cells of *Mycobacteria* growing in human macrophages. Science 260: 984-986.

Park JS, Tamayo MH, Gonzalez-Juarrero M, Orme IM, Ordway DJ (2006) Virulent clinical isolates of *Mycobacterium tuberculosis* grow rapidly and induce cellular necrosis but minimal apoptosis in murine macrophages. Journal of leukocyte biology 79: 80-86.

Parwati I, van Crevel R, van Soolingen D (2010) Possible underlying mechanisms for successful emergence of the *Mycobacterium tuberculosis* Beijing genotype strains. Lancet Infect Dis 10: 103-111.

Pascopella L, Collins FM, Martin JM, Lee MH, Hatfull GF, et al. (1994) Use of *in vivo* complementation in *Mycobacterium tuberculosis* to identify a genomic fragment associated with virulence. Infection and immunity 62: 1313-1319.

Pasula R, Wright JR, Kachel DL, Martin WJ (1999) Surfactant protein A suppresses reactive nitrogen intermediates by alveolar macrophages in response to *Mycobacterium tuberculosis*. Journal of Clinical Investigation 103: 483-490.

Paul S, Laochumroonvorapong P, Kaplan G (1996) Comparable growth of virulent and avirulent *Mycobacterium tuberculosis* in human macrophages in vitro. Journal of Infectious Diseases 174: 105-112.

Pearson ML, Jereb JA, Frieden TR, Crawford JT, Davis BJ, et al. (1992) Nosocomial Transmission of Multidrug-resistant *Mycobacterium tuberculosis*: A Risk to Patients and Health Care Workers. Annals of internal medicine 117: 191-196.

Perenboom RM, Van Schijndel A, Beckers P, Sauerwein R, Van Hamersvelt HW, et al. (1996) Cytokine profiles in bronchoalveolar lavage fluid and blood in HIV□seronegative patients with *Pneumocystis carinii* pneumonia. European journal of clinical investigation 26: 159-166.

Perskvist N, Long M, Stendahl O, Zheng L (2002) *Mycobacterium tuberculosis* promotes apoptosis in human neutrophils by activating caspase-3 and altering expression of Bax/Bcl-xL via an oxygen-dependent pathway. The Journal of Immunology 168: 6358-6365.

Petrovsky N, McNair P, Harrison LC (1994) Circadian rhythmicity of interferon-gamma production in antigen-stimulated whole blood. Chronobiologia 21: 293.

Philips JA, Rubin EJ, Perrimon N (2005) Drosophila RNAi screen reveals CD36 family member required for *Mycobacteria*l infection. Science 309: 1251-1253.

Phyu S, Jureen R, Ti T, Dahle UR, Grewal HMS (2003) Heterogeneity of *Mycobacterium tuberculosis* isolates in Yangon, Myanmar. Journal of clinical microbiology 41: 4907-4908.

Piddock LJV (2006) Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clinical Microbiology Reviews 19: 382-402.

Pitulle C, Dorsch M, Kazda J, Wolters Jr, Stackebrandt E (1992) Phylogeny of rapidly growing members of the genus Mycobacterium. International journal of systematic bacteriology 42: 337-343.

Portevin D, Gagneux S, Comas I, Young D (2011) Human macrophage responses to clinical isolates from the *Mycobacterium tuberculosis* complex discriminate between ancient and modern lineages. PLoS pathogens 7: e1001307.

Powrie F, Coffman RL (1993) Inhibition of cell-mediated immunity by IL4 and IL10. Research in immunology 144: 639-643.

Prigozy TI, Sieling PA, Clemens D, Stewart PL, Behar SM, et al. (1997) The mannose receptor delivers lipoglycan antigens to endosomes for presentation to T cells by CD1b molecules. Immunity 6: 187-197.

Pym AS, Brodin P, Brosch R, Huerre M, Cole ST (2002) Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines Mycobacterium bovis BCG and Mycobacterium microti. Molecular microbiology 46: 709-717.

Qian L, Van Embden JDA, Van Der Zanden AGM, Weltevreden EF, Duanmu H, et al. (1999) Retrospective Analysis of the Beijing Family of*Mycobacterium tuberculosis* in Preserved Lung Tissues. Journal of clinical microbiology 37: 471-474.

