Chapter 5

Immunologic and pathogenic effect of
*M. tb:*lspA↑ recombinant construct
in mouse model
5.1. Introduction

The success of *M. tuberculosis* as a chronic and persistent pathogen depends to great extent on its ability to manipulate the host immune response in diverse and paradoxical ways. (Lancioni CL et al., 2011) *M. tuberculosis* is an intracellular pathogen that infects phagocytic antigen-presenting cells (APCs) in the lung, including alveolar macrophages, lung macrophages and dendritic cells (DC) and triggers potent proinflammatory and antigen-specific T cell responses, while at the same time, it displays a wide array of immune evasion strategies, such as inhibition of intracellular killing and antigen processing (Lancioni CL et al., 2011; Bhatt K and Salgame P, 2007; Gehring AJ et al., 2004; Harding CV and Boom WH, 2010; Pecora ND et al., 2006). Understanding the complex mechanisms by which *M. tuberculosis* regulates the host immune response is essential to developing more effective drug treatments and tuberculosis vaccines. In tuberculosis, innate and T cell mediated immunity plays major role in the control of bacterial propagation and protection against disease.

A major virulence feature of pathogenic *M. tuberculosis* is its ability to parasitize the host’s scavenger cells, and more particularly macrophages, which are niche cells for mycobacterial infection. Yet, they may also contribute to elimination of the bacilli via a number of mechanisms, including successful phagosome acidification and maturation (Russell DG, 20016; Jordao L et al., 2008). However, *M. tuberculosis* ensures its survival within host macrophages by arresting the maturation pathway that leads to phagosome-lysosome fusion, thus avoiding the phagolysosome which is rich in acid hydrolases capable of microbicidal degradation, thereby creating a suitable environment for bacillary survival and replication.

In contrast to innate mechanisms, the specific or adaptive immune response requires the specific recognition of foreign antigens. The innate immune system has a profound influence on the type of acquired immune mechanisms generated, and vice versa. The specific immune response executes several of its effector functions via the
activation of components of the innate immunity. Specific immune responses can be divided into cell mediated mechanisms, which include T cell activation and effector mechanisms, and the humoral immune response, consisting of B cell maturation and antibody production (Caruso AM et. al., 1999). Both mechanisms are not mutually exclusive, and T helper cells are required for antibody maturation, isotype switching and memory. B cells also function as antigen presenting cells by activating T cells in a specifically driven manner.

In previous chapter, we have seen that the effect of overexpression of LspA in the biology of *M. tuberculosis*. In the present chapter we have reported the immunologic and pathogenic effect of *M. tb:ospA↑* recombinant constructs in a mouse model. Balb/c mice were infected with a low dose aerosol challenge with *M. tuberculosis* H37Rv and *M.tb:ospA↑*. The animals were sacrificed at various time points and intracellular growth and survival of the *ospA* overexpressed *M. tuberculosis* were measured in the lungs and spleen tissue of the challenge animals. The pathogenic effect was determined by histopathological examination of the internal organs. In addition, the immunologic effect of the *ospA* overexpression was investigated by assaying for the various Th1- Th2 cytokines in the sera of the infected animals.
5.2. Materials and Methods

5.2.1. Culture Media and Antibiotics

Middlebrook 7H9 broth (Difco) and 7H11 agar (Difco) were used to culture *M. tuberculosis*. OADC (oleic acid, albumin [bovine, fraction V], dextrose, catalase [Difco]) and glycerol (SRL) were used as supplement. Antibiotics: Amphotericin B (Sigma, USA), Cycloheximide (Sigma, USA), Trimethoprim (Sigma, USA), Polymyxin B phosphate (Sigma, USA), Vancomycin (Sigma, USA) and Carbenicilline (Sigma, USA), were used in the medium to avoid the fungal and bacterial contamination. DMSO (Sigma, USA) and Chloroform (SRL) were used to dissolve antibiotics.

