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. *Mycobacterial* 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 intratracheally (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 Mycobacterial 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 (Hernandez-Pando et al 1996, Hernandez-Pando et al, 1997, Hernandez-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 NH₄Cl, 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 $2 \times 10^5$ 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°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.
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.
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 *M. tuberculosis* 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 effector mechanism in humans (Chane 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\textsuperscript{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 \textit{in vivo} and \textit{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 \textit{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 \textit{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.