RESULTS

Identification and functional characterization of infected cell associated molecules in malaria is central not only to understand the basic mechanism involved in parasite survival and growth but also in selection of targets for controlling the disease. In recent years, a plethora of target antigens have been identified from Plasmodium genome using advanced proteomics technologies in combination with bioinformatics tools (Florens et al., 2004; Hiller et al., 2004). However, due to high complexity, only few molecules expressed on infected cell surface e.g. PfEMP-1, stevor, rifins and vacuolar H\textsuperscript{+} ATPase (Deitsch and Hviid, 2004; Kyes et al., 2001; Marchesini et al., 2005; Petter et al., 2007) have been partially characterized. Further, most of these molecules suffer from allelic heterogeneity between strains, antigenic variation within a single strain and high sequence polymorphism at critical target epitopes (Blythe et al., 2004; Craig and Scherf, 2001; Florens et al., 2004).

Phage display has evolved as a major tool to investigate host-pathogen interactions in malaria (Fairlie et al., 2008; Ghosh et al., 2002; McIntosh et al., 2007). Antibody and peptide libraries have been used widely in malaria research for epitope mapping and antibody engineering, as well as in the identification of peptide mimics and protein–protein interactions (Ghosh et al., 2002; Lauterbach et al., 2003; Li et al., 2002). Phage display derived mimotope sequences for functional antibodies and pathological antigens have been used to a good effect for both diagnosis and disease prevention (Beckmann et al., 2005; Fairlie et al., 2008; Greenwood et al., 1991; Mukai et al., 2006; Zhang et al., 2001). This system is contributing rapidly towards the identification and development of novel pharmaceutical entities to combat malaria (Beckmann et al., 2005; Fairlie et al., 2008; Greenwood et al., 1991; Mukai et al., 2006; Zhang et al., 2001).

Earlier studies of the lab demonstrated the occurrence of MSP-1 and RhopH3 not only on parasite membrane but also on infected cell surface (Bhattacharjee, 2003). Among the panels of MAbs generated, against *P.falciparum* and *P.berghei* parasite and respective infected cell membrane (Choudhury et al., 1997; Indu, 1998), the MAbs F10 and D2 were found to be reactive with MSP-1 and RhopH3 respectively. Important features pertaining to the reactivity
of these antibodies were: i) both of these MAbs react with *P. berghei* parasite and infected cell surface (Bhattacharjee, 2003) ii) unlike MAbD2, MAbF10 is specifically taken up by parasite and infected cell *in vivo* and *in vitro* (Bhattacharjee, 2003; Chaudhary, 1996; Owais et al., 1995), iii) MAbF10 recognize three different high molecular weight forms of MSP-1 (MSP<sub>230</sub>, MSP<sub>195</sub>, MSP<sub>156</sub>) while MAbD2 recognize RhopH3 (105 kDa) (Bhattacharjee, 2003). In subsequent studies, another MAbC3 recognizing high molecular weights of MSP-1 (mentioned above) was found to be growth inhibitory (Tiwari, 2008). Taken together these observations and recent reports describing the utility of phage display system, the aims and objectives of this study were three folds: i) dissection of the structurally/functionally important epitope(s) of MSP-1 and RhopH3; ii) selection of phage peptides reactive to *P. falciparum* infected erythrocytes and associated molecules i.e. vacuolar H<sup>+</sup> ATPase (V-H<sup>+</sup> ATPase), and, iii) to check if any of these peptides arrests blocs the growth of intracellular parasite.

### 4.1 Dissection of structurally/functionally important epitopes of MSP-1 and RhopH3

Antibodies specific to MSP-1 have been found to inhibit the invasion of RBC by the parasite, thus affecting its growth in host (Blackman *et al.*, 1994; Dodoo *et al.*, 2008). Secondly, failure to form *rhopH3* disruption mutant have implicated the essentiality of RhopH3 for plasmodium survival (Cowman *et al.*, 2000). In this part of study, phage display system was first used to identify the epitopes and/or mimotopes defining the regions on infected cell associated MSP-1 and RhopH3 recognized by MAbs F10 and D2 respectively. These peptides were then taken to set the platform for checking their immunogenic potential. Attempts were then further extended to identify the mimotopes recognized by growth inhibitory MAbC3, which could eventually be used as vaccine candidate.

#### 4.1.1 Mapping of epitopes reactive to MAbF10 and MAbD2

In order to screen antibody specific phage peptides, MAbs F10 and D2 were coated on ELISA plate for overnight at a concentration of 50μg/ml, so that even low affinity phage peptides could be selected, and incubated with 1x10<sup>11</sup> pfu of 12 mer linear phage display peptide library. After washing, the antibody bound phage peptides were eluted under acidic
conditions. The pan eluate was titered to determine the number of antibody bound phage peptides. 1st eluate was amplified for next round of panning so as to enrich the antibody

![Bar graph](image.png)

(A)

![Peptide sequence alignment](image.png)

(B)

Fig 5: Panning of random 12 mer phage display peptide library onto MAbF10. Panel A: 12 mer random phage display peptide library was panned onto the wells of 96 well plate coated with MAbF10. The bound phages were eluted under acidic conditions, neutralized and amplified for next round of panning. Titer was determined as described in materials and methods. Panel B: clustal X alignment of peptide sequences (mimotopes) obtained after third round of panning on MAbF10 where mimotope names are given on the left of their relative amino acid sequences. Gaps are introduced to maximize the alignment.
Fig 6: Panning of random 12 mer phage display peptide library onto MAbD2. Panel A: 12 mer random phage display peptide library was panned onto the wells of 96 well plate coated with mAbD2. The unbound phages were washed off and antibody bound phages were eluted under acidic conditions, neutralized and amplified for next round of panning. Titer was determined as described in materials and methods. Panel B: clustal X alignment of peptide sequences (mimotopes) obtained after third round of panning on MAbD2 where mimotope names are given on the left of their relative amino acid sequence. Gaps are introduced to maximize the alignment.
specific phage peptides. Two more rounds of panning were performed with increased stringency i.e. decreasing the antibody concentration to 10μg/ml and increasing the tween 20 concentration to 0.5% in second round. While in third round of panning, phage number was decreased to 1x 10^10 so that only specific and high affinity phage peptides could be selected. Panel eluates of both rounds were amplified and titered. As seen in Figs 5 and 6, panel A, there was increase in titer of antibody bound phage peptides with successive rounds of panning. Also, there was enrichment of specific phage peptides for both antibodies after 3rd round of panning. Plaques were randomly picked from the titer plate so that antibody bound phage clones could be characterized.

4.1.2 Sequence analysis of antibody specific phage peptides

Randomly picked plaques were amplified in E.coli and further processed for isolation of DNA followed by its sequencing. The sequences were retrieved by sequence analysis software Chromas and all sequences were aligned by clustal X alignment software to see the identity or similarity among them. In case of MAbF10, out of twenty peptide sequences, eleven were found to be different and one among these, F10p11, was found in ten clones indicating its sequence preference for binding to MAbF10 (Fig 5, panel B). In case of MAbD2 specific phage peptides, sequence analysis and alignment revealed 19 different sequences in 25 sequences selected (Fig 6, panel B). D2p2 was found in 4, D2p3 in 3 and D2p8 in 5 phage clones. Two motifs E/F(Y)GRPW and TWWP were present in most of these sequences.

To find the actual antigenic mimics out of these peptides, homology search was done using Blast available at www.ncbi.nlm.nih.gov. It revealed ~60% homology of peptide FlOp4 (LYPLSNLESLPG) to MSP1 of P.berghei (Fig 7). Homology search for D2p1 (YLGPLEDTNLGY) resulted in ~60% sequence homology to RhopH3 (Fig 8) indicating that these might represent the epitope regions reactive to MAbs FlO and D2 respectively.

When MAbF10 specific phage peptides were aligned with MSP-1 of P.berghei using clustalX alignment software, all the sequences were aligned to 24-45 amino acid region in N-terminus of protein (Fig 9, panel A). Similar alignment was also observed for the peptide
Fig 7: Retrieved hit of BLAST search with mimotope F10p4 sequence. The mimotope F10p4 reactive with MAbF10 is depicted as query while subject indicates the retrieved hit. Identities are represented by single letter code for the respective amino acid. Radical change between query and the subject is shown by a gap.

Fig 8: Retrieved hit of BLAST search with mimotope D2p1 sequence. The mimotope D2p1 reactive with MABD2 is depicted as query while subject indicates the retrieved hit. Identities are represented by single letter code for the respective amino acid. Radical change between query and the subject is shown by a gap.
Fig 9: Sequence alignment of MAbF10 reactive mimotopes with MSP-1. Selected mimotopes obtained through panning on MAbF10 were aligned by clustal X alignment software with MSP-1. Mimotopes aligned in the region 24-45 amino acids in MSP-1 of P.berghei (Panel A). Panels B and C show the individual alignment of mimotope F10p4 and F10p11 respectively with MSP-1. (*) and (:) represent identical and similar amino acids respectively. While, (.) indicates conservative substitutions and gaps are introduced to maximize the alignment.
F10p4, where 11 residues of 12-mer peptide were identical/similar to this region (Fig 9, panel B). LESL motif found to be common in peptide F10p4 and *P. berghei* MSP-1. On the other hand, peptide F10p11 which occurred dominantly, suggesting its preferential binding to MAbF10, aligned with 450-480 amino acid region of the protein (Fig 9, panel C).

In case of MAbD2 specific phage peptides, clustalX alignment with RhopH3 revealed that all sequences aligned to 685-700 amino acid region of *P. berghei* RhopH3 protein (Fig10, panel A). Further, clustalX alignment of peptide D2p1 with RhopH3 protein revealed the presence of eight identical residues in 670-692 amino acid region of RhopH3, indicating that this region might be the epitope of MAbD2 (Fig 10, panel B). Thus, different sequences, obtained for peptides reactive to MAbs F10 and D2, might represent the peptide mimics (mimotopes) of the original epitope, as they share some amino acids among themselves. It is not uncommon to find a number of peptides with different sequences binding to a single MAb that recognizes a discontinuous or conformational epitope (Meloen *et al.*, 2000). These different sequences, although binding to same antibody, may display different levels of antigenicity, which was checked as mentioned below.

### 4.1.3 Antigenicity of MAb specific phage peptides

#### 4.1.3.1 Reactivity of MAb specific phage peptides

As different peptides specific to MAb were obtained so it was pertinent to check their antigenicity in terms of their binding with respective MAbs. The criterion for selecting different peptides for checking their antigenicity was based on the differences in their sequences. For MAbF10, peptides F10p1, 2, 4, 5, 6, 7, 8, 10, 11, 14 and F10p16 (Fig 5, panel B) were selected while in case of D2, D2p1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 13 and p16 (Fig 6, panel B) were taken. First, sandwich ELISA was performed, where antibody was coated at a concentration of 10μg/ml and incubated with different phage peptides in varying number from 1x 10^{12} to 6.25x10^{5}. In case of MAbF10, out of different F10 specific phage peptides, F10p4 showed higher level of reactivity even at a phage number of 3.9x10^{6}, F10p1, p8 and p11
followed by F10p14 and p16 had comparable good levels of binding with the antibody and F10p5, F10p6 followed by F10p2, F10p4 and F10p7 exhibited moderate binding. Control peptide D2p1 (panned against RhopH3 MAb), showed negligible reactivity thus further confirming that the binding is peptide specific (Fig 11).