5.2.2. Experimental Animals

Specific-pathogen-free Balb/c mice (female, 6–8 weeks old) were procured from Central Drug Research Institute (CDRI), Lucknow, India. Animals were kept in Biosafety Level-3 facility of National JALMA Institute for Leprosy and Other Mycobacterial Diseases (NJIL&OMD), Agra, India under specific-pathogen-free conditions. All animal experiments were approved by the Institutional Animal Ethics Committees of NJIL & OMD. All animal experiments were performed according to the National Regulatory Guidelines issued by CPSEA (Committee for the Purpose of Supervision of Experiments on Animals), Ministry of Environment and Forest, Govt. of India.

5.2.3. Mycobacterial Culture Preparation for Aerosol Exposure to Balb/c Mice

**Primary culture:** A loopful of *M. tuberculosis* H37Rv and *M.tb:lspA↑* from 7H11 agar plates were inoculated into 3ml of 7H9 broth containing 0.2% tween80, 1%ADC in a 30ml screw capped culture tube, and incubated at 37°C in shaker incubator at 200 rpm for 4 days till the culture became turbid and homogenous.

**Secondary culture:** Contamination of primary culture was checked before secondary inoculation. After that 1% of primary culture was inoculated into 100ml 7H9 broth media containing 0.2% tween80, 1%ADC in a 500ml screw capped culture flask , and incubated at 37°C in shaker incubator with 200 rpm for 5 days till OD<sub>600</sub> of culture reached 0.8-1.0.
Preparation of stocks to be used for challenge experiment: Culture was harvested by centrifugation at 8000rpm/4°C/30min. Supernatant was discarded carefully because *M. tuberculosis* pellet tends to be loose. Pellet was resuspended in 100ml ice cold 1X PBS and harvested by centrifugation at 8000rpm/4°C/30min. This step was repeated twice and the pellet of 100ml culture was resuspended in 10ml ice cold 1X PBS and dispended into screwcapped cryovials as an aliquots of 1ml and stored at -80°C.

CFU analysis of the prepared culture stocks: 900µl of 1X PBS was added in each tube and 100 µl of stock was added in vial 1, mixed properly followed by serial 10 fold dilutions. 100 µl of each dilution was plated on 7H11 agar plate. Plates were incubated for 3weeks at 37°C (Fig 5.1).

Calculation of CFU/ml = No. of colonies in a particular dilution X Dilution factor X 10

![Fig 5.1: Serial dilution and plating on 7H11 agar plate of *M. tuberculosis* H37Rv stock](image)

5.2.4. Aerosol Exposure to Balb/c Mice

Mice were infected with a low dose aerosol challenge with *M. tuberculosis* H37Rv and *M.tb:lspA↑* because mice remain more susceptible to *M. tuberculosis* infection initiated via the respiratory route than in intravenous route (North RJ et. al., 2001).
**Inhalation exposure system:** The Inhalation Exposure System (Glas-Col, Terre Haute, IN)) (Fig5.2) provides reproducible animal exposure to droplet-borne contaminants and infectious agents and also produces deep-lung infections that more closely simulate real-world circumstances. The animals (Balb/c mice) were placed in a compartmented mesh basket within the chamber (10 mice in each chamber) and animals were exposed to $10^6$ bacilli of *M. tuberculosis* H37Rv and *M.tb:lspA↑*. The cycle of the aerosol inhalation exposure system consist of 4 steps:

**Preheat cycle:** 900sec. or 15min, Nebulizing cycle: 2700sec or 45min, Cloud decay cycle: 1800sec or 30min and Decontamination cycle: 900sec or 15min. After aerosol exposure mice were kept for different time points (1day, 2weeks, 4weeks, 6weeks, 8weeks and 10weeks) under specific-pathogen-free conditions at room temperature. This part of the work was done at NJIL&OMD, Agra.

![Fig 5.2: Inhalation Exposure System used in aerosol infection](image)

**5.2.5. Serum Separation**

After aerosol infection blood was collected from eye vein of mice with the help of .5mm capillaries in 1.5ml tube. Tubes were incubated undisturbed at 37°C for 2 hrs to coagulate the blood, spun at 2000 rpm for 30 minutes and the supernatant containing pale yellow serum was collected in a fresh 1.5ml tube. This serum was used in ELISA for cytokine analysis.
5.2.6. Cytokine Assay

The cytokines (TNF-α, IFN-γ, IL-12, IL-10, IL-6 and IL-4) were assayed in the serum collected from Balb/c mice infected with *M. tuberculosis* H37Rv and *M.tb:*lspA↑ at various time point namely 1day, 2weeks, 4weeks, 6weeks, 8weeks and 10weeks) by using solid phase sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (Diaclone Research, France).