Rajavelu P, Das SD (2007) A correlation between phagocytosis and apoptosis in THP-1 cells infected with prevalent strains of *Mycobacterium tuberculosis*. Microbiology and immunology 51: 201.

Rakotosamimanana N, Raharimanga V, Andriamandimby SF, Soares J-L, Doherty TM, et al.(2010) Variation in gamma interferon responses to different infecting strains of *Mycobacterium tuberculosis* in acid-fast bacillus smear-positive patients and household contacts in Antananarivo, Madagascar. Clinical and Vaccine Immunology 17: 1094-1103.

Ramaswamy S, Musser JM (1998) Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tubercle and Lung Disease 79: 3-29.

Rao KR, Ahmed N, Srinivas S, Sechi LA, Hasnain SE (2006) Rapid identification of *Mycobacterium tuberculosis* Beijing genotypes on the basis of the *Mycobacteria*l interspersed repetitive unit locus 26 signature. Journal of clinical microbiology 44: 274-277.

Raviglione MC, Pio A (2002) Evolution of WHO policies for tuberculosis control, 1948-2001. The Lancet 359: 775-780.

Ravn P, Munk ME, Andersen AB, Lundgren B, Lundgren JD, et al. (2005) Prospective evaluation of a whole-blood test using *Mycobacterium tuberculosis*-specific antigens ESAT-6 and CFP-10 for diagnosis of active tuberculosis. Clinical and diagnostic laboratory immunology 12: 491-496.

Reed MB, Pichler VK, McIntosh F, Mattia A, Fallow A, et al. (2009) Major *Mycobacterium tuberculosis* lineages associate with patient country of origin. Journal of clinical microbiology 47: 1119-1128.

Reiner SL, Locksley RM (1995) The regulation of immunity to *Leishmania major*. Annual review of immunology 13: 151-177.

Reisner BS, Gatson AM, Woods GL (1995) Evaluation of *Mycobacteria* growth indicator tubes for susceptibility testing of *Mycobacterium tuberculosis* to isoniazid and rifampin. Diagnostic microbiology and infectious disease 22: 325-329.

Remus N, El Baghdadi J, Fieschi C, Feinberg J, Quintin T, et al. (2004) Association of IL12RB1 polymorphisms with pulmonary tuberculosis in adults in Morocco. Journal of Infectious Diseases 190: 580-587.

Riedel DD, Kaufmann SH (1997) Chemokine secretion by human polymorphonuclear granulocytes after stimulation with *Mycobacterium tuberculosis* and lipoarabinomannan. Infection and immunity 65: 4620-4623.

Roberts LJ, Baldwin TM, Curtis JM, Handman E, Foote SJ (1997) Resistance to Leishmania major is linked to the H2 region on chromosome 17 and to chromosome 9. The Journal of experimental medicine 185: 1705-1710.

Roch F, Bach MA (1990) Strain differences in mouse cellular responses to Mycobacterium lepraemurium and BCG subcutaneous infections. I. Analysis of cell surface phenotype in local granulomas. Clinical and experimental immunology 80: 332.

Rodrigues LC, Diwan VK, Wheeler JG (1993) Protective effect of BCG against tuberculous meningitis and miliary tuberculosis: a meta-analysis. International Journal of Epidemiology 22: 1154-1158.

Rojas M, Olivier M, Gros P, Barrera LF, Garcia LF (1999) TNF-alpha and IL-10 modulate the induction of apoptosis by virulent *Mycobacterium tuberculosis* in murine macrophages. J Immunol 162: 6122-6131.

Rook GAW, Hernandez-Pando R (1996) The pathogenesis of tuberculosis. Annual Reviews in Microbiology 50: 259-284.

Roque S, Nobrega C, Appelberg R, Correia-Neves M (2007) IL-10 underlies distinct susceptibility of BALB/c and C57BL/6 mice to Mycobacterium avium infection and influences efficacy of antibiotic therapy. The Journal of Immunology 178: 8028-8035.

Rossouw M, Nel HJ, Cooke GS, van Helden PD, Hoal EG (2003) Association between tuberculosis and a polymorphic NF kB binding site in the interferon γ gene. The Lancet 361: 1871-1872.

Rouse DA, DeVito JA, Li Z, Byer H, Morris SL (1996) Site□directed mutagenesis of the katG gene of *Mycobacterium tuberculosis*: effects on catalaseperoxidase activities and isoniazid resistance. Molecular microbiology 22: 583-592.