Fig 10: Sequence alignment of MAbD2 reactive mimotopes with RhopH3. Selected mimotopes obtained through panning on MAbD2 were aligned by clustalX alignment software with RhopH3. Mimotopes aligned in the region 670-692 amino acids in RhopH3 of *P.berghei* (Panel A). While Panel B shows the individual alignment of mimotope D2p1 with RhopH3. (*) and (: ) represent identical and similar amino acids respectively. While (.) indicates conservative substitutions and gaps are introduced to maximize the alignment.
Fig 11: Reactivity pattern of MAbF10 specific phage peptides with MAbF10 in ELISA. The antibody (10μg/ml) coated on 96 well plate was incubated with serial dilutions of different phage peptides (represented by numbers) followed by detection with peroxidase conjugated anti-phage (M13) antibody as described in material and methods. Phage peptide D2p1 (reactive to MAbD2) was taken as negative control.

Fig 12: Reactivity pattern of MAbD2 specific phage peptides with MAbD2 in ELISA. The antibody (10μg/ml) coated on 96 well plate was incubated with serial dilutions of different phage peptides (represented by numbers) followed by detection with peroxidase conjugated anti-phage (M13) antibody as described in material and methods. Phage peptide F10p4 (reactive to MAbF10) was taken as negative control.
Among MAbD2 specific phage peptides, D2p7 followed by D2p6 showed much higher levels of reactivity and D2p2, p5, p8, p9 and p13 have comparable good reactivity with MAbD2, while D2p4 and D2p10 exhibited moderate levels of binding. On the other hand peptides, D2p16 and D2p21 had very low levels of reactivity (Fig 12). Interestingly, peptides having identical motifs behaved similarly in binding with MAbD2 (Fig 6, panel B). Thus, although peptides having different sequences reacted to MAbs F10 and D2, yet these peptides exhibited different levels of antigenicity.

Since each amino acid residue in peptide/protein sequence might contribute towards its antigenicity/immunogenicity (Kulkarni-Kale et al., 2005). So, a limited attempt was made to analyze the abundance of any particular amino acid in these peptides. First, the antigen-antibody crystal structures were retrieved from Protein Data Bank (PDB). PDBsum and Rasmol were used to analyze these complexes and then amino acid composition analysis was done by web server Copid available at www.imtech.res.in/raghava/copid to determine the amino acid residues binding to antigen binding site of antibody. Analysis revealed that mainly, Gly, Pro, Ser/Thr were found to predominate in crystal structures (Table 4.1).

**Table 4.1: Amino acid composition of different mimotopes**

<table>
<thead>
<tr>
<th>Epitopes/ mimotopes</th>
<th>Amino acids</th>
<th>Leu</th>
<th>Pro</th>
<th>His</th>
<th>Ser</th>
<th>Thr</th>
<th>Tyr</th>
<th>Phe</th>
<th>Trp</th>
<th>Glu</th>
<th>Ala</th>
<th>Gly</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAbD2 specific phage peptides</td>
<td>Leu</td>
<td>85.7</td>
<td>100</td>
<td>21.4</td>
<td>78.5</td>
<td>92</td>
<td>64.2</td>
<td>42.8</td>
<td>85.7</td>
<td>78.5</td>
<td>50</td>
<td>14.2</td>
</tr>
<tr>
<td>MA bF10 specific phage peptides</td>
<td>Pro</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>100</td>
<td>30</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>Crystal structures</td>
<td>Ser</td>
<td>51.7</td>
<td>68.9</td>
<td>31.0</td>
<td>58.6</td>
<td>58.6</td>
<td>55.1</td>
<td>31.0</td>
<td>48.2</td>
<td>37.9</td>
<td>41.3</td>
<td>68.9</td>
</tr>
</tbody>
</table>

* Frequency of amino acids with varying percentage in peptides, selected through phage display for MAbD2, MAbF10 and in epitope regions binding to antibody as revealed by X-ray crystallography was determined by Copid available at www.imtech.res.in/raghava/copid.
While amino acid composition analysis of different peptide sequences, binding to these two MAbs F10 and D2, also showed that the residues Pro and Ser/Thr also dominated in majority of these peptide epitopes /mimics obtained by panning (Table 4.1). These results further validated the observed antigenic properties of the selected phage peptides.

### 4.1.3.2 Specificity of the epitope of MAbF10 and MAbD2

Different mimotopes were obtained for each of the monoclonal antibodies F10 and D2 and out of these, F10p4 and D2p1 gave homology to MSP-1 and RhopH3 respectively (Figs 7 and 8). Since in case of MAbF10, majority of peptides aligned in 24-45 amino acid region of MSP-1 while F10p11 aligned in different region 450-480 amino acid of MSP-1. In case of MAbD2, only one region of RhopH3, between 670-692 amino acids was recognized as probable epitope. So, to further confirm the antigenicity and specificity of these identified epitopes/mimotopes, competitive inhibition assay was performed using custom synthesized peptides corresponding to phage peptides as well as their aligning regions in respective proteins. Thus, for MSP-1, MAbF10 reactive phage peptide sequences F10p4, F10p11 and their corresponding MSP-1 aligning regions [MSP-1(24-45) and MSP-1(450-80)] were taken, and MSP-1(900-915) aligning with F10p8 served as control. While for RhopH3, MAbD2 reactive phage peptide sequence D2p1 and its corresponding aligned region [RhopH3(670-692)] were taken. Peptides F10p4 and F10p11 were able to inhibit the binding of antibody with *P.berghei* extract with increase in concentration and up to 80% abolishment of the binding could be seen at the highest peptide concentration of 800 µg/ml (Fig. 13, panel A), suggesting the specificity of peptide F10p4 and F10p11 in terms of binding with antibody. Further, MSP-1(24-45) (24-45 amino acid region in N terminus) also inhibited the binding of antibody, whereas, other regions of MSP-1, MSP-1(450-480) aligning with F10p11 and MSP-1(900-915) aligning with F10p8 did not inhibit the binding of MAbF10 to native antigen. Thus, indicating that MSP-1(24-45) might constitute an epitope of MSP-1 for MAbF10. Similarly, in case of MAbD2, both D2p1 peptide and its corresponding aligning region, RhopH3 (670-692), inhibited the binding of MAbD2 with parasite (Fig 13, panel B).
Fig 13: Competitive inhibition by custom synthesized peptide(s) on binding of MAbs F10 (Panel A) and D2 (Panel B) with *P. berghei* extract. Panel A: MAbF10 (5μg/ml) was pre-incubated with increasing concentrations of peptides (100-80 μg/ml) representing respective MAb binding phage peptides or corresponding protein sequences (as mentioned in boxes). While in Panel B, MAbD2 (5μg/ml) was pre-incubated with increasing concentrations of peptides. The mix was added to *P.berghei* extract coated micrtiter 96 well ELISA plate followed by detection of binding with goat anti-mouse IgG-HRP conjugate as described in materials and methods. D2p1 and F10p4 peptides were used as negative control with MAbs F10 and D2 respectively.
Above results were further substantiated by checking the affinity of both peptide regions of MSP-1 i.e. MSP-1\(_{(24-45)}\) and MSP-1\(_{(450-480)}\) for MAbF10 using biacore. For this, MAbF10 was immobilized on to sensor chip at 300 RU and both peptides were passed as analyte onto the immobilized surface at different concentrations (100 μg/ml to 1 mg/ml). MSP-1\(_{(24-45)}\) showed increase in level of binding with the increase in concentration of the peptide with a \(K_D\) value of 8nM (Fig 14, panel A). However, no such binding could be observed in case of MSP-1\(_{(450-480)}\) aligned with F10p11 (Fig 14, panel B). Thus, above results indicated the epitopes as well as mimotopes (antigenic mimics) of MSP-1 and RhopH3 reactive to MAbF10 and MAbD2 respectively. Immunogenic potential of these phage bearing sequences were further tested as described below.

4.2 Immunogenicity of phage peptides

Earlier studies have reported that phage displayed peptides can induce humoral immune response in animals (Bastien et al, 1997; Meola, 1995). These mimotopes do not necessarily have sequence homology with antigen but may have sufficient conformational homology to induce high affinity antibodies that can bind to mimotopes as well as natural antigen (Collins, 1997). Therefore, focus of this part of study was to check the immunogenic potential of identified mimotopes of antibodies. As described in above section, numbers of mimotopes were identified corresponding to the epitopes, on MSP-1 and RhopH3, recognized by MAbs F10 and D2 respectively. These mimotopes exhibited different levels of antigenicity. In earlier study of the lab mimotopes corresponding to the epitope on MSP-1, recognized by a growth inhibitory antibody MAbC3, were identified (Tiwari, 2008). Since MAbF10 is also anti-MAP-1 antibody, but does not affect the parasite growth. So mimotopes of both these antibodies were selected for checking; a) the difference, if any, in their immunogenicity and, b) suitability of any mimotope for selection as vaccine target, based its capacity to induce protective antibodies. The criteria for selecting the phage peptides for checking their immunogenic potential was based on their levels of antigenicity. In case of MAbF10, F10p4, p5, p7, p8 and p11 were taken (Fig 9) while for MAbC3; C3p1, p5, p7 and p10 mimotopes were selected from a pool of phage peptides reactive to MAbC3 (Tiwari, 2008). Immunization of mice was
done with above selected phage peptides and the antisera collected after second booster, were tested for antibody reactivity and specificity.

Fig 14: Characterization of the binding of peptides to MAbF10 by surface plasmon resonance. The peptides MSP-1(24-45) (Panel A) and MSP-1(450-480) (Panel B) were injected at different concentrations in the range of 100μg/ml to 1 mg/ml over MAbF10 immobilized CM5 sensor chip surface. Data represent resonance units plotted as a function of time. The sensogram was analyzed by Biaevaluation 3.0 (BIAcore) software as described in materials and methods.
4.2.1 Specificity of phage peptide sera

Reactivity of anti-phage peptides antisera was first tested with corresponding phages. This was followed by checking its specificity with purified antigen and parasite. Attempt was also made to localize the binding sites of the antibodies generated, as described below.

4.2.1.1 Reactivity of phage sera with phage peptides

For checking the reactivity of anti-phage peptide antisera with corresponding phage peptides, western blotting was performed. For this, phage peptides were resolved by SDS-PAGE under reducing conditions and transferred on to PVDF membrane followed by incubation with their corresponding sera. Antisera raised against F10p4, F10p7, F10p8 and F10p11 reacted well with 42kDa corresponding to pIII coat protein displaying peptides. While in case of p5, no band appeared at 42kDa indicating that peptide specific immune response was not generated (Fig 15, panel A). Upper and lower bands corresponding to other coat proteins of the phage were also recognized by all antisera including that of F10p5. Similarly, in case of MAbC3 reactive phage peptides, unlike C3p7, antisera could be generated against C3p1, C3p5 and C3p10 phage peptides (Fig 16, panel A). No reactivity was observed at 42kDa with control sera in any of the phage peptide. These sera generated against the peptides were further assayed for their reactivity with native antigens.

4.2.1.2 Reactivity of phage sera with MSP-1 and parasite extract

Since MAbs F10 and C3 recognize 3 different forms of MSP-1; MSP-1\textsubscript{(230)}, MSP-1\textsubscript{(195)} and MSP-1\textsubscript{(156)} (Bhattacharjee, 2003). Therefore, in order to test whether the corresponding phage sera also exhibit similar reactivity, western blotting was performed taking affinity purified MSP-1 of \textit{P.berghei} (Figs 15 and 16, panel B) while reactivity with parasite extract was tested by ELISA (Figs 15 and 16).

