

SUMMARY OF THE THESIS

Pathogenic mycobacteria are facultative intracellular parasites that are capable of surviving and multiplying in phagocytes. This success is most likely inherent in the unusual properties of the mycobacterial cell envelopes. Several cell envelope components have been shown to impair host immunological responses further substantiating the implication of mycobacterial cell envelopes and their components in the pathogenesis of mycobacterial infections. The control of tuberculosis cannot be achieved without further basic molecular characterization of individual cell envelope components of the bacilli. Such studies aid in our understanding of interaction of individual components with host cells as well as for the identification of the candidate molecules to use as drug targets and vaccines. In this study, attempts were made to characterize mycobacterial cell envelope associated hypothetical probable lipoprotein, Lppi, and mycobacterial DNA binding protein, MDP1. Attempts were also made to set a platform for studying the specificity/cross reactivity of mycobacterial capsular polysaccharides.

Gene *lppi* (Rv2046) from *Mycobacteria tuberculosis* (H37Rv), so far unexplored, was taken for its molecular characterization. Lppi is a hypothetical, mycobacterial cell wall anchored probable lipoprotein, sharing no conserved domains outside the genus mycobacteria. The gene was PCR amplified, cloned and expressed in *E. coli* with N-terminal histidine tag at a molecular mass of 23.5kDa in SDS-PAGE. However anti-histidine mAb did not recognize the N-terminal his-tag in the expressed protein.

The common feature of lipoproteins is the presence of a conserved consensus sequence called a lipobox ([LV] [ASTVI] [GAS] C) with a universally conserved cysteine at position +1. It directs the processing of the prolipoprotein to form the mature acylated protein. Clustal X analysis of *lppi* gene derived from different mycobacterial species revealed that at positions 13–17 of the precursor protein of *lppi* is the sequence IAGCS, which is similar to the consensus sequence for lipidation and cleavage by signal peptidase II based on predicted and known lipoproteins of Gram-negative and Gram-positive bacteria. The particular lipobox was found to be 100% identical with that of *lprG* (Rv1411c). Thus, the protein was expressed with C-terminal his tag. The *lppi* gene indeed possessed the N-terminal signal sequence which was getting chopped off, as evidenced by the observations that acylated *lppi* with C-terminal his-tag was expressed, at slightly higher molecular size compared to N-terminal his-tagged protein, and reacted with anti-his antibody unlike expressed N-terminal his tagged *lppi*.

In *E. coli*, expression of Acy.*lppi* with C-terminal his-tag could be observed at 25.5 kDa in SDS-PAGE as compared to the predicted size of 22 kDa, characteristic of bacterial

lipoproteins due to the post translational modification (acylation) in lipoproteins and the same was purified from the *E. coli* in the presence of 0.5 - 1% triton X-100 in binding/ lysis and wash buffers confirming the lipid modification of this recombinant lppi (Acy.lppi), in the heterologous expression host *E. coli*. The difference in the mobilities of Acy.lppi in SDS-PAGE, ran under non-reducing and reducing conditions, suggested the presence of intra molecular disulphide linkages in Acy.lppi. The non acylated form of lppi was expressed and purified from the cytosolic fractions of *E. coli*, only after incorporation of wobble changes at the 5' end of the gene, in the absence of tritonX-100 in buffers, thus confirming the absence of lipid moiety in non-acylated lppi as expected theoretically. SDS-PAGE of purified Acy.lppi and NAcy.(W)lppi proteins revealed only minor difference in the mobilities of the two which might be attributed to the acylation of Acy.lppi. Further, purified acylated and non-acylated lppi were found to be antigenically similar with respect to the reactivity with rabbit anti-acylated lppi antibodies in western blotting.

Immunoblotting of cell wall proteins of different mycobacterial species, with rabbit anti-Acy.lppi (*E. coli* expressed) antibodies, revealed expression of the lppi in the cell wall proteins of different virulent and nonvirulent mycobacterial species under both non-reducing and reducing conditions. Under nonreducing condition, in *M. microti* the diffused band was highlighted at 24-29kDa, *M. smegmatis* at 37 and 24kDa, *M. tuberculosis* H37Rv at 66, 45 and 24kDa, *M. tuberculosis* H37Ra at 24kDa and *M. bovis* at 66, 29 and 24kDa. Whereas, under reducing condition a band at 25.5kDa, analogous to Acy.lppi expressed in *E. coli*, was observed in both *M. tuberculosis* complex members viz. *M. microti*, *M. tuberculosis* H37Rv and *M. bovis* and non virulent species *M. tuberculosis* H37Ra and *M. smegmatis*. However, in *M. smegmatis*, another band at 37kDa was also recognized. Further, lppi was also found to be localized on the cell surface of both *M. tuberculosis* complex members viz. *M. microti*, *M. tuberculosis* H37Rv and *M. bovis* and non virulent species *M. tuberculosis* H37Ra and *M. smegmatis*.