Runyon EH (1970) Identification of *Mycobacteria*l pathogens utilizing colony characteristics. American journal of clinical pathology 54: 578-586.

Rusch-Gerdes S, Domehl C, Nardi G, Gismondo MR, Welscher H-M, et al. (1999) Multicenter evaluation of the *Mycobacteria* growth indicator tube for testing susceptibility of *Mycobacterium tuberculosis* to first-line drugs. Journal of clinical microbiology 37: 45-48.

Russell DG (2001) *Mycobacterium tuberculosis*: here today, and here tomorrow. Nature Reviews Molecular Cell Biology 2: 569-586.

Rutledge BJ, Rayburn H, Rosenberg R, North RJ, Gladue RP, et al. (1995) High level monocyte chemoattractant protein-1 expression in transgenic mice increases their susceptibility to intracellular pathogens. The Journal of Immunology 155: 4838-4843.

Sarkar R, Lenders L, Wilkinson KA, Wilkinson RJ, Nicol MP (2012) Modern Lineages of *Mycobacterium tuberculosis* Exhibit Lineage-Specific Patterns of Growth and Cytokine Induction in Human Monocyte-Derived Macrophages. PloS one 7: e43170.

Sartain MJ, Dick DL, Rithner CD, Crick DC, Belisle JT (2011) Lipidomic analyses of *Mycobacterium tuberculosis* based on accurate mass measurements and the novel Mtb LipidDB□. Journal of lipid research 52: 861-872.

Sassetti CM, Boyd DH, Rubin EJ (2003) Genes required for *Mycobacteria*l growth defined by high density mutagenesis. Molecular microbiology 48: 77-84.

Scanga CA, Mohan VP, Yu K, Joseph H, Tanaka K, et al. (2000) Depletion of CD4+ T cells causes reactivation of murine persistent tuberculosis despite continued expression of interferon γ and nitric oxide synthase 2. The Journal of experimental medicine 192: 347-358.

Schaefer, W. B., C. L. Davis, et al. (1970). "Pathogenicity of transparent, opaque, and rough variants of Mycobacterium avium in chickens and mice." The American review of respiratory disease 102(4): 499-506.

Schirm J, Oostendorp LA, Mulder JG (1995) Comparison of Amplicor, in-house PCR, and conventional culture for detection of *Mycobacterium tuberculosis* in clinical samples. Journal of clinical microbiology 33: 3221-3224.

Schlesinger LS (1993) Macrophage phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. The Journal of Immunology 150: 2920-2930.

Schlesinger LS, Bellinger-Kawahara CG, Payne NR, Horwitz MA (1990) Phagocytosis of *Mycobacterium tuberculosis* is mediated by human monocyte complement receptors and complement component C3. The Journal of Immunology 144: 2771-2780.

Schlesinger LS, Kaufman TM, Iyer S, Hull SR, Marchiando LK (1996) Differences in mannose receptor-mediated uptake of lipoarabinomannan from virulent and attenuated strains of *Mycobacterium tuberculosis* by human macrophages. The Journal of Immunology 157: 4568-4575.

Schweizer A, Kornfeld S, Rohrer J (1996) Cysteine34 of the cytoplasmic tail of the cation-dependent mannose 6-phosphate receptor is reversibly palmitoylated and required for normal trafficking and lysosomal enzyme sorting. The Journal of cell biology 132: 577-584.

Seah GT, Scott GM, Rook GAW (2000) Type 2 cytokine gene activation and its relationship to extent of disease in patients with tuberculosis. Journal of Infectious Diseases 181: 385-389.

Senaldi G, Yin S, Shaklee CL, Piguet P-F, Mak TW, et al. (1996) Corynebacterium parvum- and Mycobacterium bovis bacillus Calmette-Guerin-induced granuloma formation is inhibited in TNF receptor I (TNF-RI) knockout mice and by treatment with soluble TNF-RI. The Journal of Immunology 157: 5022-5026.

Sertl K, Takemura T, Tschachler E, Ferrans VJ, Kaliner MA, et al. (1986) Dendritic cells with antigen-presenting capability reside in airway epithelium, lung parenchyma, and visceral pleura. The Journal of experimental medicine 163: 436-451.