Results of western blotting with MSP-1 revealed that like MAbF10, antisera generated against F10p4 and F10p11 reacted well with MSP-1\textsubscript{(230)}, MSP-1\textsubscript{(195)} and MSP-1\textsubscript{(156)} (Figs 15, panel B). However, F10p5, F10p7 and F10p8 antisera did not recognize any of these three
Fig 15: Immunoreactivity of MAbF10 specific phage peptide sera. Panel A: The reactivity of different MAbF10 specific phage peptide sera (at 1:4000 dilution) was tested with corresponding phages F10p4 (lane 1), F10p5 (lane 3), F10p7 (lane 5), F10p8 (lane 7) and F10p11 (lane 9), while C10p1 phage sera (raised against mimotope reactive to anti-M. microti MAb C10B5) was used as negative control (lanes 2, 4, 6, 8 and 10). Panel B shows the reactivity of anti-phage antisera (F10p4, lane 1), (F10p5, lane 2), (F10p7, lane 3), (F10p8, lane 4) and (F10p11, lane 5) with affinity purified MSP-1 from P. berghei. Immunoblotting was performed as described in materials and methods.
Fig 16: Immunoreactivity of MAbC3 specific phage peptide sera. Panel A: The reactivity of different MAbC3 specific phage peptide sera (1:4000 dilution) was tested with corresponding phage peptides antisera C3p1 (lane 1), C3p5 (lane 3), C3p7 (lane 5) and C3p10 (lane 7), while C10p1 phage sera (raised against mimotope reactive to anti-\textit{M. microti} MAbC10B5) was used as negative control (lanes 2, 4, 6, 8). Panel B shows the reactivity of anti-phage antisera (C3p1, lane 1), (C3p5, lane 2), (C3p7, lane 3) and (C3p10, lane 4) with affinity purified MSP-1 from \textit{P. berghei}. Immunoblotting was performed as described in materials and methods.
forms. Similarly, like MAbC3, sera generated against C3p1, C3p5 and C3p10 recognized three different forms of MSP-1 as mentioned above (Fig 16, panel B). The cross reactivity of phage peptide antisera was assayed with native antigen (MSP-1) by ELISA where *P. berghei* extract was coated O/N and incubated with serial dilutions of antisera against phage peptides. Different levels of immunogenicity was displayed by these phage peptide antisera with maximum immunoreactivity of peptide F10p4 specific sera followed by F10p11 specific sera. Antiserum to C10p1 was used as negative control which didn’t show reactivity with *P. berghei* extract indicating the specificity of sera towards MSP-1 (Fig 17). MAbC3 recognize the epitope which is well conserved in both *P. berghei* and *P. falciparum* (Tiwari, 2008). Therefore, antisera for mimotopes of MAbC3 were checked for their reactivity with both *P. berghei* and *P. falciparum* extracts. Results showed higher levels of immunoreactivity of C3p1 antisera followed by that of C3p10 and C3p5 in case of both *P. falciparum* (Fig 18, panel A) and *P. berghei* parasite extract (Fig 18, panel B).

These results showed that the antigenic as well as immunogenic mimics of the epitope recognized by MAbF10 were phage peptides F10p4 and F10p11, while phage peptides C3p1, C3p5 and C3p10 were the mimics of the epitope recognized by MAbC3.

4.2.1.3 Localization of binding sites of phage peptide sera in infected erythrocytes

Confocal microscopy was done to further confirm the specificity of anti-phage peptides antisera by assessing the binding pattern on infected erythrocytes. The cells in suspension were blocked with 1% BSA and then probed with antisera followed by subsequent treatment with goat anti-mouse antibodies conjugated to Alexa594. For co-localization of erythrocyte membrane binding sites, rabbit anti-mouse NRBC membrane antiserum was used which was then subsequently probed with goat anti-rabbit antibodies conjugated to Alexa488. For parasite localization, nuclear staining was done with DAPI. Results showed that, like MAbF10, antisera to phage peptides F10p4 (Fig 19, row II) and F10p11 (Fig 19, row III) stained predominantly intracellular structures as well as surface structures (Fig 19, row I), unlike control antiserum (Fig 19, row IV).
Fig 17: Immunoreactivity of MAbF10 specific phage peptide sera with *P. berghei* extract in ELISA. *P. berghei* extract (10 µg/ml) was coated on 96 well plate, blocked and incubated with different dilutions of MAbF10 specific phage peptide antisera (F10p4, F10p5, F10p7, F10p8 and F10p11), while C10p1 phage sera (raised against mimotope reactive to anti-*M. microti* MAbC10B5) was used as negative control followed by incubation with HRP-labeled secondary antibody. Colour was developed as described in materials and methods.
Fig 18: Immunoreactivity of MAbC3 specific phage peptide sera with *P. falciparum* (Panel A) and *P. berghei* (Panel B) extracts in ELISA. Parasite extracts (10 μg/ml) were coated on 96 well plate, blocked and incubated with different dilutions of MAbC3 specific phage peptide (C3p1, C3p5, C3p7 and C3p10) sera, while C10p1 phage sera (raised against mimotope reactive to anti-*M. microti* MAbC10B5) was used as negative control followed by incubation with HRP-labeled secondary antibody. Colour was developed as described in materials and methods.
Fig 19: Fluorescence micrograph showing the localization of MSP-1 on *P.berghei* infected erythrocytes. Localization of MSP-1 on IRBCs was analyzed by incubating the infected cells with MAbF10 (5μg/ml, row I) and its reactive mimotopes (F10p4; row II), (F10p11; row III) and control phage (C10p1; row IV) antisera (at 1:200 dilution) along with rabbit anti-NRBC membrane antiserum (1:200 dilution). The sera binding was localized by using goat anti-mouse Ig conjugated to Alexa594 (Panel A) and goat anti-rabbit Ig conjugated to Alexa488 (Panel B). Panel C represents phase contrast image of the field corresponding to field B while Panel D shows parasite counterstained by nuclear stain DAPI. Merged image is depicted in Panel E.
Similarly *P. falciparum* infected erythrocytes probed with specific sera to C3p1 (Fig 20, row I), C3p5 (Fig 20, row II) and C3p10 (Fig 20, row III) showed significant fluorescence while control phage antiserum (Fig 20, row IV) did not react. Further, in a mixed population of uninfected and infected cells, no binding of sera could be seen with uninfected normal RBC, indicating that observed fluorescence was specifically due to MSP-1 (Figs 19 and 20, panel E). Further, co-localization of rabbit anti-NRBC membrane antibody binding sites (Figs 19 and 20, panel B) with that of anti-peptide sera binding sites (Figs 19 and 20, panel A) further validated the MSP-1 association with infected cell surface, as observed in our earlier lab studies using MAbs F10 and C3 (Bhattacharjee, 2003; Tiwari, 2008).

### 4.2.2 Effect of mimotope specific sera on in vitro growth of *P. falciparum* parasite

Few phage peptides, specific to anti-MSP-1 reactive MAbs F10 and C3, were found immunogenic and the antisera so generated reacted with MSP-1, parasite extract and infected cell surface. Therefore, it was pertinent to check the growth inhibitory potential of these phage peptides antisera, for which [³H]-Hypoxanthine incorporation assay was performed. *P. falciparum* culture was synchronized by sorbitol treatment, adjusted to 1% parasitaemia and then incubated in presence and absence of test or control antisera at different dilutions (1:10 and 1:50). As shown in Fig 19, MAbF10 mimotopes (F10p4 and F10p11) specific antisera did not show any effect on the *in vitro* growth of *P. falciparum* while sera obtained from mice immunised with the mimotope C3p1 showed 50% growth inhibition of *P. falciparum*, when taken at a dilution of 1:10, unlike, pre-immune serum taken at the same dilution. Thus, among mimotopes of MSP-1, antisera against C3p1 significantly inhibited the *in vitro* growth of *P. falciparum*, like MAbC3, thereby mimicking the functional epitope of MAbC3. Thus in nutshell, this part of study demonstrated following: i) The epitopes of MAbF10 and MAbD2 are localized towards N and C termini of MSP-1 and RhopH3 respectively. 2) Among the mimotopes selected for MSP-1 few exhibited both antigenicity and immunogenicity and 3) One mimotope C3p1 was capable of generating growth inhibitory antibodies.
Fig 20: Fluorescence micrograph showing the localization of MSP-1 on *P. berghei* infected erythrocytes. Localization of MSP-1 on IRBCs was analyzed by incubating the infected cells with MAbC3 reactive mimotopes (C3p1; row I), (C3p5; row II), (C3p10; row III) and control phage (C10p1; row IV) antisera (at 1:200 dilution) along with rabbit anti-NRBC membrane antiserum (1:200 dilution). The sera binding was localized by using goat anti-mouse Ig conjugated to Alexa594 (Panel A) and goat anti-rabbit Ig conjugated to Alexa488 (Panel B). Panel C represents phase contrast image of the field corresponding to field B while Panel D shows parasite counterstained by nuclear stain DAPI. Merged image is depicted in Panel E.
Fig. 21: In vitro *P. falciparum* growth inhibitory potential of mimotope sera: The sera were incubated with synchronized *P. falciparum* in vitro culture and growth inhibition was measured by tritiated hypoxanthine incorporation assay at (1:10 and 1:50 dilutions of sera). EGTA was taken as positive control. Percent growth inhibition was calculated as described in methods. Figure is a representation of three identical experiments.
4.3 Selection and characterization of P. falciparum infected erythrocytes specific phage peptides

Selective identification of malaria infected cell for targeting anti malarial drugs have been a pivotal goal in controlling the disease. Despite the reports of various dramatic changes in the membranes of IRBCs in the last few decades, few antimalarial drugs have been developed through the targeting of such changes. Antibody mediated selective identification of molecules associated with infected cell has been the focus of the lab (Bhattacharjee, 2003; Indu, 1998; Tiwari, 2008). Phage display system has also been recently used not only in mapping the protein–protein interactions that are important in plasmodium biology, but also in the identification of molecules that might be exploited in the design of therapeutic agents or vaccines (Fairlie et al., 2008; Lanzillotti and Coetzer, 2004). Due to specific mapping of immunoreactive regions within a protein, this strategy obviates the often-difficult expression of corresponding full-length recombinant protein by providing defined peptides for testing and formulations of diagnostic preparation (Lanzillotti and Coetzer, 2008; Li et al., 2008).

Scattered attempts have also been made to exploit phage display system to identify: a) small peptide probe for selective delivery of cytotoxic agents to malaria infected RBCs (IRBC) (Eda et al., 2004) and b) mimotopes of IRBC surface molecules by screening a phage display library on pool of sera from malaria-infected individuals (Infected human sera, IRS) recognizing the surface changes of IRBC membrane (Eda and Sherman, 2005). In view of these, the objectives of this section were: i) identification of peptides selectively recognizing infected cell, ii) checking if these peptide reactive components are conserved in different species of plasmodium and, iii) testing if any of these peptide can affect growth of intracellular parasite.