PCR amplification, cloning, expression of lppi in homologous expression host, *M. tuberculosis* H37Ra revealed the presence of Acy.lppi in the pellet/membrane fraction, whereas NAcy.lppi was detected in the soluble/cytosolic fraction which was what theoretically expected. Also, *M. tuberculosis* Ra expressed Acy.lppi appeared at 25.5kDa, having similar molecular size as that of *E. coli* origin Acy.lppi. However the mycobacterial NAcy.lppi was found to be of much lower molecular size as compared to NAcy.(W)lppi protein of *E. coli* origin. Attempts to purify *M. tuberculosis* H37Ra expressed recombinant

Acy.lppi and NAcy.lppi in sufficient quantity for immunological characterization were not successful.

E. coli expressed Acy.lppi and NAcy(W).lppi, when immunized in mice, induced IgG1/IgG2a ratio of 10:1 and 3.7:1 respectively, in second booster sera when checked at dilution of 1:10k, indicative of good Th2 type immune response. Although in this study detailed immunological characterization of lppi was not done, yet IgG1/IgG2a ratio seen upon immunization with Acy.lppi was indicative of strong Th2 type of immune response. FACS analysis revealed significant promoter activity in the 1kb region upstream of lppi coding region in *M. tuberculosis* Ra harboring lppi promoter-*gfp* fusion construct. It showed two fold higher peak than positive control of pSC301, harboring SOD promoter-*gfp* construct. Analysis of the promoter activity under different *in vitro* stress conditions, mimicking intracellular environment of infected macrophages viz., nitrosative stress, oxidative stress, acidic environment, and starvation conditions, revealed higher induction of the lppi promoter as compared to that of the SOD promoter. In line with these observations, confocal images of macrophages infected with *M. tuberculosis* Ra harboring lppi promoter-*gfp* transcriptional fusion construct at different post infection time period, till 96 h also showed strong lppi promoter activity. Acy.lppi or NAcy.lppi were not found to be trafficked inside macrophages and remained localized in phagolysosome containing bacteria.

With a long term goal to check the specificity/cross reactivity of carbohydrate determinants on mycobacteria surface and to provide a comprehensive information about capsular polysaccharides of pathogenic microorganism, anti-polysaccharide antibodies and related information from a single source, a database was generated in collaboration with Dr. G.P.S. Raghava's group at IMTECH. First, the structure of the database was established in the form of HaptenDB, by including important parameters like: 1) hapten molecules, 2) carrier molecules, 3) conjugation methods (ways to raise antibodies against particular group of haptens), 4) specificity and cross-reactivity of raised antibody, 5) application of antibodies e.g. in constructing cost-effective and simple detection kits. All the data for HaptenDB were collected from published literature. A simple search in the HaptenDB database against a hapten molecule displays a summary of all records where either an antibody is raised against that hapten or some other antibody, raised against somewhat different hapten but having significant cross-reactivity with the searched hapten. Next, HaptenDB was upgraded to CarboDB, by including entries for major polysaccharides associated with mycobacterial cell wall e.g. glucans and arabinomannans and similar

polysaccharides from other microorganisms. Entries related to their microbial origin, function, antigenic nature, proposed utility etc. were also included in the database.

-As a test case, database revealed that glucan is not only present in different mycobacterial species but also in many human pathogenic fungi. Anti-glucan monoclonal antibody 24c5 reacted equally well with *M. microti* and *M. tuberculosis* H37Rv in ELISA and IFA without any observed reactivity with *Candida albicans* as checked in ELISA, thus indicating that with respect to the epitope of mAb 24c5, glucans of these two species of mycobacteria are same, but differ from that of *Candida albicans*. On the other hand, anti-arabinomannan monoclonal antibody 9d8 did not react with *M. microti*, unlike *M. tuberculosis* H37Rv, indicating that arabinomannan differs in these two species. These findings were further corroborated by the observations that: i) unlike mAb 9d8 (anti-arabinomannan antibody), mAb 24c5 (anti-glucan antibody) coating of *M. microti* overcomes the mycobacteria mediated inhibition of NF κ B activation, thus modulating the intracellular survival of mycobacteria.

