Summary & Conclusions
SUMMARY AND CONCLUSION

The infection of erythrocytes by malaria parasite significantly alters the physiological functioning and cellular biology of the host cell. These parasites induced physiological alterations of the erythrocyte membrane help the parasite to gain access to the nutrients from extracellular milieu, which are not otherwise taken up by normal erythrocytes. The exact molecular mechanism by which the malaria parasite alters the host erythrocyte membrane has not been conclusively elucidated. However, these structural and functional modifications of the host cell are not only essential for parasite survival but also responsible for clinical manifestation of the disease. An array of parasite-derived molecules is expressed on infected cell membrane but the functionality of most of these proteins remains elusive. Selective identification of infected cell associated components for the development of vaccine and targeting anti malarial drugs have been a pivotal goal in controlling the disease. In this context, phage display has been used to good effect to investigate host-pathogen interactions in the malaria parasite. Phage display system has been recently exploited not only in mapping the protein–protein interactions that are important in Plasmodium biology, but also towards the identification of parasite molecules that might be exploited in the design of isolate therapeutic agents or vaccines. Using phage display system, the objectives of this study were: identification of structurally/ functionally important conserved epitopes/ mimotopes of MSP-1, RhopH3 and vacuolar ATPase, expressed on infected cell, ii) Selection of peptides specifically reactive to *P. falciparum* infected cell and iii) to check if any of these peptides is capable of inhibiting parasite growth.

Three monoclonal antibodies F10, D2 and C3 were selected, from the panel generated in the lab, and based on their following characteristics: i) all these three MAbs react with the parasite infected cell surface, ii) MAb D2 is anti RhopH3 antibody, while MAb F10 and C3 recognize high molecular weight forms of MSP-1(MSP-1230, MSP-195, MSP-1156), iii) MAbs F10 and C3 are taken up by the parasite and infected cell and, iv) among these MAbs, C3 is *P. falciparum* growth inhibitory. Phage peptides reactive to these antibodies were selected in order to find respective epitopes and mimotopes.

Three rounds of panning on MAbF10 and D2 with 12 mer phage display peptide library resulted in enrichment of antibody specific phage peptides as indicated by increase in titer.
with successive rounds of panning. Number of different sequences representing peptide mimics of the original epitope were obtained for each of the antibody. In case of MAbF10, one of the peptide F10p11 (SHRLPRMAEGPS) was present in many of phage clones while most of the phage peptides selected for MAbD2 shared two motifs, EF/Y(GR)PW and TWWP. Inspite of difference in amino acid sequences, all phage peptides bound specifically to respective antibody. Amino acid composition analysis of peptide mimics revealed that Leu, Pro and Ser were present in higher frequency. BLAST search of F10p4 (LYPLSNLESLP) using revealed ~70% homology to MSP1 of P.bergherie while in case of MAbD2; D2p1 (YLGPLEDTNLGY) showed ~60% sequence homology to RhopH3.

F10p4, when aligned with MSP1 of P.bergherie using clustalX alignment software, clustered at 24-45 a.ac region in N-terminus of the protein. While, D2p1 aligned in 670-692 a.ac region in RhopH3 of P.bergherie, indicating that the epitopes of MAbs F10 and D2 might be present in N and C terminus of their corresponding proteins, MSP-1 and RhopH3 respectively. While, F10p11, the highly frequent peptide was aligning in 450-480 a.ac region of MSP-1 (MSP-1\textsubscript{450-480}). Custom synthesized phage peptides F10p4, F10p11 and F10p4 aligned regions of MSP-1 i.e. MSP-1\textsubscript{25-40} were able to inhibit the binding of MAbF10 with P.bergherie extract in dose dependent manner with maximum inhibition was seen at peptide concentration of 800μg/ml. While F10p11 aligning region, MSP-1\textsubscript{450-480} and another region, MSP-1\textsubscript{900-915} (used as control) did not have any effect on binding of MAbF10. This result was further substantiated by Biacore results which showed increase in level of binding of peptide MSP-1\textsubscript{24-45} with immobilized MAbF10 (K\textsubscript{D} value of 8nM), whereas no such binding could be observed in case of MSP-1\textsubscript{450-480} aligned with F10p11. Similar inhibition assay in case of MAbD2 revealed that D2p1 and RhopH3 (670-692) were able to inhibit the binding of MAbD2 with RhopH3 in the parasite extract. Thus, these results identified epitopes as well as mimics i.e. mimotopes on MSP-1 & RhopH3.