4.3.1 Panning of phage display peptide library on IRBCs

The attempts were made to isolate phage peptides that specifically bind to the surface of IRBCs. For this, phage displayed peptide library expressing random 7 mer peptides,
constrained by disulfide bonding of two cysteine residues (NEB, Beverly, MA), was used. It is assumed that these peptides are stable and tend to participate in structurally biased protein-protein interactions and also their receptor binding affinities often become more favorable (Li and Roller, 2002). Since it has been shown that drastic membrane changes occur on the IRBC surface at the later stages of parasite development in erythrocytes (Brand et al., 2003; Eda and Sherman, 2002), so percoll enriched trophozoite and schizont stages of IRBC were used for the panning of phages. In first round of panning, \(1 \times 10^{11}\) phage peptides from the library were absorbed with \(1 \times 10^7\) normal red blood cells (NRBC) so as to remove NRBC binding phage peptides. The unbound phages were then incubated with equal number of percoll enriched *P. falciparum* infected erythrocytes at high parasitemia (50–90%) followed by elution under acidic conditions. Those were then amplified in *E. coli* for next round of panning. The phages were incubated with NRBCs in every round of panning so as to remove normal cell reactive phage peptides. NRBC and IRBC specific phage eluates were titrated on to LB/IPTG/Xgal plates to determine the number of bound phage peptides. As shown in Fig 22, panel A, there was no significant increase in titer of IRBC bound phages in 3rd round revealing the enrichment of IRBC specific phage peptides. The results showed that, there was increase in ratio of IRBC bound phages to the total phages used with subsequent round of panning, as revealed by the recovery of phages. Neither the recovery of phage after panning nor the binding specificity of phages to IRBCs increased after the third panning (Fig 22, panel B).

4.3.2 Specificity of phage peptide binding to infected cell

To check the specificity of panned phage peptides towards IRBC, first whole cell ELISA was performed. *P. falciparum* infected erythrocytes were incubated with phage peptides of each pan eluate followed by subsequent treatment with mouse- anti M13-HRP conjugate. Equal number of NRBCs was also taken to check the binding of these eluates. The results showed almost similar levels of binding of 1st amplified phage eluate with infected (Fig 23, panel A) as well as normal erythrocytes (Fig 23, panel B), while the reactivity of 2nd and 3rd amplified phage eluates successively increased for IRBCs with a
concomitant decrease in the level of binding with NRBCs, suggesting the enrichment of IRBC specific phage peptides.

![Graph showing changes in phage binding](image)

Fig 22: Panning of 7mer cysteine constrained random phage display peptide library onto *P. falciparum* infected erythrocytes. Panel A: Peptide library was first panned onto suspension of normal red blood cells (NRBCs) and unbound phages were then incubated with *P. falciparum* infected erythrocytes (IRBCs) at 50-90% parasitemia. The bound phages were eluted and amplified for next rounds of panning. Specific enrichment of IRBC reactive phages in comparison to NRBC reactive phages was observed in successive rounds of panning. Panel B represents the recovery of phages from the surface of IRBCs and the ratio of phage peptides bound to IRBCs and NRBCs. Later was calculated by dividing the pfu of phages bound to IRBCs by the pfu of phage bound to NRBCs in each panning.
Fig. 23: Reactivity pattern of *P. falciparum* infected erythrocyte specific phage eluates with IRBCs and NRBCs in whole cell ELISA. *P. falciparum* IRBCs (Panel A) and NRBCs (Panel B) coated 96 well plate was incubated with $1 \times 10^{11}$ pfu of different phage amplified eluates, followed by detection with peroxidase conjugated anti-M13 antibody, as described in materials and methods. The figure is representative of three identical experiments.
Secondly, binding of pan eluates (containing phage peptides) with IRBCs was also checked by flow cytometry. For this, cells were incubated with different pan eluates and binding was analyzed after staining with antibody to phage M13. The analysis of Mean Fluorescence Intensity (MFI) revealed that there was no difference in the levels of binding of 1st eluate of IRBC bound phages (MFI 1086.82; Fig 24, panel A) and NRBC specific amplified phage eluates (MFI 968.55; Fig 24, panel C). However, significantly higher level of binding of 3rd IRBC amplified phage eluate (MFI 1721.61; Fig 24, panel B) as compared to 3rd NRBC amplified phage eluate (MFI 153.24; Fig 24, panel D) indicated the enrichment of IRBC specific phage peptides after three rounds of panning. Control phage peptide did not show any binding to IRBCs suggesting the specificity of binding (MFI 7.52; Fig 24, panel E).

4.3.3 Characterization of IRBC specific phage clones

As there was enrichment of IRBC specific phage peptides after three rounds of panning with IRBCs, individual plaques (~ 61) were randomly picked from 3rd round titer plate for their further characterization. The plaques were amplified for their sequence retrieval and analysis, localization of their binding sites in infected erythrocytes and functional characterization in terms of their effect on survival of *P. falciparum*.

4.3.3.1 Sequence analysis of isolated phage clones

Plaque DNA was isolated and sequenced for their classification. Upon sequence retrieval, different phage peptides were obtained which were then aligned by ClustalX alignment software to see the sequence identity/similarity between them. As shown in Fig 25, out of 61 phage peptides, number of peptides exhibited overlapping sequences, e.g. the sequences I3a3, I3a4 and I3a7 occurred in 4 similar phage clones. Sequences of I3a9 and I3p8 represented 5 similar clones each and I3p17 sequence was found in 7 similar clones (Fig 25). Accordingly, 6 phage peptides; I3a3, I3a4, I3a7, I3a9, I3p8 and I3p17, representing various overlapping groups, were selected for their further characterization.
Fig 24: FACS analysis of binding of IRBC and NRBC specific phage amplified eluates with \textit{P.falciparum} infected RBCs. Infected cells were incubated with IRBC specific 1\textsuperscript{st} amplified phage eluate (Panel A) and 3\textsuperscript{rd} amplified phage eluate (Panel B) or NRBC specific 1\textsuperscript{st} amplified phage eluate (Panel C) and NRBC specific 3\textsuperscript{rd} amplified phage eluate (Panel D), while Panel E shows the binding of control phage peptide with IRBCs. The cells were then treated with mouse-anti-M13 antibody followed by incubation with FITC-conjugated secondary antibody. 10,000 cells were acquired on Cell Quest pro software after live gating on log FSC and SSC parameters.
Fig 25: Sequence analysis of *P. falciparum* infected erythrocyte specific phage peptides. The plaques were randomly picked from 3\(^{rd}\) round panning plate. Their sequences were determined by ABI sequencing kit and analyzed using clustal X alignment software to determine identity/similarity among different sequences. Mimotope names (represented by I (IRBC), 3 (3\(^{rd}\) eluate number) and a 7-19/p1-28 (phage number)) are given on the left of their relative amino acid sequences. Gaps are introduced to maximize the alignment.
4.3.3.2 Binding of selected phage peptides with IRBC

Selected IRBC specific phage peptides (I3a3, I3a4, I3a7, I3a9, I3p8 and I3p17) were checked for their binding and cross reactivity with *P. falciparum* infected erythrocytes and normal erythrocytes. For this, whole cell ELISA and immunofluorescence assay were performed. IRBCs at late trophozoite and schizont stages, enriched by percoll gradient, were taken. For ELISA, phage peptides (1 x 10^{11} to 1 x 10^6 pfu) were incubated with infected as well as normal erythrocytes coated on microtiter 96 well plate followed by subsequent treatment with HRP-conjugated mouse-anti-M13 antibody. Significant difference in binding of selected phage peptides between IRBC (Fig 26, panel A) and NRBC (Fig 26, panel B) was observed. Binding of control phage peptide with IRBC and that of test phage peptides with NRBC was comparable and significantly low as compared to the test phage peptides binding with IRBC.

In immunofluorescence assay, the cells (IRBCs or NRBCs) in suspension were incubated with test phage peptides (1 x 10^{11} pfu) and subsequently probed with mouse-anti-M13 antibody followed by FITC labeled secondary antibody. NRBCs were localized by probing with rabbit anti-mouse normal red blood cell membrane antiserum, while parasite infected RBCs were counterstained with nuclear stain DAPI. Results are shown in Fig 27. Significant fluorescence was observed due to binding of IRBC specific phage peptide I3a3, I3a4, I3a7, I3a9 and I3p8 with IRBCs (Fig 27, panel B, rows I-V) whereas no observed fluorescence on NRBC (Fig 27, panel D, rows I-V) revealed that these selected phage peptides did not react with NRBC indicating their specificity towards IRBC. Inspite of good level of reactivity of I3p17 with fixed IRBCs in whole cell ELISA, no fluorescence could be seen with IRBCs (Fig 27, panel B, row VI). Further, it was observed that incubation of IRBCs and NRBCs with I3p17 phage peptide resulted in decrease in number of IRBC without any effect on NRBC, indicating selective lysis of infected cells. Therefore, for this phage peptide, fluorescence assay was performed with fixed population of IRBCs where significant fluorescence was observed on binding of I3p17 phage peptide with infected erythrocytes (Fig 27, panel F).
Thus, above results showed the IRBC specific binding of selected phage peptides and selective lysis of IRBC by I3p17.

Fig 26: Binding of selected *P. falciparum* IRBC specific phage peptides in whole cell ELISA with infected erythrocytes (Panel A) and normal red blood cells (Panel B). 1X10^5 cells were coated onto 96 well plate followed by incubation with different phage peptides at varying number (1X10^{11} to 1X10^9) at 10 fold serial dilutions and then subsequent detection with mouse-anti-M13 HRP conjugate at 1:5000 dilution, as described in materials and methods. The figure is representative of three identical experiments.
Fig 27: Localization of peptide phage binding sites in *P. falciparum* infected erythrocytes as revealed by immunofluorescence assay. *P. falciparum* IRBCs (Panel A) and NRBCs (Panel C), preincubated with selected phage peptides (I3a3; row I, I3a4; row II, I3a7; row III, I3a9; row IV, I3p8; row V, I3p17; row VI and control phage peptide; row VII, were coated on to the slide, treated with mouse anti-M13 antibody followed by goat anti-mouse Ig Alexa594 conjugate (Panels B and D). The parasite was counterstained with DAPI (Panel A and E) while NRBCs were localized by using rabbit-anti-NRBC membrane antiserum followed by goat anti-rabbit Ig Alexa488 conjugate (Panel C). Panel F represents binding of I3p17 with fixed IRBCs.
4.3.3.2 Cross reactivity of IRBC reactive phage peptides

Recent studies from our lab have suggested the functional importance of conserved domains of target molecules in plasmodium (Kaur, 2008; Tiwari, 2008). Therefore, in order to select the phage peptides reactive to conserved protein/domains on infected cell surface and subsequently to identify these molecules, cross reactivity of selected phage peptides with *P. berghei* IRBCs was checked by immunofluorescence assay. The results showed that among phage peptides 13a3, 13a4, 13a7, 13a9, 13p8 and 13p17, significant fluorescence was observed due to the binding of phage peptides 13a4 and 13a7 with *P. berghei* infected RBCs (Fig 28, panel B, rows II & III), while no fluorescence could be observed in case of 13p17 (Fig 28, panel B, row VI). Also, when fluorescence assay was done with fixed *P. berghei* IRBCs, no binding was observed (Fig 28, panel D), suggesting: i) the reactivity of 13a4 and 13a7 with region/epitope conserved in *P. falciparum* and *P. berghei* infected RBCs and, ii) specific lysis of *P. falciparum* IRBCs unlike *P. berghei* IRBC, by phage peptide 13p17.

