

SUMMARY & CONCLUSION

Studies on the surface component(s) of mycobacteria and infected macrophages are of paramount importance in understanding the pathogenesis of the disease and can also help towards the identification of the targets for therapeutic interventions. Antigenic determinants on mycobacteria have been studied by various ways including polyclonal antibodies, monoclonal antibodies and patient sera. These antigens have been linked with diagnosis, vaccine development and pathogenesis of the disease. The induction of mycobacterial genes during infection as well as altered expression of various adhesion molecules on infected cell surface has been demonstrated, but nothing much has been done to identify and characterize bacterial/host cell components specifically present on the infected cell. The available genome sequence of *M. tuberculosis* and the upcoming attempts unraveling genome of various mycobacterial species like *M. microti*, *M. avium*, *M. leprae* and *M. smegmatis* are providing blue fingerprints to compare and explore the potential therapeutic and vaccine targets.

Earlier studies in lab using *M. microti* as a model system pointed towards the association of mycobacterial antigenic determinants of three monoclonal antibodies (mAbs) namely C2B5, C10B5 and C3G5 on the surface of infected macrophages. In continuation, the present study aimed at the characterization of mycobacterial cell surface antigens and those present on the infected cell surface using above mentioned antibodies and mAbs E7B2 and D5G8, generated in the present study. Also, bioinformatics analysis of different mycobacterial genomes with emphasis on cell wall associated molecules of *M. tuberculosis* complex shortlisted few target molecules, among which cutinases seemed to be an important unexplored attractive target for therapeutic intervention was selected for this study.

Immuno-blotting with *M. microti* crude extract, cytosolic and cell membrane fraction revealed that both mAbs E7B2 and D5G8 identified a protein of molecular mass 38 kDa in all the three tested mycobacterial fractions. mAb D5G8 also recognized 66 kDa molecule, in addition to 38 kDa in crude extract. With the membrane fraction, D5G8 recognized 66 kDa, 38 kDa, 23 kDa and 18 kDa molecules, whereas mAb E7B2 reacted with 38 kDa and 24 kDa molecules. mAbs C2B5, C10B5, C3G5 and D5G8 showed good reactivity with both *M. microti* whole cells and cell wall fraction. 19kDa molecule recognized by mAb C10B5 and 66 kDa doublet recognized by mAb C3G5 were found to be lipoproteins. While, 38 kDa, 40 kDa and 42 kDa molecules recognized by mAbs E7B2,

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D5G8 and C2B5 respectively did not seem to possess lipid attachment. mAbs D5G8 and C3G5 also reacted with extracellular secreted proteins of *M. microti* and *M. tuberculosis*.

Flow cytometric analysis using Alexa labeled secondary antibodies also showed the reactivity of mAbs C3G5, C2B5 and C10B5 with *M. microti* infected macrophage cell surface. Confocal microscopy further confirmed this reactivity, where mAb C2B5 showed punctuate type fluorescence while, mAb C10B5 and C3G5 showed uniform fluorescence across the infected cell surface. Pronase treatment of mycobacterial cell surface completely abolished the binding of mAb C10B5, indicating that the binding epitope resides in a protein. While, binding of mAb C2B5 was not affected by pronase treatment. Carbohydrate did not constitute epitope of any mAb C3G5, C2B5 and C10B5 reactive to infective cell surface.

mAb C10B5 specifically reacted with the members of *M. tuberculosis* complex and *M. avium* without any reactivity with cell walls of *M. smegmatis*, *M. phlei*, *M. fortuitum*, *M. vaccae* and *M. kansasii*. 19 kDa protein reactive to C10B5 was observed in cell walls of *M. tuberculosis*, *M. bovis* BCG and *M. microti*, while recognizing an additional band at 24 kDa in *M. bovis* BCG. Also, this antibody did not react with extracts of *E. coli* and *S. typhimurium*. The C10B5 reactivity with infected macrophages was not due to non-specific interactions as revealed by immunoprecipitation of biotinylated cell surface-associated 19 kDa protein. Following experimental results established that mAb C10B5 reactive 19 kDa is different from well-characterized 19 kDa protein (HYT6 reactive), reported earlier.

- (i) In contrast to LpqH (19 kDa, Rv3763), mAb C10B5 reactive 19 kDa protein was found to be non-secretory.
- (ii) Electrophoretic mobility was different under non-reducing conditions.
- (iii) Specific immunoprecipitation of respective proteins by HYT6 and C10B5, without any cross reactivity.

Attempts for N-terminal sequencing of the C10B5 affinity purified 19 kDa were not successful. Increasing the yield to some extent, by using denaturing or non-denaturing conditions also did not help. Thus, the high affinity of mAb C10B5 and lipoprotein nature of 19 kDa protein posed major hindrance in establishing its identity by N-terminal sequencing. In second approach, *M. microti* genomic library was constructed in λ ZAP II vector having an initial titer of 4.45×10^5 pfu/ μ g vector DNA, which rose to 5.6×10^9

pfu/ μ g upon one round of amplification. The average insert size in library was 3.01 kbp and ratio of recombinants to non-recombinants was 98%. Initial immunoscreening with polyclonal antisera showed all library clones to be reactive with anti-*M. microti* lysate antisera. While immunoscreening with mAb C10B5 yielded positive phages in the primary round. However, none of the positive plaque reacted with mAb C10B5 during the secondary screening.

