CHAPTER 5

Summary and conclusion

M. tuberculosis is one of the most successful human pathogen by virtue of its remarkable ability to adapt to several adverse conditions. Over the course of its life cycle in vivo, *M. tuberculosis* is exposed to a plethora of environmental stress conditions. Temporal regulation of genes expressing a wide variety of proteins involved in sensing and responding to such conditions is, therefore, crucial for *M. tuberculosis* to establish an infection. Lipoproteins are membrane anchored proteins often recognised to be crucial in cell wall related functions and in immune-modulation of the host. The lipoproteins of *M. tuberculosis* constitute an important fraction of proteins with significant potential to modulate host immune responses. Though they are regarded as important virulence factors, majority of them remain uncharacterized with regard to their physiological and cellular functions. In this context the present study has been focused on a novel lipoprotein LprI of *M. tuberculosis*, LprI, in order to gain insights on its functionality in bacillus and understand its relevance in the cellular metabolism of the host.

As a first step to understand the functions of LprI, various features of LprI have been analysed using bioinformatic tools to develop a rationale for pursuing experimental studies. In the genome of *M. tuberculosis*, *lprI* gene (at the locus Rv1541c) is present downstream of *glbN* (at the locus Rv1542c) which encodes a truncated haemoglobin (HbN). Moreover, *lprI* and *glbN* share an operon and co-transcribe. Interestingly, genome-wide investigation of *lprI* and *glbN*, conducted across various species, confines

their presence exclusively in several pathogenic strains of mycobacteria. This indicates that these two genes may have some functional correlation with respect to virulence, which may be a prerequisite during pathogenic cycle of *M. tuberculosis*. *M. tuberculosis* HbN has already been associated with an exquisite mechanism to relieve the toxicity of nitric oxide (NO) and nitrosative stress. It has been characterized both basic biochemically and biophysically. Recently, HbN has been found to undergo glycosylation at its C-terminus localize at the surface of *M. tuberculosis*. HbN leads to an increase in the infectivity and intracellular survival; and also brings about changes in the levels of pro- and anti-inflammatory cytokines.

Computational assessment of various features and motifs of LprI of *M. tuberculosis* has been carried out which helped to affirm it as a lipoprotein. The presence of lipobox motif, LSAC, supposedly targets it to the membrane. Experimental investigations through ultracentrifugation and Triton X-114 (Tx-114) phase partitioning validated the membrane and cell-surface localization of LprI.

A distinct difference in the molecular weight of LprI expressed in different hosts has been observed through immunoblot analysis. Molecular weight of LprI from mycobacterial strains has been observed to be significantly higher as compared to that of LprI purified from *E.coli*. This is presumed to be a manifestation of post-translational modifications (PTMs) in mycobacterial strains as the proteins expressed in *E. coli* do not undergo PTMs. Since, a large number of lipoproteins have been recognised to be modified through O-glycosylation, presence of putative glycosylation sites in LprI have been searched using the NetOglyc program. Potential glycosylation sites have been predicted at 9 Ser/Thr residues. Experimental verification of this prediction has been carried out using site-directed mutagenesis of 3 Thr residues i.e. Thr24, Thr28 and Thr117, chosen on the basis of proximity to Pro residues. These mutants of LprI displayed a relatively faster migration on SDS-PAGE with respect to the wild-type LprI when expressed in *M. smegmatis* confirming presence of glycosylation on these residues. Thus, LprI is glycosylated at several positions and is a highly glycosylated protein. Glycosylation is an important determinant of immune-recognition and protein–protein interactions between host and the pathogen. The implications of glycosylation in LprI have not been ascertained as yet, but might be important for some specific function.