To identify the components of infected cell surface reactive to different phage peptides western blotting was performed. *P. falciparum* and *P. berghei* extracts were run on SDS-PAGE followed by immunoblotting with different phage peptides. 13p17 phage peptide recognized bands at 80 kDa and 83 kDa and a broad band above 40 kDa in *P. falciparum* extract (Fig 29, panel A) and not in *P. berghei* extract (Fig 29, panel C) or normal red blood cell extract (Fig 29, panel B), further substantiating the above observation that 13p17 recognition of *P. falciparum* IRBC is specific. Further, the reactivity pattern of 13a4 was different in both species as it recognised two bands at 75kDa and 80kDa in *P. falciparum* and two proteins at 30 and 40 kDa position in *P. berghei*. 13a7 reactivity showed a major band at 42 kDa and a minor band at 75 kDa while, in *P. berghei*, smear pattern of reactivity was observed with a clear band at 30 kDa. Neither of the phage peptide reacted with NRBC extract indicating that recognition is IRBC specific. Thus, 13a3 and 13a4 recognised different protein components in both *P. berghei* and *P. falciparum* while 13p17 reacted with *P. falciparum* only.

Although studies identified the conserved components recognised by phage peptides yet the detailed analysis of these molecules could not be carried out because of paucity of purified
material. However, growth inhibitory potential of these peptides was checked and has been described in later section.

Fig 28: Localization of peptide phage binding sites in *P. berghei* infected erythrocytes as revealed by immunofluorescence assay. *P. berghei* IRBCs, preincubated with selected phage peptides (I3a3; row I, I3a4; row II, I3a7; row III, I3a9; row IV, I3p8; row V, I3p17; row VI and control phage peptide; row VII, were coated on to the slide, treated with mouse anti-M13 antibody followed by goat anti-mouse Ig Alexa594 conjugate (Panel B). The parasite was counterstained with DAPI (Panel A and C). Panel D shows binding of I3p17 with fixed IRBCs.
Fig 29: Reactivity pattern of IRBC specific phage peptides with IRBC and NRBC extracts by western blotting. The parasite extracts of *P. falciparum* (Panel A), *P. berghei* (Panel C) and human NRBCs extract (Panel B) were run on 10% SDS-PAGE and blotted onto nitrocellulose membrane followed by incubation with selected IRBC specific phage peptides; l3a3(lane 1), l3a4(lane 2), l3a7(lane 3), l3a9(lane 4), l3p8 (lane 5) and l3p17(lane 6) and then binding was detected by HRP conjugated mouse-anti-M13 antibody as described in materials and methods.
4.3.4 Hemolytic potential of IRBC specific phage peptide I3p17

Preliminary experiments as mentioned above indicated the lytic potential of I3p17 phage peptide without any effect on normal red blood cells. Therefore, experiments were performed to further substantiate this observation.

4.3.4.1 Hemolytic assay

The hemolytic assay was performed to check the hemolysis of *P. falciparum* infected erythrocytes by selected phage peptides following the method described earlier (Arvinder, 1994). IRBC (2x10^7 cells) were incubated with I3p17 and other phage peptides at 37°C for 120 minutes and then centrifuged. The supernatant containing released hemoglobin (Hb) was taken for measuring its O.D at 550 nm. The relative percentage lysis was calculated by taking O.D of released Hb in presence of phage peptides, compared to that observed in water (100%). The result showed the lysis of IRBCs (Fig 30, panel A) in presence of I3p17 phage peptide (52%) indicating the hemolytic potential of I3p17 phage peptide in comparison to normal red blood cell where no hemolysis was obtained (Fig 30, panel B). Also, other phage peptides I3a3, I3a4, I3a7, I3a9, I3p8 and control phage peptide did not lyse IRBC, thus indicating the selective lysis due to I3p17 (Fig 30, panel A). The finding was further substantiated by Evan’s blue dye exclusion assay for which the IRBCs were incubated with Evan’s blue and then blue stained cells were counted on light microscope. Results showed almost 55% cells got lysed in presence of phage peptide I3p17, while negligible lysis was observed in case of I3a7 and control phage peptide (Fig 30, panel C).

4.3.4.2 Dose and time dependent lysis of infected erythrocytes

Since above results showed the lysis of 50-55% IRBC in presence of phage peptide I3p17, so experiments were performed to determine the kinetics of lysis in time and dose dependent manner. For this, cells (IRBC, 2x10^7) were incubated with increasing phage number from 1x10^9 pfu to 1x10^13 pfu at 37°C, while taking normal erythrocytes as control. As seen in Fig 31, panel A, % lysis increased with increase in phage number and maximum lysis (50%) was observed at 1x10^{12} pfu, which was significantly higher as compared to that
Fig 30: Hemolytic effect of *P. falciparum* infected erythrocyte reactive phage peptides. Infected erythrocytes (Panel A) as well as normal erythrocytes (Panel B) were incubated with 1X 10^{12} pfu of different IRBC reactive phage peptides at 37°C for 120 minutes. Hemoglobin (Hb) released in the supernatant was measured at O.D of 550 nm, while the pellets obtained after incubating the cells with I3α3 and I3p17 were stained with Evan’s blue to determine the lysed cell population (Panel C). The % hemolysis was calculated as described in materials and methods. The figure is representative of three identical experiments.
observed with NRBC (7% lysis). Surprisingly, ten fold further increase in phage number i.e. 1x10^{13} pfu did not result in increase in IRBC lysis (Fig 31, panel A).

For monitoring time dependent lysis, *P. falciparum* infected RBC (2x10^7) as well as normal RBCs were incubated with 1x10^{12} pfu at 37°C for increasing time intervals (30-150 minutes). % relative lysis increased with time and maximum lysis of around 48% was achieved at 120 minutes (Fig 31, panel B) with negligible effect (5% lysis) on NRBCs.

### 4.3.4.3 Effect of lysis on cell viability

To further see the effect of hemolytic phage peptide on the viability of *P. falciparum* infected erythrocytes, cell viability assay was performed by propidium iodide (PI) staining. Upon cell death, IRBC membrane permeability increases as compared to intact IRBC, thereby, allowing PI to go inside the cell, which is measured as Mean Fluorescence Intensity (MFI) by flow cytometry. 1x10^6 cells were incubated with phage peptide I3p17 for 120 minutes at 4°C as well as 37°C. After incubation with PI at 2μg/ml for 15 minutes at 4°C, the cells were analyzed by flow cytometry. The analysis of (MFI) showed that amount of PI inside the IRBC incubated with I3p17 phage peptide was higher at 37°C (MFI 957.37; Fig 32, panel D) than IRBCs without any phage peptide (MFI 27.38; Fig 32, panel F) or with control phage peptide, I3a7 (MFI 31.85; Fig 32, panel E), indicating the IRBC specific lysis. Secondly, PI was higher in IRBCs incubated with I3p17 phage peptide at 37°C than at 4°C (MFI 15.40; Fig 32, panel A). These results showed a significant decrease in viability of IRBC, upon treatment with I3p17 phage peptide at 37°C, thus indicating lysis.

### 4.3.4.4 Lytic potential of I3p17 phage peptide at different stages of infected erythrocytes

Results presented in above section 4.3.4.1 showed the partial lysis of IRBC by phage peptide I3p17 even at higher number of the phage thus indicating that some specific population of IRBCs was undergoing lysis. Therefore, stage specific binding/lysis of IRBC with I3p17 phage peptide was checked by confocal microscopy. *P. falciparum* infected erythrocytes, taken at ring/early trophozoite and late trophozoite/schizont stages were
incubated with I3p17 phage peptide and control phage peptide as described in section 4.3.3.2.

Fig 31: Effect of phage number and time on hemolysis of *P.falciparum* infected erythrocytes. IRBCs or NRBCs (2x10^7) were incubated with different phage number of I3p17 or different time periods. After incubation, released hemoglobin in the supernatant was measured at 550 nm. **Panel A:** Cells were treated with increasing number of I3p17 phage peptide (1X10^8 to 1X 10^{13} pfu) and incubated at 37°C for 120 minutes. **Panel B** shows the incubation of cells (2x10^7) with 1X 10^{12} pfu for different time intervals (0-150 minutes) at 37°C. Hemoglobin in the supernatant was measured at 550 nm and % hemolysis was calculated as described in materials and methods. The figure is the representation of three identical experiments.
Fig 32: Effect of I3p17 phage peptide on the viability of *P.falciparum* infected erythrocytes as analysed by propidium iodide (PI) staining. 1x10^6 cells were incubated with phage peptides; I3p17 (Panels A and D) or I3a7 (Panels B and E) at either 4°C (Panels A and B) or 37°C (Panels D and E) for 120 minutes. Panels C and F show the cells without any treatment with phage peptide at 4°C and 37°C respectively. After incubation, cells were treated with PI (2 µg/ml) for 15 minutes and then acquired on Cell Quest pro software for flow cytometric analysis. The figure represents two identical experiments.
For co-localization of RBC membrane and phage peptide binding, rabbit anti-mouse normal red blood cell membrane antiserum, was used while parasite infected RBCs were counterstained with nuclear stain DAPI. After incubation, cell associated fluorescence pattern was monitored in order to assess the cell lysis. Results showed that in case of I3p17, cells at ring/early trophozoite were intact indicated by RBC membrane fluorescence (Fig 33, panel B, row II), which got merged with the fluorescence due to binding of phage peptide on RBC membrane (Fig 33, panel A, row II). While cells at late trophozoite and schizont got lysed, as no fluorescence due to membrane and phage binding could be seen (Fig 33, panels A & B, row I). On the other hand, significant fluorescence was observed due to binding of non lytic, but IRBC binding phage peptide, I3a7 with both early ring/trophozoite (Fig 33, panels A & B, row IV) and late trophozoite/schizont stage IRBCs (Fig 33, panels A & B, row III). These results indicated that late trophozoites/schizont stage IRBCs were susceptible for lysis by phage peptide I3p17.

4.3.5 Effect of synthetic peptide on IRBC

After establishing the hemolytic potential of I3p17 phage peptide, lysis of IRBCs was checked using free peptide (7 mer cyclic peptide) corresponding to I3p17 phage. IRBCs and NRBCs (2x10^7) were incubated with two concentrations of peptide (200μg/ml and 1mg/ml) at 37°C for 120 minutes, followed by measurement of released hemoglobin at 550 nm. Results presented in Fig 34, panel A shows around 35% lysis of IRBCs at 1 mg/ml concentration of the free peptide, whereas no such effect was observed on NRBCs (Fig 34, panel B). Thus, like phage I3p17, its corresponding free peptide also exhibited IRBC lysis.