Screening random phage peptide library while mapping of C10B5 reactive epitope yielded many peptides with consensus sequence -[S] [HLV] [FY] [SLV] ISSTYS [RST] [ST]-. The mimotope "HFEGSPKYSRS" upon blast search indicated that 19.6-kDa *M. tuberculosis* pyrazinamidase (PZase) might be the C10B5 antigen. The epitope of mAb C10B5 was mapped to 11-residue linear sequence HFSGTPDYSSS of mycobacterial pyrazinamidase with fair identity and positivity of 63.3% and 72% respectively. Following experimental evidences further strengthened the interpretation that C10B5 reactive 19 kDa protein is mycobacterial pyrazinamidase

- (i) Anti-*M. microti* cell wall antiserum reactivity with rPZA showed pyrazinamidase to be localized in cell wall fraction, in concordance with mAb C10B5 reactivity with cell wall of *M. microti*.
- (ii) Anti-phage peptide p1 and p2 (C10B5 reactive) antisera reacted with the rPZA in ELISA, 19 kDa protein of *M. microti* cell wall preparation and mAb C10B5 immunoprecipitated 19 kDa protein.
- (iii) The mAb C10B5 reactive epitope "HFSGTPDYSSS" was localized of the surface of pyrazinamidase protein model.

During insilico analysis of mycobacterial genomes, for mycobacterial cell surface molecules, so far unexplored cutinases were shortlisted. *M. tuberculosis* Rv genome analysis revealed seven cutinase determinants, Cut1 (Rv1758), Cut2 (Rv2301), Cut3 (Rv3451), Cut4 (Rv3452), Cut5 (Rv3724A and Rv3724B), Rv3802c and CFP21(Rv1984c). Serine motif P-x-[STA]-x-[LIV]-[IVT]-x-[GS]-G-Y-S-[QL]-G (PROSITE motif PS00155 – PNSRIVLGGYSQ/LG) and an aspartate /histidine motif C-x(3)Dx[IV]CxG[GST]x(2)[LIVM]x(2,3)H(PS00931CAPDDPICSGGGNIMAH) were found to be conserved in all mycobacterial cutinases, thus forming the functional catalytic triad, peculiar of cutinases, lipases and esterases, all being members of α/β hydrolase super-family. The structural organization of Cut5 gene of *M. tuberculosis* Rv was

peculiarly distinct when compared to *M. microti* and *M. bovis* Cut5 gene. Besides, bioinformatics analysis revealed that Cut5 protein was the only cell surface associated protein. Clustal x analysis of Cut5 genes of *M. tuberculosis* Rv and *M. bovis* showed that Cut5A possess a unique stretch of 13 amino acids residues at C-terminus whereas Cut5B protein has a unique stretch of 21 amino acids present at the N-terminus of Cut5B. Homology searches with Cut5 revealed similar homologs in *M. smegmatis*, *M. avium*, *M. africanum* and *M. marinum* genomes. Interestingly, phylogenetic analysis showed fungal cutinases form cluster B.2.2 that is well separated from that of mycobacterial cutinases cluster B.2.1, indicating that their function might be different.

Attempts made in expressing Cut5 gene proved futile. *E. coli* BL21(DE3) cells containing Cut5B gene upon induction with 1mM IPTG, over-expressed a 23-kDa protein, in accordance with the expected size. Culturing under different temperatures ranging from 16°C –37°C revealed only 10% of the over-expressed protein was present in soluble fraction while the remaining was going to inclusion body. Immuno-blot analysis with anti-His antibody confirmed the association of His tag with the expressed protein. Purification of native Cut5B protein from the soluble fraction using nickel affinity column revealed low yield of 23 kDa Cut5B protein along with some contaminating proteins. The purification from inclusion body, following on-column refolding gave good yield of almost pure protein, possessing lipase activity on both short chain and long chain hydrocarbons i.e. tributyrin and olive oil.

Polyclonal antisera raised against Cut5B exhibited good titer. Immunofluorescence analysis using this antiserum revealed the presence of Cut5B on *M. microti* and *M. tuberculosis* Rv cells. Immunoblotting with different sub cellular fractions of *M. microti* further identified 29-kDa Cut5 protein specifically present only in the cell wall fraction. In accordance with blast search results, immunoblotting experiment showed following Cut5 homologs in different mycobacterial species: 23 kDa and 29 kDa proteins in *M. tuberculosis* Rv cell wall; 29-kDa protein in the cell wall preparations of *M. tuberculosis* Ra, *M. smegmatis* and *M. avium subsp avium* and 25-kDa protein in cell walls of *M. bovis* BCG and *M. vaccae*. Limited attempts explored the immunological potential of Cut5B protein. Significant lymphoproliferation of leukocytes leading to IFN- γ and IL-2 production was observed indicating that cutinase Cut5B can be a putative candidate for exploring its protective role in further studies.

Thus, the present study shows for the first time: (i) the presence of mAb C10B5 reactive 19 kDa protein on the mycobacteria and infected cell surface. This molecule is present in *M. tuberculosis* complex and *M. avium*. Further, evidences presented in the study indicate that this protein might be pyrazinamidase and, (ii) the existence of cell wall associated Cut5B and Cut5 in mycobacteria.