5.2.7. CFU Analysis of Lung and Spleen Tissue

After aerosol exposure, three mice from each group (infected with *M. tuberculosis* H37Rv and *M.tb:*lspA↑) were sacrificed at each time point and lungs and spleens were removed and examined grossly. Both of the organs were then homogenized in PBS, acid fast staining of the homogenates was performed and serial dilutions were plated on Middlebrook 7H11 agar (BD Biosciences, USA) plates containing antibiotics [Amphoterecin B (Sigma, USA), Cycloheximide (Sigma, USA), Trimethoprim (Sigma, USA), Polymyxin B phosphate (Sigma, USA), Vancomycin (Sigma, USA)] , supplemented with 2% ADC (BD Biosciences, USA) and incubated at 37°C for 3 weeks. Numbers of CFU was determined after 3 weeks of incubation.

5.2.8. Histopathology of Lung and Spleen

A portion of lung and spleen from infected mice was stored in 10% neutral buffered formalin. They were embedded in paraffin, sectioned and stained for haematoxylin and eosin and examined by Olympus IX71 light microscopy. Lung and spleen of healthy mice were also examined for histopathological analysis.

5.2.9. Statistical Analysis

The statistical analysis was done by paired t test for CFU analysis and two way ANOVA with Bonferroni comparison for cytokine analysis using Graph Pad Prism software version 5.0 (San Diego, CA, USA). All results are presented as mean ± SE values of triplicate wells. A two tailed p value < 0.5 is considered statistically significant.
5.3. Results

5.3.1. Gross Examination of Balb/c Mice

Balb/c mice were challenged with $10^6$ bacilli of wild type of *M. tuberculosis* and *M. tb:lspA↑* via aerosol route and examined grossly after ten weeks of infection. The mice challenged with *M. tb:lspA↑* looked considerably sick as compared to mice challenged with wild type of *M. tuberculosis*. Hair loss was significantly higher in mice infected with *M. tb:lspA↑* as compared to wild type as shown in Figure 5.3.

![Fig 5.3](image-url)

**Fig 5.3:** Gross examination of Balb/c mice after 10 weeks of infection. A-B: Healthy Balb/c mice. C-E: Mice infected wild type H37Rv. F-H: Mice infected *M. tb:lspA↑*.

5.3.2. Gross Examination of Lungs Removed from Experimental Mice

Gross organ examination of lungs after infection revealed that, the lung surface of mice infected with the *M. tb: lspA↑* showed a greatly increased number of discrete well-circumscribed granulomas (Fig 5.4 A) as compared to the lungs of wild type H37Rv.
infected mice (Fig 5.4 B). The lungs infected with the *M. tb: lspA↑* showed progressively enlarged granulomatous lesions with sharp circumscribed borders and necrosis of alveoli, whereas the lungs of wild type infected mice showed progressively enlarged granulomas that were diffuse, poorly circumscribed, and coalescing.

**Fig 5.4(A):** Gross examination of lungs removed from Balb/c mice

**Fig 5.4(B):** Comparative gross examination of lungs of infected mice after 10 weeks. **A:** Lungs of Balb/c mice infected with *M. tb: lspA↑*. **B:** Lungs of Balb/c Mice infected with *M. tuberculosis* H37Rv
5.3.3. CFU Analysis of Lung and Spleen Tissue

The lungs and spleen homogenates of aerosol infected Balb/c mice at different time points of infection (day1, 2weeks, 4weeks, 6weeks, 8weeks and 10weeks) were plated on Middlebrook 7H11 agar plates (Fig 5.5) and found that CFU in the lungs and spleen of mice (Table 5.1 & 5.2) infected with M. tb: lspA↑ was 2 fold greater than the CFU in the lungs and spleen of Balb/c mice infected with wild type M. tuberculosis as shown in Fig 5.6 and 5.7 respectively.