4.3.6 Effect of selected IRBC specific peptides on survival of *P.falciparum*

After establishing the specific binding of selected IRBC reactive phage peptides, their effect on *in vitro* parasite growth was checked. The growth of *P.falciparum* was first checked in presence of free peptides corresponding to cell surface reactive phages i.e. I3a3, I3a4, I3a7, I3a9, I3p8 and I3p17.
Fig 33: Binding of lytic phage peptide I3p17 with different stages of *P.falciparum* infected erythrocytes. Either ring/early trophozoites (rows II and IV) or late trophozoites/schizonts (rows I and III) were incubated with phage peptide I3p17 (rows I and II) or I3a7 (rows III and IV) for 120 minutes at 37°C, followed by treatment with mouse anti-M13 antibody. The phage peptide binding and NRBC membrane antiserum binding were co-localized by using goat anti-mouse Ig Alexa594 conjugate (Panel A) or goat anti-rabbit Ig Alexa488 conjugate (Panel B) respectively at 1:600 dilution. Panel D represents phase contrast image of the field corresponding to field B. Panel C shows parasite counterstained by DAPI, while merged image is depicted in Panel E.
Fig 34: Hemolytic potential of free peptide corresponding to phage l3p17. IRBC (Panel A) or NRBC (Panel B) were incubated with test or control peptides at two different concentrations and released hemoglobin was measured at 550 nm. % hemolysis was calculated as described in materials and methods. The figure represents two identical experiments.
This was followed by testing growth inhibitory peptide at different doses Hypoxanthine incorporation assay was done as previously described. Synchronized *P. falciparum* parasite was cultured at different parasitemia (1-5%) in the absence or presence of different peptides. Only free peptide I3p17 was able to inhibit the growth of *P. falciparum* in *in vitro* culture at a concentration of 1 mg/ml by 38% (Fig 35) and none of the other peptides showed any effect on survival of parasite. Further, the percent growth inhibition by I3p17 peptide was found to increase with increasing parasitaemia and reached to 55% at peptide concentration of 1mg/ml (Fig 36) without any effect of control peptide at higher concentrations. Thus, I3p17 phage peptide which bound to *P. falciparum* infected erythrocytes caused the selective lysis of schizont stage infected RBCs and lead to decrease in survival of parasite as shown by growth inhibition assay.
Fig. 35: In vitro growth inhibitory potential of *P.falciparum* infected erythrocyte reactive free peptides. *In vitro* culture of *P.falciparum* was synchronized at 1% parasitaemia and the peptides were added to the culture at a concentration of 1 mg/ml. Growth inhibition was measured by tritiated hypoxanthine incorporation assay. 5mM EGTA was taken as positive control and percent growth inhibition was calculated as described in materials and methods.

Fig 36: In vitro growth inhibitory potential of I3p17 free peptide at different concentrations and parasitemia. *In vitro* culture of *P.falciparum* was synchronized at two different parasitemia 2% and 5%. The lytic I3p17 free peptide and control I3a7 free peptides were added to the culture at different concentrations (50 μg/ml to 1mg/ml) and growth inhibition was measured by tritiated hypoxanthine incorporation assay. EGTA was taken as positive control and percent growth inhibition was calculated as described in materials and methods.
4.4 Functional dissection of malaria infected cell surface associated target molecule

Results from previous sections demonstrated that phage display derived peptides specific to antibodies can be successfully used for generating protective antibodies and also in targeting the parasite derived molecules present on the surface of infected cell. Earlier reports in the literature have demonstrated that high-affinity ligands for protein targets could be selected from phage display peptide libraries (Cwirla et al., 1990), and more recent investigations have identified peptides capable of disrupting protein contacts by binding to a single preferred protein–protein interaction surface or “hot spot”. Hot spots occupy a small portion of the protein interface and are the energetic focus of the protein–protein interaction (Thanos et al., 2003). Targeting this region with a peptide inhibitor may provide a useful reagent to assist small-molecule drug discovery (Arkin and Wells, 2004; DeLano et al., 2000). Therefore, in view of the broad success of this approach, 7 mer cysteine constrained library was used to select the short cyclic peptide(s) that bound to the functional domain(s) of the target protein thus disrupting their function and then looking for its effect on the survival of parasite so as to reveal the essentiality of protein. In this section, the focus was to select the protein so as to target it by phage display. The selected protein was cloned, over expressed and purified. The purified protein was used to screen its binding peptides from phage display peptide library, followed by their characterization for eventually testing their parasite growth inhibitory potential.

4.4.1 Selection of target protein on malaria infected erythrocytes

An array of plasmodium derived antigens are exported on infected cell membrane (Craig and Scherf, 2001) which could be the targets for vaccine design. Therefore, analysis was done on parasite derived proteins which have been proposed to be present on the surface of infected erythrocytes (as reviewed in Table 2.1). The criteria for selecting a target protein were: a) presence on infected red blood cell membrane, b) conserved nature in different species of plasmodium and, c) its function. Due to high complexity, only few infected cell surface molecules have been characterized, including PfEMP-1, stevor, rifins, and vacuolar H⁺-ATPase (Deitsch and Hviid, 2004; Kyes et al., 2001; Marchesini et al., 2005; Petter et al., 2007). Most of these molecules such as PfEMP-1, rifins, stevors etc. are present on infected
cell membrane with well known function but suffer from antigenic diversity/polymorphism or clonal antigenic variations (Hisaeda et al., 2004) which cause great hindrance to target them. Accordingly, other proteins e.g. Exp-2 (Export 2 protein), PIESP-1, PIESP-2, V-H⁺ ATPase present on infected cell membrane were selected for their sequence analysis by Blast homology available at www.ncbi.nlm.nih.gov. As shown in Table 4.2, no conservation of Exp-2 and PIESP-2 was found in different species of plasmodium as indicated by differences in E-value. While PIESP-1 was relatively conserved but its function is not yet known. On the other hand, presence of V-H⁺ ATPase protein in different species of plasmodium (P.falciparum, P.berghei, P.yoelii, P.chabaudi and P.vivax) with closer E-values and >97% identity revealed its functional significance in the parasite. Besides, it is the first functional enzyme reported on infected cell membrane (Marchesini et al., 2005). It is rather intriguing that after its discovery, nothing much has been done as regards to its functional importance in terms of parasite survival. Accordingly, V-H⁺ ATPase of P.falciparum was selected as possible target for its characterization and development of peptide based parasite growth inhibitor(s) and detailed analysis.

4.4.2 V-H⁺ ATPase as target protein

The role of V-H⁺ ATPase has been implicated in the maintenance of the intracellular pH (pHi) of infected erythrocyte important for maximum uptake of metabolites into infected erythrocytes. Localization studies using antiserum to B subunit of V-H⁺ ATPase has revealed its presence on surface of infected cell membrane (Marchesini et al., 2005). No such ATPase is present on NRBC membrane. Recently, there has been a growing evidence for the existence of B subunit diversity among V-ATPases and it has been reported that these are expressed differently in organelles and tissues (Nishi and Forgac, 2002; Sun-Wada et al., 2003; Toyomura et al., 2003; Ueda et al., 2003). Moreover, functional properties vary among various vacuolar H⁺ ATPases. Different cells and tissue specific isoforms of B subunit of V-H⁺ ATPase make them a suitable drug target. Sequence comparison of V-H⁺ ATPase from different species of plasmodium by using clustal X showed that this protein is conserved in rodent parasite e.g. P.berghei, P.yoelii as well as in human malaria parasite e.g. P.vivax, P.falciparum (Fig 37).
<table>
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*Gi represents accession no of the protein and E-value shows the significance of homology*
Fig 37: Multiple sequence alignment of vacuolar-H\(^+\) ATPase subunit B protein from different species of plasmodium, illustrating the degree of identity/similarity. ClustalX (1.81) multiple sequence alignment of subunit B protein sequences of P.falciparum (Pf), P.vivax (Pv), P.berghei (Pb), P.yoelii (Py) and P.chabaudi (Py). Identical amino acids are represented by asterisk (*), conserved residues by colon (:) and semi conserved residues by dot (.). The red coloured box I represents the Walker A motif homologous position and box II represent walker B motif.
The conserved blocks composed of long stretches of sequences are clustered throughout except in initial N-terminal region of 1-22 amino acids where replacement by similar amino acids and conservative substitutions were found. V-H\(^+\) ATPase sequence showed that five cysteine residues are conserved in all plasmodium species while one more cysteine is present in B subunit of \textit{P.vivax} and \textit{P.falciparum} ATPase. Bioinformatics analysis revealed the presence of transmembrane domain in N terminal region (a.a 9-27), one of the prerequisite for membrane association in general. Further, to assess the overall structural conservation of B subunit, the putative conserved domains were searched using NCBI Conserved Domain Search. (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The sequence showed the presence of walker A homologous position and walker B motif, the characteristics of nucleotide binding proteins.

Next, B subunit was cloned and overexpressed to screen the peptides specific to it. Simultaneously, attempts were also made to check if this subunit, because of presence of walker motif, exhibits any ATPase activity. If yes, can that be exploited for development of specific inhibitors eventually leading to the control of parasitemia?

4.4.3 Cloning and expression of B subunit of V-H\(^+\) ATPase

\textit{P. falciparum} V-H\(^+\) ATPase subunit B encoding gene is 1485 nucleotides which comprise of 2 exons of 1-109 base pairs (0.109 Kb) and 585-1960 base pairs (1.375Kb) with one middle intron of 110-584 base pairs (0.474 Kb). Overlap extension PCR strategy was followed to clone full length sub unit B of V-H\(^+\) ATPase (1.485 Kb) from \textit{P. falciparum} genomic DNA. Based on the database of \textit{P. falciparum} genomic sequence at NCBI (www.ncbi.nlm.nih.gov), primers were designed for first fragment (1-109 base pairs) and second fragment (585-1960 base pairs) with the overlap in reverse primer of first fragment and forward primer of 2\(^{nd}\) fragment as mentioned in section 3.1.4 (Table 3.3). Forward primer with Nhe1 and reverse primer with Xho1 were designed. The two gene fragments of 0.109 Kb (Fig 38, panel A, lane 2) and 1.38 Kb (Fig 38, panel A, lane 3) respectively were amplified from \textit{P. falciparum} genomic DNA using PCR and then both PCR products were used as template for third PCR resulting in amplification of full length sub- unit B of V-H\(^+\) ATPase gene (Fig 38, panel A, lane 4). The amplified PCR product was then cloned in pET28a with N terminal his tag (Fig 38, panel B,
Fig 3.8: PCR amplification and cloning of *P. falciparum* subunit B of V-H⁺ ATPase gene with N terminal his tag. The gene was amplified from genomic DNA of *P. falciparum* by overlap extension PCR strategy. Panel A shows PCR amplified 1st (lane 2), 2nd (lane 3) and full length (lane 4) gene fragment of 0.109 Kb, 1.38 Kb and 1.484 Kb corresponding to first exon, second exon and full length coding sequence respectively. Panel B shows double digestion of pET28a positive clone releasing subunit B gene insert of 1.48 Kbp. Lane 1 in Panels A and B shows 2 log DNA ladder.
lane 2) and the nucleic acid sequence was determined using an automated sequencer to check the authenticity of the cloned gene.

The cloned fragment was transformed into *E. coli* BL21(DE) competent cells and expression was checked at 25°C. SDS-PAGE analyses of recombinant vector having B subunit construct revealed no expression of protein of 56kDa in cells with or without IPTG induction at any of the temperatures 37°C, 25°C and 16°C (Fig 39, panel A). Considering codon biasness of *P. falciparum* genome and different codon preference as compared to *E. coli*, expression was checked in Rosetta and BL21 codon plus at different temperatures which showed the over expression of protein corresponding to molecular weight of 56kDa in BL21 codon plus strain at 25°C (Fig 39, panel B, lane 4) and at all three temperatures in rosetta strain of *E. coli* (Fig 39, panel C, lanes 3-5). The expressed recombinant protein (rB subunit) was checked for presence of histidine tag in western blotting by anti-histidine antibody which reacted with a protein of 56kDa in rosetta and BL21 codon plus (Fig 39, panel D, lanes 1 and 2) while no reactivity in control cell lysate (Fig 39, panel D, lane 3) was observed.