PFAM based analysis of LprI sequence revealed presence of a MliC (membrane bound lysozyme inhibitor of the c-type lysozyme) motif. This has been an interesting revelation, as this motif has so far been recognised in the gram-negative pathogens only. Sequence and structure-based analysis brought forth several important features of LprI. The sequence of MliC motif in LprI showed high similarity with those of other MliC proteins. It also showed a high similarity with the conserved regions which are the basis of lysozyme binding in other MliC proteins. A homology based model structure of LprI has been superimposed with the known structure of a MliC protein from *P. aeruginosa* ($MliC_{pa}$). The structure of LprI showed high structural conservation with $MliC_{pa}$. In $MliC_{pa}$, the key conserved region, SGSGAKY, inserts into the active-site cleft of HEWL (hen egg white lysozyme) and interacts with its Glu35 and Asp52 residues. The structural analysis indicated that the analogous region in LprI, SGSGARY, forms a loop that extends into HEWL in a similar manner as the loop of $MliC_{pa}$. Conservation of the residues at the interacting region indicates that LprI might be adopting a similar mechanism as that of $MliC_{pa}$ for binding to lysozyme. Furthermore, $MliC_{pa}$ is known to exist as a dimer in its crystal structure and its dimeric form is stabilized by the hydrophobic residues present at a specific region of the protein. Several proteins of the MliC family are predicted to form dimers on the basis of the presence of hydrophobic residues at similar positions. A comparison of the structure of LprI with $MliC_{pa}$ and its sequence based analysis with other MliC/PliC proteins suggested that LprI might also be functional as a dimer. Gel filtration profile of the recombinant LprI protein purified from *E. coli* has provided evidence in favour of its dimeric existence.

The physical interaction of LprI with HEWL has been studied using fluorescence spectroscopy. The fluorescence spectrum of an equimolar mixture of HEWL and LprI has been found to differ significantly from that expected when adding the fluorescence emission spectra of the individual proteins. An overall 15.5% quenching of the fluorescence was measured with maximal quenching at 344 nm. In addition to its specific physical interaction with HEWL, LprI has been found to be a potent inhibitor of HEWL enzymatic activity. An agar-based gel diffusion assay has been conducted to determine the effect of HEWL on *M. luteus* cells both in the presence and absence of recombinant LprI protein. Formation of a clear zone indicative of *M. luteus* cell lysis by

HEWL is observed. However, in the presence of equimolar amount of LprI, the lytic activity of HEWL is completely abrogated and no clear zone appeared which confirmed the functional activity of the MliC motif in LprI. The observation led us to examine lysozyme inhibition activity of LprI in mycobacterial strains. Concentration dependent effect of HEWL on the wild-type and LprI overexpressing mycobacterial strains (*M. smegmatis*, *M. tuberculosis* (*H37Ra* and *H37Rv*)) has been analysed by an *in vitro* growth profile and REMA plate assay. Presence of LprI in *M. smegmatis* conferred protection against the lytic activity of HEWL that was apparent in both the assays. Use of *M. smegmatis* recombinant strains complemented with a copy of *lprI* is advantageous for unambiguous validation of the lysozyme inhibition activity of LprI and provides an alternative to the knock-out system. Similarly, the enhanced expression of LprI in *M. tuberculosis* strains has been observed to abrogate the lytic activity of HEWL and confers better tolerance against it in comparison to the wild-type strains in both the assays. The inhibitory activity of LprI has also been checked against human lysozyme (hLZ). Under *in vitro* conditions the mycobacterial strains exhibited lesser sensitivity to the action of hLZ in comparison to HEWL. However, this observation does not suggest that hLZ may not be able to act on the bacilli under *in vivo* conditions. Two possible reasons to support this presumption are: (i) Differences in the amino acid sequences of HEWL and hLZ (57% identitiy and 76% similarity) might contribute to differences in their enzymatic activities by affecting the affinities for substrates. (ii) In the physiological milieu, the activity of lysozyme is affected and enhanced due to various synergistically acting factors. Therefore, to get better insights into functional relevance of LprI experiments need to be carried out under *in vivo* conditions.

The above results establish that LprI carries a functionally active MliC motif which might be providing significant protection to *M. tuberculosis* from the lytic activity of lysozyme. The role of LprI in abrogating the lytic activity of lysozyme to aid the survival of bacilli under *in vitro* conditions might have crucial implications during infection of *M. tuberculosis*. The emergence of lysozyme inhibitors is an evolutionarily significant phenomena and its identification in mycobacteria is a interesting finding. The designing of new anti-microbial molecule for targeting the lysozyme inhibition activity of LprI would be beneficial. Designing of a molecule that binds to the MliC

motif of LprI with a better efficacy than lysozyme would eventually enable the later to act unrestrained on the cell wall and bring about its lysis.