Setia MS, Steinmaus C, Ho CS, Rutherford GW (2006) The role of BCG in prevention of leprosy: a meta-analysis. The Lancet infectious diseases 6: 162-170.

Shiratsuchi H, Johnson JL, Ellner JJ (1991) Bidirectional effects of cytokines on the growth of Mycobacterium avium within human monocytes. The Journal of Immunology 146: 3165-3170.

Silver RF, Li Q, Ellner JJ (1998) Expression of Virulence of *Mycobacterium tuberculosis*within Human Monocytes: Virulence Correlates with Intracellular Growth and Induction of Tumour Necrosis Factor Alpha but Not with Evasion of Lymphocyte-Dependent Monocyte Effector Functions. Infection and immunity 66: 1190-1199.

Sinsimer D, Huet G, Manca C, Tsenova L, Koo MS, et al. (2008) The phenolic glycolipid of *Mycobacterium tuberculosis* differentially modulates the early host cytokine response but does not in itself confer hypervirulence. Infect Immun 76: 3027-3036.

Sohn H, Lee KS, Kim SY, Shin DM, Shin SJ, et al. (2009) Induction of cell death in human macrophages by a highly virulent Korean isolate of *Mycobacterium tuberculosis* and the virulent strain H37Rv. Scandinavian journal of immunology 69: 43-50.

Soini H, Pan X, Amin A, Graviss EA, Siddiqui A, et al. (2000) Characterization of *Mycobacterium tuberculosis* isolates from patients in Houston, Texas, by spoligotyping. Journal of clinical microbiology 38: 669-676.

Sola C, Filliol I, Gutierrez MC, Mokrousov I, Vincent Vr, et al. (2001) Spoligotype database of *Mycobacterium tuberculosis*: biogeographic distribution of shared types and epidemiologic and phylogenetic perspectives. Emerging Infectious Diseases 7: 390.

Sola C, Horgen L, Devallois A, Rastogi N (1998) Combined numerical analysis based on the molecular description of *Mycobacterium tuberculosis* by four repetitive sequence-based DNA typing systems. Research in microbiology 149: 349-360.

Somoskovi A, Parsons LM, Salfinger M (2001) The molecular basis of resistance to isoniazid, rifampin, and pyrazinamide in *Mycobacterium tuberculosis*. Respir Res 2: 164-168.

Sousa AO, Mazzaccaro RJ, Russell RG, Lee FK, Turner OC, et al. (2000) Relative contributions of distinct MHC class I-dependent cell populations in protection to tuberculosis infection in mice. Proceedings of the National Academy of Sciences 97: 4204-4208.

Sporri R, e Sousa CR (2005) Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. Nature immunology 6: 163-170.

Sreevatsan S, Pan XI, Stockbauer KE, Connell ND, Kreiswirth BN, et al. (1997) Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. Proceedings of the National Academy of Sciences 94: 9869-9874.

Stein CM, Zalwango S, Chiunda AB, Millard C, Leontiev DV, et al. (2007) Linkage and association analysis of candidate genes for TB and TNFα cytokine expression: evidence for association with IFNGR1, IL-10, and TNF receptor 1 genes. Human genetics 121: 663-673.

Steingart KR, Ng V, Henry M, Hopewell PC, Ramsay A, et al. (2006) Sputum processing methods to improve the sensitivity of smear microscopy for tuberculosis: a systematic review. The Lancet infectious diseases 6: 664-674.

Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, et al. (1998) An antimicrobial activity of cytolytic T cells mediated by granulysin. Science 282: 121-125.

Stuart LM, Deng J, Silver JM, Takahashi K, Tseng AA, et al. (2005) Response to Staphylococcus aureus requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain. The Journal of cell biology 170: 477-485.

Supply P, Lesjean S, Savine E, Kremer K, van Soolingen D, et al. (2001) Automated high-throughput genotyping for study of global epidemiology of *Mycobacterium tuberculosis* based on *Mycobacteria*l interspersed repetitive units. J Clin Microbiol 39: 3563-3571.

Supply P, Magdalena J, Himpens S, Locht C (1997) Identification of novel intergenic repetitive units in a *Mycobacteria*l two‐component system operon. Molecular microbiology 26: 991-1003.