4.4.4 Purification of recombinant subunit B of V-H+ ATPase

Expression of recombinant subunit B (rB subunit) of V-H+ ATPase protein was then checked in soluble and pellet fractions of cell lysate. Sub cellular fraction analysis of recombinant protein on SDS-PAGE revealed that most of the protein was in inclusion bodies in Rosetta and BL21 codon plus strains of *E. coli* (Fig 40, panel A, lanes 1 and 3). In order to have protein in soluble form, heat shock was given to the culture so as to activate the chaperons which will facilitate the refolding of the protein. For this, culture (~ 0.5 O.D) was incubated at 42°C followed by induction at temperatures 25°C and 37°C. Western blot with mouse anti-his antibody showed the presence of protein in soluble fraction of BL21 codon plus at 25°C (Fig 40, panel B, lane 2) and 37°C (Fig 40, panel B, lane 4) although in lesser amount, but no protein was observed in soluble fraction of rosetta strain (Fig 40, panel B, lanes 3, 5 and 7). Accordingly, the protein was purified from soluble fraction of transformed BL21 codon plus by Ni-NTA chromatography. Initially, SDS-PAGE analysis showed the presence of most of the protein in the flow through indicating no binding of protein to column.
This could be due to buried histidine tag of the protein. Sequence analysis of subunit B of V-H\(^{+}\) ATPase revealed that N terminal sequence has transmembrane domain which could be the

![Expression of rB subunit of P.falciparum V-H\(^{+}\) ATPase.](image)

**Fig 39:** Expression of rB subunit of *P.falciparum* V-H\(^{+}\) ATPase. SDS-PAGE analysis of rB subunit of V-H\(^{+}\) ATPase in BL21 DE3 (Panel A), BL21 codon plus (Panel B) and Rosetta strain (Panel C) of *E.coli* at 16\(^{\circ}\)C (lanes 3), 25\(^{\circ}\)C (lanes 4) and 37\(^{\circ}\)C (lane 5) with IPTG induction and without IPTG induction (lanes 2). Lane1 in Panels A, B and C shows the vector control (*E.coli* cell lysate). Panel D shows the western blot analysis of *E.coli* cell extract expressing N terminal his tagged recombinant subunit B in Rosetta (lane 1) and BL21 codon plus (lane 2) at 25\(^{\circ}\)C. Lane 3 represents vector (*E.coli* cell lysate) control. Components with their respective molecular weights (in kDa) are indicated.
Fig 40: Purification $V-H^+$ ATPase $\theta B$ subunit protein by Ni-NTA chromatography under native conditions. Panel A: Analysis of solubility of $V-H^+$ ATPase $\theta B$ subunit; SDS-PAGE followed by coomassie staining of $\theta B$ subunit protein induced at $25^0C$ in rosetta (lanes 1 and 2) and BL21 codon plus (lanes 3 and 4) strains of $E.coli$ in the inclusion body (lanes 1 and 3) and soluble fraction (lanes 2 and 4). Panel B: Immunoblotting, using anti-his antibody, of soluble fractions of the $\theta B$ subunit protein after heat shock treatment in both BL21 codon plus (lanes 2, 4 and 6) and rosetta strains (lanes 3, 5 and 7) of $E.coli$ at $25^0C$ (lanes 2 and 3), $37^0C$ (lanes 4 and 5) and $16^0C$ (lanes 6 and 7) while lane 1 shows the reactivity of $E.coli$ cells expressing $\theta B$ subunit with anti-his antibody and used as positive control. Panel C: $\theta B$ subunit of $V-H^+$ ATPase purified by Ni-NTA chromatography, was analyzed on 10% SDS-PAGE; lanes 1 and 2 represents $V-H^+$ ATPase $\theta B$ subunit and control cell lysate while lane 3 shows the purified protein.
reason of hidden nature of histidine tag. Therefore, the purification was carried out in presence of 0.5% triton X 100 in lysis buffer (McBride et al., 2003). The eluate, when checked by SDS-PAGE, showed the presence of purified protein at the expected molecular weight of 56kDa (Fig 40, panel C, lane 3) with a minor band at 30kDa which might be the contamination of *E. coli*. The purified recombinant protein (rB subunit) was then used for further characterization.

### 4.4.5 Specificity of anti-rB subunit antiserum

The sequence analysis of B subunit indicated its conservation in different Plasmodia (Fig 37). Therefore, in order to check whether conserved regions in B subunit constitute epitopes, and thus cross reactivity among different species of malaria parasite, mouse-anti-sera was generated against rB subunit. This antiserum (at 1:5000 dilution) reacted well with rB subunit in immunoblotting (Fig 41, panel A, lane 1). The anti-his antibody reactivity to rB subunit, at 56kDa served as positive control (Fig. 41, panel A, lane 3). The reactivity of antiserum with recombinant protein was also checked by ELISA, which indicated that a good titer antiserum was generated against rB subunit (Fig 41, panel B).

The antibodies formed against rB subunit recognized *P. falciparum* parasite having native V-H\(^+\) ATPase protein in ELISA, while no reactivity was observed with preimmune mouse serum (Fig 42, panel A). In order to test the antigenicity of conserved regions of B subunit of V-H\(^+\) ATPase in different species of plasmodium, the reactivity of antiserum was checked with other rodent parasite species i.e. *P. berghei*. Interestingly, antiserum specific to rB subunit of *P. falciparum* V-H\(^+\) ATPase also reacted well with *P. berghei* extract (Fig 42, panel B) in ELISA.

Further, the antiserum reacted very well at 56kDa protein (position of B subunit) in immunoblot of *P. falciparum* parasite extract (Fig 43, panel A, lane 1) as well as *P. berghei* extract (Fig 43, panel A, lane 2) while control sera did not show any reactivity (Fig 43, panel A, lanes 3 and 4). These results demonstrated that the antibodies generated against rB subunit of V-H\(^+\) ATPase effectively recognize the native protein in parasite. Further, these antibodies

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**Results**
cross reacted with different species of parasite thus showing that conserved region(s) in rB subunit are antigenic.

![Image of immunoblotting](A)

![Image of ELISA](B)

**Fig 41**: Reactivity of mouse anti- rB subunit antiserum: Panel A: immunoblotting of purified fractions of rB subunit with 1:5000 dilution of mouse anti-r B subunit antiserum (lane 1) and pre immune serum (lane 2), followed by incubation with secondary antibody–HRP conjugate as described in materials and methods, while reactivity with anti-histidine antibody (lane 3) was used as positive control. Panel B: reactivity of anti-rB subunit antiserum with purified rB subunit protein in ELISA. 2 µg/ml of the recombinant protein was coated on 96 well plate and incubated with increasing dilutions of anti-rB subunit antiserum or preimmune serum followed by incubation with peroxidase labeled secondary antibody as described in materials and methods.
Fig 42: Reactivity of anti-rB subunit of V-H\(^+\) ATPase antiserum with *P. falciparum* (Panel A) and *P. berghei* (Panel B) parasite lysate in ELISA. The parasite lysate (10\(\mu\)g/ml) was coated in 96 well plate and incubated with increasing dilutions of anti-r B subunit antiserum followed by incubation with peroxidase labeled secondary antibody as described in materials and methods.
Fig. 43: Specificity of anti-rB subunit antiserum. *P. falciparum* (lanes 1 and 3) and *P. berghei* parasite lysate (lanes 2 and 4) were separated by SDS-PAGE under reducing conditions, transferred to nitrocellulose membrane, probed with 1:5000 dilution of anti-rB subunit antiserum (lanes 1 and 2) or preimmune mouse serum at 1:5000 dilution (lanes 3 and 4) followed by incubation with goat anti-mouse HRP conjugate secondary antibody. As described in materials and methods.
4.4.6 Localization of the B sub unit of V-H⁺ ATPase in infected erythrocytes

V-H⁺ ATPase has been reported to be present on the surface of *P. falciparum* infected erythrocytes (Marchesini et al., 2005) but no information is available for other species of plasmodium. Since above results showed the cross reactivity of anti-rBsubunit antiserum with *P. falciparum* and *P. berghei* parasites, therefore it was pertinent to check the localization of V-H⁺ ATPase in *P. berghei* infected erythrocyte surface using this antiserum. First, immunofluorescence assay was performed with *P. falciparum* infected erythrocytes, while co localizing the membrane binding sites with anti-NRBC membrane antibodies. This was then followed by checking the antiserum reactivity with *P. berghei* infected erythrocytes. Results showed the specific binding of antiserum on *P. falciparum* infected cell surface (Fig 44, panel B, row II), which got merged with fluorescence due to anti-NRBC membrane antiserum binding (Fig 44, panel D, row II), indicating the presence of protein on *P. falciparum* infected erythrocyte surface (Fig 44, row I). Binding was also observed on parasite as well as cytoplasm of infected cell. No binding could be seen on normal red blood cell further emphasizing the specificity of antiserum towards plasmodium. Further the pattern of fluorescence due to sera binding was quite similar in *P. berghei* (Fig. 44, row I, panels B and D) indicating its presence on infected cell. Thus, above results suggest that the conserved domain(s) of B subunit of V-H⁺ ATPase are expressed in different species of plasmodium and infected erythrocytes.

4.4.7 Characterization of ATPase activity in B subunit of V-H⁺ ATPase

Since subunit B of V-H⁺ ATPase has the motifs required for activity as described in previous section 4.4.2. So, first the experiments were performed to assess the ATPase activity in rBsubunit of V-H⁺ ATPase, so that it can be exploited for selecting peptide inhibitors. Probable role of subunit B in nucleotide binding at both regulatory and catalytic sites has been demonstrated in Yeast and few residues were identified that contribute to the V" catalytic site (Liu et al., 1996). No such information is available for plasmodium V-H⁺ ATPase. Accordingly, following experiments were done to check the activity in rB subunit of V-H⁺ ATPase of *P. falciparum*. 
Fig 44: Fluorescence micrograph showing the localization of V-H⁺ ATPase in *P. berghei* (row I) and *P. falciparum* infected erythrocytes (row II): Intact cells were treated with 1:500 dilution of mouse anti-rB subunit antiserum and simultaneously with 1:200 dilution of rabbit-anti NRBC membrane antiserum followed by incubation with goat anti-mouse Ig conjugated to Alexa594 (Panel B) and goat anti-mouse Ig conjugated to Alexa488 (Panel A) respectively at 1:600 dilution. For nuclear staining, the parasite was counterstained with DAPI (Panel C). Panel D represents the merged image. Row III represents the binding of preimmune sera with intact *P. berghei* erythrocytes.
4.4.7.1 Activity assay

The activity was determined using a colorimetric assay which is based on measurement of phospho-ammonium molybdate complex formed by released $\text{P}_i$ in the reaction. Assay was carried out at pH 7.4 and $2\text{mM} \text{Mg}^{2+}$ (Choi and Mego, 1988). Results showed the hydrolysis of ATP by B subunit of *P. falciparum* (Fig. 45, panel A) while no activity could be seen after heat inactivation of the protein at $95^\circ\text{C}$ for 15 minutes, indicating that rB subunit exhibited the enzyme activity.

Plasmodium V-H$^+$ ATPase requires ammonium chloride (NH$_4$Cl) for the activity (Choi and Mego, 1988). Therefore, activity of rB subunit was determined in its absence so as to rule out any contamination of *E.coli* associated ATPase. Result showed the abolishment of ATPase activity in absence of NH$_4$Cl while canine kidney V-H$^+$ ATPase (used as positive control) was insensitive to the presence or absence of NH$_4$Cl (Fig 45, panel A) as reported earlier. These results further supported the observation that rB subunit of V-H$^+$ ATPase exhibits plasmodium V-H$^+$ ATPase specific activity.