The enhanced expression of LprI has not exhibited any effect on growth characteristics of *M. tuberculosis* under *in vitro* conditions. Pathogenesis of M tuberculosis is directed by its ability to reside within the macrophages where a plethora of environmental stressors, such as reactive nitrogen species (RNS) and reactive oxygen intermediates (ROI), low pH, and hydrolases and the expression of several crucial genes is regulated in response to these stresses. Knowledge about the temporal regulation of genes involved in sensing and responding to environmental stressors is important in understanding the ability of *M. tuberculosis* to persist inside the host macrophages. RT-PCR analysis of the expression of *lprI* in murine macrophages indicated a significant amplification in a time-dependent manner. Furthermore, $MliC_{pa}$ is known to exist as a dimer in its crystal structure and its dimeric structure is stabilized by hydrophobic interactions between two protomers of $MliC_{pa}$. Hydrophobic residues present at specific regions of the protein chain are responsible for these interactions. Furthermore, several proteins of the MliC family are predicted to form dimers on the basis of the presence of these hydrophobic residues at similar positions. A comparison of the structure of LprI with $MliC_{pa}$ and its sequence based analysis with other MliC/PliC proteins suggested that it might also be functional as dimer. Gel filtration profile of the recombinant LprI protein purified from *E. coli* has provided evidence in favour of its dimeric existence

LprI has a distinct lysozyme inhibition property and its expression level augments rapidly during the course of infection in macrophages. In view of these, LprI had been expected to confer an enhanced survival to the bacterium while it resides in the macrophages. To study the implications of the co-presence of LprI and HbN as a unit, *M. smegmatis* has been engineered to express the complete *glbN-lprI* operon. Survival studies have been carried out with a focus to understand the effect of enhanced production of LprI, HbN and LprI-HbN together, in *M. smegmatis* using the RAW 267.4 or THP1 cell lines. However, any significant change in the survival of bacilli due to LprI overexpression could not be observed. An increase in infectivity and intracellular survival has been observed in case of HbN and LprI-HbN overexpressing cells. Since, LprI alone does not lead to any positive effect on survival, in case of cells overexpressing LprI-HbN, the enhanced survival could be due to a positive influence of

HbN. The absence of any beneficial effects on the intracellular survival due to LprI might be explained on the basis of the low concentration of lysozyme present in the secondary cell lines used in the experiments. This concentration of lysozyme present in the secondary cells seems inadequate to cause lysis of even the wild-type *M. smegmatis* strain. Therefore, cells overexpressing LprI show a similar pattern of survival as that of wild-type *M. smegmatis* strain due to the lack of any challenging concentration of lysozyme in the cellular environment. Under actual physiological conditions, lysozyme is present at high concentration in different tissues and is able to mount a stronger attack on the cell surface by synchronizing its action with other molecules. Synergistic action of lysozyme with lactoferrin which is also present at high concentrations is well documented. Therefore, the possibility of LprI to confer an increased survival to the bacillus cannot be excluded on the basis of survival studies carried out in cell lines. In this regard, it is anticipated that further investigations using animal models infected with mycobacterial strains either overexpressing LprI or with the deletion of *lprI* would be more informative.

Enhanced expression of LprI in *M. smegmatis* and *M. tuberculosis* led to a dissimilar pattern in the secretion of pro-inflammatory cytokines, IL-6 and IL-12. Any definite conclusions could not be drawn from the observed levels of these cytokines for the LprI expressing *M. smegmatis* cells. However, LprI-HbN expressing *M. smegmatis* cells consistently displayed a pattern in which both IL-6 and IL-12 levels have increased with respect to wild-type at all time-points. This suggests that the LprI-HbN gene assembly acts to cast a pronounced pro-inflammatory response. Surprisingly, in case of *M. tuberculosis,* reduced levels of IL-6 and IL-12 are observed for LprI-overexpressing cells at all times points. This suggests that LprI evokes an effect which is apparently anti-inflammatory in nature.

In *M. smegmatis,* the HbN overexpression led to increased nitric oxide (NO) production and any significant increase could not be observed due to LprI. However, the resultant level of NO for the strain carrying both LprI and HbN together has been the highest. This is an interesting observation which suggests that when both LprI and HbN are present together host cells respond with elevated NO production. In case of *M. tuberculosis*, the augmented expression of both LprI and HbN lead to increase in NO levels in comparison to the wild-type strain. In case of HbN, this increase is consistent

with a previous study (Arya *et al.*, 2013). But it appears to be a unique phenomenon indicative of the fact that LprI shows enhanced effect in the presence of even basal levels of functionally active HbN. This has lent support from increased levels noted for LprI-HbN, whereas, LprI alone has not shown any increased NO levels. These observations indicate that LprI and HbN do have some degree of cooperation with respect to the production of NO.