Table 5.1: CFU of M.tb:lspA↑ and M. tuberculosis H37Rv in lungs

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Average CFU in Lungs</th>
<th>M.tb:lspA↑</th>
<th>H37Rv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>49 ± 6.66</td>
<td>40 ± 6.11</td>
<td></td>
</tr>
<tr>
<td>2 week</td>
<td>1.46 X 10^6 ± 5.69e+005</td>
<td>2.52 X 10^5 ± 6.68e+004</td>
<td></td>
</tr>
<tr>
<td>4 weeks</td>
<td>4.33 X 10^6 ± 5.30e+005</td>
<td>1.38 X 10^5 ± 2.81e+005</td>
<td></td>
</tr>
<tr>
<td>6 weeks</td>
<td>6.95 X 10^5 ± 1.23e+006</td>
<td>2.34 X 10^5 ± 6.03e+005</td>
<td></td>
</tr>
<tr>
<td>8 weeks</td>
<td>3.67 X 10^6 ± 4.77e+006</td>
<td>1.39 X 10^5 ± 5.51e+006</td>
<td></td>
</tr>
<tr>
<td>10 weeks</td>
<td>5.0 X 10^7 ± 8.27e+006</td>
<td>2.61 X 10^7 ± 9.61e+006</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2: CFU of M.tb:lspA↑ and M. tuberculosis H37Rv in spleen

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Average CFU in Spleen</th>
<th>M.tb:lspA↑</th>
<th>H37Rv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>00</td>
<td>00</td>
<td></td>
</tr>
<tr>
<td>2 week</td>
<td>00</td>
<td>00</td>
<td></td>
</tr>
<tr>
<td>4 weeks</td>
<td>5.89 X 10^7 ± 1.86e+004</td>
<td>3.22 X 10^7 ± 1.21e+004</td>
<td></td>
</tr>
<tr>
<td>6 weeks</td>
<td>2.26 X 10^7 ± 2.20e+005</td>
<td>1.28 X 10^7 ± 1.71e+005</td>
<td></td>
</tr>
<tr>
<td>8 weeks</td>
<td>3.45 X 10^6 ± 1.56e+005</td>
<td>2.47 X 10^6 ± 8.15e+004</td>
<td></td>
</tr>
<tr>
<td>10 weeks</td>
<td>2.49 X 10^7 ± 1.85e+006</td>
<td>7.32 X 10^7 ± 3.62e+005</td>
<td></td>
</tr>
</tbody>
</table>
Fig 5.6: CFU counts in lung of Balb/c mice infected by aerosol route with $10^6 M. tb: lspA↑$ and wild type $M. tuberculosis$ H37Rv. High CFU was observed in mice infected with $M. tb: lspA↑$ as compare to wild type $M. tuberculosis$ H37Rv.

Fig 5.7: CFU counts in Spleen of Balb/c mice infected by aerosol route with $M. tb: lspA↑$ and wild type $M. tuberculosis$ H37Rv. High CFU was observed in mice infected with $M. tb: lspA↑$ as compared to wild type $M. tuberculosis$ H37Rv.
5.3.4. Lung and Spleen histopathology

We examined the lung and spleen pathology in *M. tuberculosis* H37Rv and *M. tb: lspA↑* infected mice.

**Lungs:** Microscopically, at 10 weeks after infection, the lungs of *M. tuberculosis* infected mice showed collections of foamy macrophages accompanied by perivascular and peribronchiolar lymphocytes. Focal collection of foamy histiocytes and some granulomatous cells were also seen but they were not well defined. In spite of the presence of these histopathological indications, functional lung tissue was also present. In contrast, in *M. tb: lspA↑* infected mice, the convergence of lesions resulted in an extensive consolidation of well-defined granulomas in the lung tissue. Numerous foamy histocytes, neutrophils, perivascular and peribronchiolar lymphocytes and macrophages were seen throughout the lungs as shown in Fig 5.8.

**Spleen:** The Spleen of *M. tuberculosis* infected mice showed few giant cells in intrafollicular area. In contrast, *M. tb: lspA↑* infected mice showed numerous giant cells in intrafollicular area and reactive germinal centers were seen as shown in Fig 5.9.