4.4.7.2 Effect of substrate concentration on the activity

The reaction was carried out with rB subunit at different concentrations of ATP ranging between from $10 \mu\text{M}$ to $10 \text{mM}$ for 40 minutes at $37^\circ\text{C}$. This activity of rB subunit increased with increase in ATP concentration reaching maximum at $2.5\text{mM}$ (Fig 45, panel C). ATPases are well known for their dependence on cofactors such as magnesium, calcium, manganese etc. So the activity of rB subunit was checked in presence of different metal ions at the concentration of $2.5 \text{mM}$. As seen in Fig 45, panel C,

4.4.7.3 Effect of metal ions on the activity

rB subunit exhibited activity, although at varied levels, in presence of different metal ions i.e. Mg$^{2+}$ and Mn$^{2+}$ ions were more potent cofactors e.g. rB subunit activity at $2.5 \text{mM}$ concentration of Mg$^{2+}$ and Mn$^{2+}$ was two fold higher as compared to that seen at same concentration of Ca$^{2+}$. No activity was observed in presence of copper ions (Fig 45, panel C).
Fig 45: ATPase activity in recombinant subunit B of *P. falciparum* vacuolar proton ATPase.

**Panel A**: ATPase activity assay; different amounts of rB subunit or standard ATPase from canine kidney (sATPase) were incubated with ATP and amount of released Pi was determined by malachite green based detection as described in materials and methods. Activity was also checked after heat inactivation at 95°C for 15 minutes or in absence of MgCl₂ and NH₄Cl. **Panel B**: activity at different substrate concentrations and **Panel C**: the effect of different metal ions on the activity of rB subunit protein. The figure is representative of three identical experiments.
Thus, these results further demonstrated that recombinant B subunit (rB subunit) of *P. falciparum* V-H\(^+\) ATPase exhibited ATPase activity. In following section, phage peptides reactive to rB subunit, were selected for subsequent testing their potential towards parasite growth inhibition.

### 4.4.8 Panning of phage display peptide library on to the recombinant protein

Although recent studies have demonstrated importance of V-H\(^+\) ATPase as drug target (Huss and Wieczorek, 2009; Keeling *et al.*, 1997) in cancer and other inflammatory disorders but in case of plasmodium biology, essentiality of this enzyme is still unclear. By panning peptide libraries on the receptors for erythropoietin and thrombopoietin, peptides have been isolated that are able to act as both agonists and antagonists (Cwirla *et al.*, 1997; Wrighton *et al.*, 1996). With this background literature, attempt was made to check whether rB subunit reactive phage peptides could be used for inhibiting parasite growth.

rB subunit was coated onto the 96 well ELISA plate at pH 7.4, and the screening of 7 mer cysteine constrained phage displayed peptide library was done following the same procedure as described in section 4.1. Results presented in Fig 46, panel A, showed the successive enrichment of ATPase specific phage peptides as indicated by increase in phage titer with successive rounds of panning.

### 4.4.9 Sequencing of protein specific phage peptides

The plaques corresponding to protein bound phage peptides were randomly picked from 3\(^{rd}\) pan titer plate, amplified in *E.coli* ER2738 followed by their DNA isolation and then subjected to automated sequencing as described in section 3.2.14.1 Twenty different sequences were obtained which were aligned by clustalX analysis software to see the identity/similarity among them. As seen in Fig 46, panel B, different sequences were obtained and CWSFPGWSC (ATPase10) was presented in majority of peptide sequences. The different sequences obtained were checked for their reactivity with rATPase and the criterion for selecting different peptides was based on the differences in their sequences. Thus, the sequences selected for further characterization were; ATPase1, ATPase2, ATPase3, ATPase4, ATPase5, ATPase6, ATPase7, ATPase10, ATPase13, ATPase18, ATPase19 and ATPase20.
Fig 46: Panning of random 7 mer cysteine constrained phage display peptide library onto rB subunit of V-H⁺ ATPase. Panel A: 7 mer random phage display peptide library was panned onto the wells of 96 well plate coated with rB subunit protein. The unbound phages were washed off and protein specific bound phages were eluted under acidic conditions, neutralized and amplified for next round of panning. Titer was determined as described in materials and methods. Panel B: ClustalX alignment of peptide sequences (mimotopes) obtained after three rounds of panning on rB subunit where mimotope names are given on the left of their relative amino acid sequence. Gaps are introduced to maximize the alignment.
4.4.10 Binding of selected phage peptides with recombinant protein

ELISA was done to check the binding of above selected phage peptides. rB subunit was coated on to the plate and was incubated with serial dilution of different phage peptides (1x10^{11} to 1x10^{4} pfu) followed by subsequent detection with anti-M13 antibody-HRP conjugate. As seen in Fig 47, ATPase2 and ATPase10 phage peptides showed almost similar and higher levels of reactivity with the protein. ATPase3, ATPase19 and ATPase20 followed by ATPase1, ATPase13 and ATPase18 had moderate levels of binding, while ATPase5, ATPase6 and ATPase7 had comparatively much lower levels of binding with the protein whereas no binding was observed with ATPase4 phage peptide. Also, control phage peptide did not react with the protein indicating that binding is peptide specific. Thus, the results indicated that the sequences which were aligning close to each other behaved similarly in terms of binding to recombinant protein.

4.4.11 Binding of selected phage peptides with P.falciparum parasite

Since phage peptides were screened with rB subunit of P.falciparum, therefore binding of these peptides was checked with native V-H^+ ATPase of P.falciparum by ELISA and immunofluorescence assay. ELISA was performed using P.falciparum parasite extract. The results showed that phage peptides also reacted with the parasite in similar pattern (Fig 48) as observed with purified protein (Fig 47). The comparison of reactivity at 2x10^{7} pfu of each phage peptide revealed that ATPase2 and ATPase10 followed by ATPase3, 19 and 20 exhibited comparatively better binding like in ELISA with recombinant protein.

Next, for demonstration of binding of these phage peptides on intact P.falciparum infected erythrocytes, fluorescence microscopy was performed, taking phage peptides ATPASE1, ATPase3, ATPase5, ATPase6, ATPase10 and ATPase13 based on differences in their reactivity behavior in ELISA with rB subunit and P.falciparum extract. As shown in Fig 49, distinct fluorescence was observed in P. falciparum intact IRBCs incubated with phage peptide ATPase1, ATPase3, ATPase10 and ATPase13 while no fluorescence could be seen in other phage peptides ATPase5 and ATPase6 and also with control phage peptide. These
Fig. 47: Reactivity pattern rB subunit of V-H⁺ ATPase panned phage peptides with rB subunit in ELISA. rB subunit of ATPase (2µg/ml) was coated on 96 well plate and incubated with serial dilutions of different phage peptides (represented by numbers) followed by subsequent detection with mouse-anti-M13 HRP conjugate at 1:5000 dilution as described in materials and methods. The figure is representative of three identical experiments.

Fig. 48: Binding of rB subunit specific phage peptides with P.falciparum extract in ELISA. P.falciparum extract (10µg/ml) was coated onto 96 well plate and incubated with serial dilutions of different rB subunit specific phage peptides (represented by numbers) followed by subsequent detection with mouse-anti-M13 HRP conjugate at 1:5000 dilution as described in material and methods. The figure is representative of three identical experiments.
Fig 49: Localization of rB subunit specific phage peptide binding sites in *P.falciparum* infected erythrocytes as revealed by immunofluorescence assay. *P.falciparum* IRBCs, preincubated with selected rB subunit specific phage peptides (ATPase1; row I, ATPase3; row II, ATPase5; row III, ATPase6; row IV, ATPase10; row V, ATPase13; row VI and control phage peptide; row VII were coated on to the slide, treated with mouse anti-M13 antibody followed by goat anti-mouse Ig Alexa594 conjugate at 1:600 dilution (Panel B). The parasite was counterstained with DAPI (Panel A).
results suggested that peptides might bind to different regions of rB subunit thus behaving
differently in their binding with infected erythrocytes. Thus phage peptides reacted with both
recombinant purified B subunit of V-H\textsuperscript{+} ATPase and the parasite.

4.4.12 Functional characterization of selected peptides

Earlier studies from the literature have demonstrated that the ligands identified through
phage display usually target functionally important sites on protein targets (Li et al, 2002). The
peptides which were reactive to rB subunit and infected cell surface, could be useful in
targeting peptide based inhibitors. In order to check this, experiments were first performed to
see the effect of these peptides on the activity of \textit{P.falciparum} rB subunit. Based on above
results, ATPase1, ATPase3, ATPase5, ATPase10 and ATPase13 phage peptides were custom
synthesized and their effect on rATPase activity was checked. This was then followed by
testing their parasite growth inhibitory potential.

4.4.12.1 Effect of selected peptides on the activity of rATPase

The effect of selected peptides was checked on the activity of rB subunit, for which
custom synthesized peptides were added to the purified enzyme, at varying concentrations and
the activity was monitored by measuring phosphoammonium molybdate complex formed by
released Pi in the reaction as described in section 4.4.7.1. As shown in Fig 50, peptides
ATPase3 and ATPase13 were effective in inhibiting the activity of rB subunit and the
inhibition was found dose dependent. Peptide ATPase13 caused abolishment of enzyme
activity by 44\% at the concentration of 250\mu g/ml and almost 86\% inhibition was seen at high
peptide concentration of 2mg/ml. Whereas, partial inhibition was observed by peptide
ATPase3 at the highest concentration of peptide used and control peptide also didn’t affect
enzyme activity, thus indicating that both peptides targeted the functionally important sites.

4.4.12.2 Effect of peptides on the survival of \textit{P.falciparum}

Since peptides screened for rB subunit; ATPase1, ATPase3, ATPase10 and ATPase13
were found to localize on infected erythrocytes and among these peptides, ATPase3 and
ATPase10 showed activity inhibition, therefore it was pertinent to assess the effect of these
peptides on parasite growth in culture. For this, hypoxanthine incorporation assay was done.
Synchronized *P. falciparum* parasite was cultured at 1% parasitemia in the absence or presence of different peptides. Results showed that the addition of peptide ATPase10 and

![Graph showing the effect of rB subunit specific peptides on ATPase activity.](image)

**Fig 50: Effect of rB subunit specific peptides on ATPase activity.** rB subunit protein was incubated with different concentrations of free peptides (250μg/ml to 2mg/ml) corresponding to selected phage peptides (mentioned in the box) for 30 minutes at 37°C followed by addition of substrate. The activity was measured as described in materials and methods.
Fig 51: In vitro growth inhibitory potential of rB subunit specific peptides: In vitro culture of *P.falciparum* was synchronized at 1% parasitemia and peptides were added to the culture at different concentrations (100 µg/ml to 1mg/ml). Growth inhibition was measured by tritiated hypoxanthine incorporation assay. 5mM EGTA was used as positive control while, I3a3 free peptide reactive to IRBC but not growth inhibitory (Fig 35) was used as negative control. Percent growth inhibition was calculated as described in material and methods. Figure is a representation of two identical experiments.
ATPase13 to doubly synchronized cultures of *P. falciparum* strain 3D7 parasites resulted in the inhibition of growth of parasite. 100 μg/ml of peptide ATPase13 resulted in 40% inhibition of parasite growth whereas 1000 μg/ml peptide showed close to 77% inhibition (Fig 51). Other peptide ATPase10 showed 60% inhibition at the concentration of 1000 μg/ml. In contrast, peptides corresponding to ATPase1, ATPase 3 and ATPase 5 did not have any effect.

Thus, number of phage peptides, reactive to rB subunit of V-·H+ ATPase, were selected. Among these, few peptides targeted the functionally important sites i.e. “hot spots” in the protein thus resulting in the disruption of protein function and also blocking parasite survival. Studies are further warranted to test weather these peptides alone or in combination can have any effect in controlling the parasitemia *in vivo*. 