Supply P, Mazars E, Lesjean S, Vincent Vr, Gicquel B, et al. (2000) Variable human minisatellite‐like regions in the *Mycobacterium tuberculosis* genome. Molecular microbiology 36: 762-771.

Tailleux L, Maeda N, Nigou Jr, Gicquel B, Neyrolles O (2003) How is the phagocyte lectin keyboard played? Master class lesson by *Mycobacterium tuberculosis*. Trends in microbiology 11: 259-263.

Tailleux L, Neyrolles O, HonorA-Bouakline Sp, Perret E, Sanchez Fo, et al. (2003) Constrained intracellular survival of *Mycobacterium tuberculosis* in human dendritic cells. The Journal of Immunology 170: 1939-1948.a

Tameris MD, Hatherill M, Landry BS, Scriba TJ, Snowden MA, et al. (2013) Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. The Lancet.

Tan T, Lee WL, Alexander DC, Grinstein S, Liu J (2006) The ESAT‐6/CFP‐10 secretion system of Mycobacterium marinum modulates phagosome maturation. Cellular microbiology 8: 1417-1429.

Tanveer M, Hasan Z, Kanji A, Hussain R, Hasan R (2009) Reduced TNF-alpha and IFN-gamma responses to Central Asian strain 1 and Beijing isolates of *Mycobacterium tuberculosis* in comparison with H37Rv strain. Trans R Soc Trop Med Hyg 103: 581-587.

Tascon RE, Soares CS, Ragno S, Stavropoulos E, Hirst EMA, et al. (2000) *Mycobacterium tuberculosis*-activated dendritic cells induce protective immunityin mice. Immunology 99: 473-480.

Theus SA, Cave MD, Eisenach KD (2005) Intracellular macrophage growth rates and cytokine profiles of *Mycobacterium tuberculosis* strains with different transmission dynamics. J Infect Dis 191: 453-460.

Thoma-Uszynski S, Stenger S, Takeuchi O, Ochoa MT, Engele M, et al. (2001) Induction of direct antimicrobial activity through mammalian toll-like receptors. Science 291: 1544-1547.

Thurnher M, Ramoner R, Gastl Gn, Radmayr C, Back Gn, et al. (1997) Bacillus Calmette-Guerin *Mycobacteria* stimulate human blood dendritic cells. International journal of cancer 70: 128-134.

Thwaites G, Caws M, Chau TTH, D'Sa A, Lan NTN, et al. (2008) Relationship between *Mycobacterium tuberculosis* genotype and the clinical phenotype of pulmonary and meningeal tuberculosis. Journal of clinical microbiology 46: 1363-1368.

Tian T, Woodworth J, Skald M, Behar SM (2005) *In vivo* depletion of CD11c+ cells delays the CD4+ T cell response to *Mycobacterium tuberculosis* and exacerbates the outcome of infection. The Journal of Immunology 175: 3268-3272.

Toossi Z, Ellner JJ (1998) The role of TGFB in the pathogenesis of human tuberculosis. Clinical immunology and immunopathology 87: 107-114.

Toossi Z, Gogate P, Shiratsuchi H, Young T, Ellner JJ (1995) Enhanced production of TGF-beta by blood monocytes from patients with active tuberculosis and presence of TGF-beta in tuberculous granulomatous lung lesions. The Journal of Immunology 154: 465-473.

Torrelles JB, Azad AK, Schlesinger LS (2006) Fine discrimination in the recognition of individual species of phosphatidyl-myo-inositol mannosides from *Mycobacterium tuberculosis* by C-type lectin pattern recognition receptors. The Journal of Immunology 177: 1805-1816.

Torrelles JB, Schlesinger LS (2010) Diversity in *Mycobacterium tuberculosis* mannosylated cell wall determinants impacts adaptation to the host. Tuberculosis 90: 84-93.

Torres M, Herrera T, Villareal H, Rich EA, Sada E (1998) Cytokine Profiles for Peripheral Blood Lymphocytes from Patients with Active Pulmonary Tuberculosis and Healthy Household Contacts in Response to the 30-Kilodalton Antigen of*Mycobacterium tuberculosis*. Infection and immunity 66: 176-180.

Trial TP (1980) Trial of BCG Vaccine in South India for Tuberculosis Prevention. Indian J Med Res 72: 1-74.