Phage peptides (mimotopes) reactive to MAbs F10 & C3 and displaying different levels of antigenicity were checked for their immunogenic potential. Following results were obtained:

1) Anti-phage peptide antisera raised against phage peptides specific to MAbF10 (F10p4, F10p7, F10p8 and F10p11) and MAbC3 (C3p1, C3p5 and C3p10) reacted well with their corresponding phage peptides. However, in case of F10p5 and C3p7 no peptide specific antibodies were generated.
2) Antisera raised against phage peptides F1op4, F1op11, C3p1, C3p5 and C3p10 exhibited similar pattern of reactivity, like MAbs F10 & C3, with affinity purified MSP-1230, MSP-1195 and MSP-1156 of *P.berghei* in western blotting.

3) Antisera of mimotopes F1op4, F1op11 and C3p1, C3p5 and C3p10 reacted well with *P.berghei* extract in ELISA. Further, MAbC3 mimotopes antisera (C3p1, C3p5 and C3p10) also reacted with *P.falciparum* extract, thus further validating conserved nature of epitope.

4) Confocal microscopy revealed that the antisera of MAbF10 reactive mimotopes (F1op4, F1op11) and MAbC3 reactive mimotopes (C3p1, C3p5 and C3p10) recognized *P.berghei* and *P.falciparum* infected cell surface respectively, thus further supporting the earlier lab observations indicating the presence of MSP-1230, MSP-1195 and MSP-1156 on infected cell surface.

5) Like original MAbC3, anti-mimotope C3p1 antisera exhibited 50% growth inhibition in *in vitro* culture thereby mimicking the functional epitope of MAbC3.

Above results conclusively demonstrated that the antigenic as well as immunogenic mimics of the epitope recognized by MAbF10 were phage peptides F1op4 and F1op11, while phage peptides C3p1, C3p5 and C3p10 were the mimics of the epitope recognized by MAbC3.

Panning of phage display peptide library onto *P.falciparum* infected erythrocytes (percoll enriched late trophozoites/schizont stage) with alternate panning with normal erythrocytes resulted in successive enrichment of phage peptides reactive to parasite infected cell. The reactivity of 2nd and 3rd amplified phage eluates successively increased for IRBCs with a concomitant decrease in the level of binding with NRBCs. Number of different sequences of IRBC reactive phage peptides were obtained out of which six peptide sequences (I3a3, I3a4, I3a7, I3a9, I3p8 and I3p17), representing multiple phage clones, were selected for checking their binding and cross reactivity with *P.falciparum* infected erythrocytes.

Binding characterization by whole cell ELISA and fluorescence microscopy revealed that these selected phage peptides (I3a3, I3a4, I3a7, I3a9, I3p8 and I3p17) specifically react with molecules associated with parasite/infected cell and not with...
NRBC. Inspite of high level of reactivity by I3p17 in whole cell ELISA with *P. falciparum* IRBCs, no fluorescence was seen while good level of fluorescence was observed with the fixed population of IRBCs. Also the repeated decrease in infected cell number after incubation with I3p17 indicated the selective lysis of *P. falciparum* IRBCs. Observed lysis of *P. falciparum* IRBCs by phage peptide I3p17 was stage specific as evident by following results:

1) Hemolytic assay with selected IRBC reactive phage peptides (I3a3, I3a, I3a7, I3a9, I3p8 and I3p17) indicated the lysis of *P. falciparum* IRBCs (52%) only in presence of phage peptide I3p17. Also no lysis of NRBC could be seen in presence of I3p17. The finding was further substantiated by Evan's blue dye exclusion assay which also revealed similar level of lysis.