5.3.5. Acid Fast Bacilli Staining of Homogenates

AFB staining of both lung and spleen homogenates of Balb/c mice infected with *M. tb: lspA↑* and *M. tuberculosis* H37Rv was done. The result showed more number of bacilli (Fig 5.10 A & B) in lungs and spleen homogenate of Balb/c mice infected with *M. tb: lspA↑* as compared to homogenate of Balb/c mice infected with wild type *M. tuberculosis* H37Rv.

5.3.6. Cytokine Analysis

The level of Th1 and Th2 type of cytokines (TNF-α, IFN-γ, IL-12, IL-10, IL-6 and IL-4) in the serum (1day, 2weeks, 4weeks, 6weeks, 8weeks and 10weeks) of infected Balb/c mice with *M. tuberculosis* H37Rv or *M.tb: lspA↑* were determined by using solid phase sandwich enzyme-linked immunosorbsorbent assay (ELISA) according to the manufacturer’s instructions (Diaclone Research, France). The levels of TNF-α and IL-12 were significantly raised in *M. tb: lspA↑* infected mice. There was no difference in the levels of IFN-γ, IL-4 and IL-10 between *M.tb: lspA↑* infected and wild type *M. tuberculosis* H37Rv. The levels of IL-6 was significantly raised in *M.tb: lspA↑* infected mice at day1 to 4week of post infection and then there was no difference in *M. tb: lspA↑* and *M. tuberculosis* H37Rv.
**Fig 5.8:** Histological sections of lung of Balb/c mice at (H & E X100) magnification. **A:** Lung of healthy Balb/c mouse. **B:** Lung of Balb/c mouse after 10 weeks of infection with H37Rv (wild type) showing a infiltration of cells (collection of lymphocytes and neutrophils) into the alveolar walls and some granulomatous lesions are found near bronchiole. **C:** Lung of Balb/c mouse after 10 weeks of infection with *M. tb:*lspA↑ showing multifocal coalescing granulomatous lesions, numerous lymphocytes and macrophages throughout the lungs.

**Fig 5.9:** Histological sections of Spleen of Balb/c mice at (H & E X100) magnification. **A:** Spleen of healthy Balb/c mouse showing lymphoid follicle. **B:** Spleen of Balb/c mouse after 10 weeks of infection with H37Rv (wild type) showing a few giant cells. **C:** Spleen of Balb/c mouse after 10 weeks of infection with *M. tb:*lspA↑ showing numerous giant cells, multinucleated cells.
**Figure 5.10(A):** Comparison of number of bacilli in lung homogenate. **A:** Lungs of mice infected with *M. tb:ispA*↑. **B:** Mice infected wild type *M. tuberculosis*. Black arrows indicates pink curved rod shaped acid fast *M. tuberculosis* seen under the microscope at 100X magnification. More number of bacilli was seen in A as compared to B.

**Figure 5.10(B):** Comparison of number of bacilli in Spleen homogenate. **A:** Spleen of mice infected with *M. tb:ispA*↑. **B:** Spleen of mice infected with wild type *M. tuberculosis*. Black arrows indicates pink curved rod shaped acid fast *M. tuberculosis* seen under the microscope at 100X magnification. There was significant increase in number of bacilli was seen in A as compared to B.
Immunologic and pathogenic effect of \( M. \text{tb:} \text{lspA} \uparrow \) recombinant construct in mouse model

**Figure 5.11:** The cytokines level in the serum of Balb/c infected with wild type H37Rv and \( M. \text{tb:} \text{lspA} \uparrow \) (\*\*\*P > 0.0001, \*\*P > 0.001, \*P > 0.05).

**Figure 5.12:** Levels of IFN\( \gamma \) in the serum of Balb/c infected with wild type H37Rv and \( M. \text{tb:} \text{lspA} \uparrow \). No significant increase in levels of IFN\( \gamma \) were observed in the serum of Balb/c infected with \( M. \text{tb:} \text{lspA} \uparrow \) as compare to wild type H37Rv.
5.4. Discussion

In this chapter, we have analyzed the survival, pathological and immunologic effects of LspA protein of *M. tuberculosis* in Balb/c mouse model. The role of LspA protein in survival was analysed by CFU study in lungs and spleen of mice and histopathology of lungs and spleen. The immunogenicity of LspA protein was studied by profiling of both pro and anti-inflammatory cytokines such as TNF-α, IFN-γ, IL-12, IL-6, IL-4 and IL-10 in the serum of mice infected with *M. tb:lspA↑* or *M. tuberculosis* H37Rv.