Tribuddharat C, Fennewald M (1999) Integron-Mediated Rifampin Resistance in Pseudomonas aeruginosa. Antimicrobial agents and chemotherapy 43: 960-962.

Trinchieri G (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nature Reviews Immunology 3: 133-146.

Tripathy NK, Chauhan SK, Nityanand S (2004) Cytokine mRNA repertoire of peripheral blood mononuclear cells in Takayasu's arteritis. Clin Exp Immunol 138: 369-374.

Tsenova L, Ellison E, Harbacheuski R, Moreira AL, Kurepina N, et al. (2005) Virulence of selected *Mycobacterium tuberculosis* clinical isolates in the rabbit model of meningitis is dependent on phenolic glycolipid produced by the bacilli. Journal of Infectious Diseases 192: 98-106.

Ulevitch RJ, Johnston AR (1978) The modification of biophysical and endotoxic properties of bacterial lipopolysaccharides by serum. Journal of Clinical Investigation 62: 1313.

Underhill DM, Ozinsky A, Hajjar AM, Stevens A, Wilson CB, et al. (1999) The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature 401: 811-815.

Valone SE, Rich EA, Wallis RS, Ellner JJ (1988) Expression of tumour necrosis factor *in vitro* by human mononuclear phagocytes stimulated with whole Mycobacterium bovis BCG and *Mycobacteria*l antigens. Infection and immunity 56: 3313-3315.

Valway SE, Sanchez MPC, Shinnick TF, Orme I, Agerton T, et al. (1998) An outbreak involving extensive transmission of a virulent strain of *Mycobacterium tuberculosis*. New England Journal of Medicine 338: 633-639.

Van der Spuy GD, Kremer K, Ndabambi SL, Beyers N, Dunbar R, et al. (2009) Changing *Mycobacterium tuberculosis* population highlights clade-specific pathogenic characteristics. Tuberculosis 89: 120-125.

Van der Zanden AGM, Kremer K, Schouls LM, Caimi K, Cataldi A, et al. (2002) Improvement of differentiation and interpretability of spoligotyping for *Mycobacterium tuberculosis* complex isolates by introduction of new spacer oligonucleotides. Journal of clinical microbiology 40: 4628-4639.

Van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, et al. (1993) Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. Journal of clinical microbiology 31: 406-409.

Van Embden JDA, Van Gorkom T, Kremer K, Jansen R, Van der Zeijst BAM, et al. (2000) Genetic variation and evolutionary origin of the direct repeat locus of *Mycobacterium tuberculosis* complex bacteria. Journal of bacteriology 182: 2393-2401.

Van Haarst JM, Hoogsteden HC, de Wit HJ, Verhoeven GT, Havenith CE, et al. (1994) Dendritic cells and their precursors isolated from human bronchoalveolar lavage: immunocytologic and functional properties. American journal of respiratory cell and molecular biology 11: 344-350.

VanHeyningen TK, Collins HL, Russell DG (1997) IL-6 produced by macrophages infected with Mycobacterium species suppresses T cell responses. The Journal of Immunology 158: 330-337.

Victor TC, de Haas PEW, Jordaan AM, van der Spuy GD, Richardson M, et al. (2004) Molecular characteristics and global spread of *Mycobacterium tuberculosis* with a western cape F11 genotype. Journal of clinical microbiology 42: 769-772.

Vidyarani M, Selvaraj P, Anand SP, Jawahar MS, Adhilakshmi AR, et al. (2006) Interferon gamma (IFN) & interleukin-4 (IL-4) gene variants & cytokine levels in pulmonary tuberculosis. Indian J Med Res 124: 403-410.

Vijayan VK (2002) Clinical aspects of tuberculosis. Indian Journal of Clinical Biochemistry 17: 96-100.

Visintin A, Mazzoni A, Spitzer JH, Wyllie DH, Dower SK, et al. (2001) Regulation of Toll-like receptors in human monocytes and dendritic cells. The Journal of Immunology 166: 249-255.

Von Groll A, Martin A, Stehr M, Singh M, Portaels Fo, et al. (2010) Fitness of *Mycobacterium tuberculosis* strains of the W-Beijing and Non-W-Beijing genotype. PloS one 5: e10191.