2) IRBC lysis was found to be temperature (37°C), time and dose dependent. However, up to 50% lysis could be seen at higher dose of phage peptides.

3) Flow cytometric analysis of propidium iodide uptake by IRBC, after treatment with I3P17, showed decrease in cell viability, indicative of lysis.

4) Confocal microscopy revealed that upon treatment of IRBC with I3p17 cells at ring/early trophozoite were intact while those at late trophozoite and schizont got lysed. I3p17 free peptide behaved similarly, and caused around 35% lysis of infected cells. The peptide also inhibited the growth of *P. falciparum* in *in vitro* culture.

Immunofluorescence assay with *P. berghei* and *P. falciparum* infected cells revealed that I3p17 only reacted with fixed *P. falciparum* IRBC, without any reactivity with fixed or unfixed *P. berghei* IRBC. On the other hand I3a4 and I3a7 reacted with *P. berghei* and *P. falciparum* IRBCs. Further, the components of *P. berghei* and *P. falciparum* by immunoblotting identified were as follows 40, 80 & 83kDa of *P. falciparum* by I3p17; 30kDa of *P. berghei* and 75& 80kDa with *P. falciparum* with I3a4; 40kDa in *P. berghei* and 42 & 75kDa of *P. falciparum*.

Subunit B of V-H⁺ ATPase was selected as target molecule based on its presence on infected cell surface and function. Sequence analysis revealed that it is conserved in different plasmodium species. Computational analysis of *P. falciparum* V-H⁺ ATPase, subunit B (494 residue) for conserved domains showed the presence of walker A and B
motifs, characteristic of nucleotide binding proteins. Overlap extension PCR resulted in amplification of full length subunit B of Vacuolar H^+ ATPase. Over expression of full length subunit B of V-H^+ ATPase (rB subunit), at expected molecular weight of 56kDa, was observed in BL21 codon plus strain of E.coli. Heat shock strategy resulted in soluble protein in BL21 codon plus cells and this was purified by Ni-NTA chromatography.

Antiserum, generated against rB-Subunit, exhibited good reactivity with purified recombinant protein as well as native V-H^+ ATPase in ELISA. Further, antiserum recognized ~56 kDa subunit B of V-H^+ ATPase protein in both P. falciparum and P. berghei, further validating the conserved nature of the protein. Confocal microscopy studies with anti-rB-subunit antiserum showed the presence of V-H^+ ATPase on infected erythrocyte membrane in P.falciparum and for the first time in P.berghei.

The presence of ATPase activity in subunit B was indicated by the presence of walker motif. Colorimetric assay based on measurement of released Pi suggested that rB-subunit protein possessed ATPase activity. The activity was observed in the presence of various divalent metal ions with maximal activity in presence of magnesium ions. Drastic reduction in the activity was observed in absence of magnesium ions and NH4Cl.

Screening of random peptide phage library on rB-subunit of V-H^+ ATPase resulted in the selection of different peptides and phage ATPase10 was present in maximum frequency. The phage peptides showed good levels of binding with both rB-subunit of V-H^+ ATPase and native P.falciparum V-H^+ ATPase, with maximal reactivity by ATPase10. Immunofluorescence studies showed the binding of ATPase1, ATPase3, ATPase10 and ATPase13 phage peptides with P.falciparum IRBCs. Among these peptides ATPase3 and ATPase13 resulted in inhibition of rB-subunit ATPase activity. Interestingly, significant in vitro growth inhibition was seen with peptides ATPase10 (60%) and ATPase13 (77%) indicating that these peptides targeted the functional regions of the protein.

Thus, important findings emanating from this study are:

i) Number of phage peptides screened for MSP-1 reactive MAbs F10 and C3 behave as antigenic as well as immunogenic mimics of native antigen.

ii) MAbC3 reactive C3p1 mimotope induced MAbC3 like antibodies, original, which inhibited in vitro growth of P.falciparum. This mimotope can be exploited as target for the development of anti-malaria peptide vaccine.
iii) The peptide I3p17 selectively lysed trophozoite/schizont stages of *P. falciparum* infected erythrocytes, and lead to decreased survival of the parasite.