The mAb E2B9 (IgG1), generated against *M. microti* cell wall protein, reacted strongly with the cell wall proteins of *M. microti* in ELISA. The antibody not only reacted with *M. microti* cell surface but also recognized *M. tuberculosis* complex members i.e. *M. tuberculosis* H37Rv, *M. bovis*, *M. avium* and avirulent species *M. smegmatis* and *M. tuberculosis* H37Ra. In western blotting with cell wall fractions of *M. microti*, *M. smegmatis*, *M. bovis* and *M. avium* this antibody revealed a common band at 29kDa. Besides, except for *M. bovis*, mAb also reacted with a lower band at 23kDa in *M. microti* and 21.5kDa in *M. smegmatis* and *M. avium*. The reactivity of mAb E2B9 with different fractions of *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra and *M. microti* recognised doublet at 29-30kDa in the cell wall fractions of all the species and in the cytosolic fraction, it recognized only single band at 30kDa in *M. tuberculosis* H37Rv and *M. tuberculosis* H37Ra. Whereas in *M. microti*, additional band at 23kDa in cell wall fraction was also found reactive along with doublet at 29-30kDa, in cytosolic fraction. Also, mAb E2B9 did not show any reactivity with secretory fraction of any mycobacterial species tested. The immunoprecipitated antigen, from the cell wall proteins of *M. microti* and cytosolic proteins of *M. tuberculosis* H37Ra, gave similar N-terminus sequence and the 15 amino acid sequence retrieved showed 100% homology with Rv2986c, also known as mycobacterial DNA binding protein 1 (MDP1) or eukaryotic histone like proteins (HUP B). Blast search of Rv2986c protein sequence in unfinished *M. microti* genome sequence available at www.sanger.ac.uk revealed identical protein but it lacked the stretch of 18

basic amino acids at C-terminus. That the mAb E2B9 is indeed directed towards MDP1 was further confirmed by its reactivity with the recombinant MDP1.

When checked in infected macrophages, using GFP tagged *M. tuberculosis* H37Ra, mAb E2B9 reactive component could be localized intracellularly. Interestingly, the antibody also reacted in the nuclear region of normal cells, barring those few cells which showed faint diffused cytosolic staining. Analysis of protein structure of Rv2986c revealed its Y shaped structure, very appropriate for DNA binding and acting as a transcriptional regulator as predicted in the literature. Chromatin immunoprecipitation (ChIP) assay performed with mAb E2B9 and *M. tuberculosis* H37Ra genome revealed the presence of MDP1 binding site in the putative promoter region of Rv0688 which codes for putative ferredoxin reductase and Rv3375 (Ami D) which codes for a amidase. Although the fragment obtained were from *M. tuberculosis* H37Ra but when blasted in NCBI, showed 100% identity with *M. tuberculosis* H37Rv genome. However, the regulation of these proteins or binding of MDP1 to upstream sequences of Rv0688 (putative ferredoxin reductase) / Rv3375 (AmiD), needs further confirmation. Nevertheless, this study provides the first indication by ChIP experiments that MDP1 might be the transcription regulator.

Thus, the present study shows for the first time:

- (i) Expression of *lppi* in both virulent and avirulent mycobacterial species and presence of N-terminal signal sequence in *lppi*, responsible for acylation and membrane trafficking.
- (ii) Strong acid inducible promoter activity in the 1kb region upstream of *lppi* coding region. Elicitation of significant Th2 immune response by *lppi* in Balb/c mice.
- (iii) *M. microti* MDP1 differs from that of *M. tuberculosis* H37Rv in lacking 18 amino acids at C-terminus. Anti-MDP1 mAb E2B9, shows binding in the perinucleolar region of human monocyte derived cell line, THP1.
- (iv) Binding of MDP1 with the putative promoter regions of Rv0688 and Rv3375 (AmiD), thus indicating its function as transcriptional regulator.
- (v) Generation of database for small molecules, HaptenDB and initial attempts for its upgradation in to the database for capsular polysaccharides of pathogenic organisms, CarboDB, which might find wide application in structural/functional epitope analysis.