Fast growing non-pathogenic strains are rapidly cleared by the host lack both LprI and HbN proteins. Interestingly, these proteins are present as a unit only in slow growing pathogenic strains of mycobacteria which are capable of entering into a dormant state. Nitric oxide mediated induction and regulation of granuloma formation has been extensively documented. Evaluation of nitric oxide levels secreted by the macrophages infected with mycobacterial strains indicated a high stimulation for NO production in the presence of both LprI and HbN. Thus, the role (if any) of these proteins in latency has been explored through an *in vitro* model of granuloma. Aggregation of peripheral blood monocytes (PBMCs) after infection with the *M. smegmatis* cells expressing LprI, HbN and LprI-HbN has been monitored. The PBMCs infected with *M. smegmatis* wild-type and LprI-expressing cells remained distinctly separated throughout the incubation period of the experiment. Interestingly, numerous cellular aggregates have been observed since the early time point after infection with HbN and LprI-HbN expressing cells. This effect has been more prominent in case of HbN-expressing cells whereas LprI-expression has been observed to be deleterious for the monocytes. In the cells expressing both LprI and HbN, the toxicity of LprI has been neutralized by the presence of HbN. This suggests that, when LprI and HbN are present together, the toxic effects caused by LprI are relieved. The involvement of LprI and HbN in the aggregation of PBMCs is an interesting and novel observation and suggests a possible role of these proteins during latency and granuloma formation.

The present study has dealt with the characterization of the structural and functional aspects of a novel lipoprotein, LprI, of *M. tuberculosis*. The immunological relevance of LprI has also been explored, nevertheless, requires further investigations for better understanding of its role in the physiology of *M. tuberculosis* are required. The genetic context of *lprI*, where it is juxtaposed with *glbN* in mycobacterial strains is interesting and unique, since this combination is exclusively present in selected pathogenic species

only. This indicates that this gene combination is involved strictly in some virulence related function(s). HbN has already been associated with crucial functions in the physiology of *M. tuberculosis*. Herein, a several-fold amplification of *glbN* and *lprI,* in a temporal fashion, has been observed post-infection of *M. tuberculosis* in murine macrophages fortifying the proposition that these genes are correlated in some manner. A unique and radical finding in LprI is the recognition of a functionally active MliC motif. The enhanced expression of LprI confers better survival to the mycobacterial strains in the presence of high concentrations of lysozyme by abrogating lytic activity of the latter. Thus, LprI exhibits a distinct lysozyme inhibition activity and this study is one of its kind to report a lysozyme inhibitor in the mycobacteria. So far, lysozyme inhibitors had been recognised in gram-negative bacteria only. Furthermore, LprI protein has been shown to possess several glycosylation sites and thus, it is a highly glycosylated protein. Also, LprI has been confirmed to remain localized on the membrane and cell-surface of the bacilli. It is speculated that surface association of LprI and the decorations in the form of glycosylation must be important determinants in some kind of host-pathogen interactions. Cell surface localization of LprI is appropriate in view of its inhibitory activity against lysozyme which targets the integrity of bacterial cells through hydrolysis of their surface. Furthermore, cell surface association of LprI ensures co-localization with HbN, which is also present at the surface, thus, enabling their interaction.

Reduced levels of IL-6 and IL-12, and increased levels of nitric oxide indicate an anti-inflammatory role of LprI during infection. Thus, even though LprI exhibits a potential defence mechanism of lysozyme inhibition, its effect on cellular responses of *M. tuberculosis* during infection have not been suggestive of pro-bacillus role when it is present alone. Another most important finding from this study is the indication obtained on a plausible role of LprI and HbN in the induction of latency as observed through aggregation of the PBMCs in an *in vitro* model of granuloma formation. The aggregation of monocytes infected with *M. smegmatis* expressing LprI and LprI-HbN is an interesting phenomenon. The identification of factors involved in promoting dormancy is significant as its represents one of the key factors in the success of *M. tuberculosis*. With the development of animal models representing a true latent

infection which closely resembles human tuberculosis, it would be worthwhile to confirm these *in vitro* findings on these proteins through an *in vivo* animal model.

Furthermore, both LprI and HbN carry out their physiological functions i.e. lysozyme inhibition and NOD activity respectively that might be crucial for the pathogenicity of the bacilli. The execution of these functions, however, is independent of their co-existence in an operon. They are capable of performing their respective roles with ease, even when they are not present together. This compels us to presume that perhaps under physiological environment their functional assistance to each other is dependent /on some specific conditions and is needed for some specialized task within the bacteria.