Wakeham J, Wang J, Xing Z (2000) Genetically determined disparate innate and adaptive cell-mediated immune responses to pulmonary Mycobacterium bovis BCG infection in C57BL/6 and BALB/c mice. Infection and immunity 68: 6946-6953.

Walburger A, Koul A, Ferrari G, Nguyen L, Prescianotto-Baschong C, et al. (2004) Protein kinase G from pathogenic *Mycobacteria* promotes survival within macrophages. Science 304: 1800-1804.

Walker D (2001) Economic analysis of tuberculosis diagnostic tests in disease control: how can it be modelled and what additional information is needed? The International Journal of Tuberculosis and Lung Disease 5: 1099-1108.

Wang H, Joseph JA (1999) Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radical Biology and Medicine 27: 612-616.

Wang J, Santosuosso M, Ngai P, Zganiacz A, Xing Z (2004) Activation of CD8 T cells by *Mycobacteria*l vaccination protects against pulmonary tuberculosis in the absence of CD4 T cells. The Journal of Immunology 173: 4590-4597.

Wangoo A, Sparer T, Brown IN, Snewin VA, Janssen R, et al. (2001) Contribution of Th1 and Th2 cells to protection and pathology in experimental models of granulomatous lung disease. The Journal of Immunology 166: 3432-3439.

Wards BJ, De Lisle GW, Collins DM (2000) An *esat6* knockout mutant of*Mycobacterium bovis* produced by homologous recombination will contribute to the development of a live tuberculosis vaccine. Tubercle and Lung Disease 80: 185-189.

Wilkinson RJ, Patel P, Llewelyn M, Hirsch CS, Pasvol G, et al. (1999) Influence of polymorphism in the genes for the interleukin (IL)-1 receptor antagonist and IL-1B on tuberculosis. The Journal of experimental medicine 189: 1863-1874.

Winkelstein JA, Marino MC, Johnston Jr RB, Boyle J, Curnutte J, et al. (2000) Chronic granulomatous disease: report on a national registry of 368 patients. Medicine 79: 155-169.

Wolf AJ, Desvignes L, Linas B, Banaiee N, Tamura T, et al. (2008) Initiation of the adaptive immune response to *Mycobacterium tuberculosis* depends on antigen production in the local lymph node, not the lungs. The Journal of experimental medicine 205: 105-115.

World Health, O. (2004). TB/HIV a clinical manual, Geneva: WHO, 2004.

WHO Tuberculosis Global Factsheet (2012)
Available: http://www.who.int/mediacentre/factsheets/fs104/en/.

WHO Tuberculosis Global Factsheet (2000)
Available: http://www.who.int/mediacentre/factsheets/fs104/en/

Wright, P. W., R. J. Wallace, et al. (1998). "Sensitivity of fluorochrome microscopy for detection of Mycobacterium tuberculosis versus nontuberculous mycobacteria." Journal of clinical microbiology 36(4): 1046-1049.

Yamada H, Mizumo S, Horai R, Iwakura Y, Sugawara I (2000) Protective Role of Interleukin-1 in *Mycobacteria*l Infection in IL-1 &agr;/&bgr; Double-Knockout Mice. Laboratory investigation 80: 759-767.

Yamamoto, T., Y. Sawada, et al. (1998). "Increased production of nitric oxide stimulated by interleukin-1beta in peripheral blood mononuclear cells in patients with systemic sclerosis." Rheumatology 37(10): 1123-1125.

Yang C-S, Lee H-M, Lee J-Y, Kim J-A, Lee SJ, et al. (2007) Reactive oxygen species and p47phox activation are essential for the *Mycobacterium tuberculosis*-induced pro-inflammatory response in murine microglia. J Neuroinflammation 4: 27.

Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, et al. (1999) Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. The Journal of Immunology 163: 1-5.

Zhang M, Gong J, Lin Y, Barnes PF (1998) Growth of virulent and avirulent*Mycobacterium tuberculosis* strains in human macrophages. Infection and immunity 66: 794-799.

Zhang M, Gong J, Presky DH, Xue W, Barnes PF (1999) Expression of the IL-12 receptor B 1 and B 2 subunits in human tuberculosis. The Journal of Immunology 162: 2441-2447.

Zhang Y, Telenti A (2000) Genetics of drug resistance in *Mycobacterium tuberculosis*: Molecular genetics of *Mycobacteria*. ASM Press, Washington, DC.