Animal experiments conducted here demonstrated the role of LspA in survival and pathogenesis of *M. tuberculosis* inside the host. For that Balb/c mice were sacrificed at 1 day, 2 weeks, 4 weeks, 6 weeks, 8 weeks and 10 weeks after aerosol challenge with *M. tb:lspA↑* recombinant strain and *M. tuberculosis* H37Rv. The CFU counts were significantly higher in lungs and spleen of the mice infected with *M. tb:lspA↑* as compared to mice infected with wild type *M. tuberculosis* H37Rv. AFB staining of lungs and spleen homogenates showed higher number of rod shaped bacilli in lungs and spleen of the mice infected with *M. tb:lspA↑* as compared to mice infected with wild type *M. tuberculosis* H37Rv supporting the CFU counts. Gross examinations of the tissues revealed that the lungs of the mice infected with the *M. tb: lspa↑* showed greatly increased number of discrete well-circumscribed granulomas as compared to the lungs of wild type H37Rv infected mice. The lungs infected with the *M. tb: lspa↑* showed progressively enlarged granulomatous lesions with sharp circumscribed borders, whereas the lungs of wild type infected mice showed progressively enlarged granulomas that were diffuse, poorly circumscribed, and coalescing.

Since *M. tb: lspa↑* increased the bacterial burden as compared to wild type, in the lungs and spleen of infected mice, we examined the lung and spleen histopathology in *M. tb: lspa↑* infected mice. At 10 weeks after infection, the lungs of wild type *M. tuberculosis* H37Rv infected mice showed few collections of foamy macrophages accompanied by perivascular and peribronchiolar lymphocytes. Focal collection of foamy histocytes and some granulomatous cells were also seen but they were not well defined. In contrast, in *M. tb: lspa↑* infected mice, the convergence of lesions resulted in an extensive consolidation of well-defined granulomas in the lung tissue. Numerous
foamy histocytes, neutrophils, perivascular and peribronchiolar lymphocytes and macrophages were seen throughout the lungs. The Spleen of *M. tuberculosis* H37Rv infected mice showed few giant cells in intrafollicular area. In contrast, *M. tb: lspA*↑ infected mice showed numerous giant cells in intrafollicular area and reactive germinal centers.

The immunogenicity of LspA was also studied by measuring Th1 and Th2 cytokines profiles such as TNF-α, IFN-γ, IL-12, IL-10, IL-6 and IL-4.

TNF-α is one of the important immunologic mediators generated by cells of the monocyte/ macrophage lineage and has been shown to have significant effects on host immunity in bacterial and parasitic infections (Beutler B and Cerami A, 1988). Tuberculosis is a chronic mycobacterial infection (of macrophages and monocytes) in which TNF-α is characteristically produced. TNF-α has been reported to be present in the pleural effusions of patients infected with *M. tuberculosis*, and released by monocytes isolated from patients with active disease (Barnes PF, 1990). TNF-α is also essential for granuloma formation (Kindler V et. al., 1989; Havell EA, 1989) which is intimately associated with resistance against mycobacteria (North RJ and Izzo A, 1993). In the present study we observed significant increased TNF-α levels production in the serum of mice infected with *M. tb: lspA*↑ as compared to wild type H37Rv after 4 weeks of infection.

Interleukin-12 (IL-12) is a heterodimeric strong pro-inflammatory cytokine that induces the production of interferon-gamma (IFN-γ), favors the differentiation of T helper 1 (Th1) cells and forms a link between innate resistance and adaptive immunity. Dendritic cells (DC) and phagocytes produce IL-12 in response to pathogens during infection. (Bright-bill HD et. al., 1999). In the present study we observed significant increased production of IL-12 in the mice infected with *M. tb: lspA*↑ as compared to wild type H37Rv at most of the time points of infection.

No significant difference was observed in the level IFN-γ, IL-4 and IL-10 cytokines. These results revealed that the effect of LspA overexpression has a strong immunogenic effect that leads to Th1 type of immune response.