Chapter 6

General discussion

6.1 Summary of results

In this work I have employed a range of techniques using recombinant *Plasmodium* proteins to identify host-pathogen protein-protein interactions occurring in malaria. These have included the use of established methodologies such as AVEXIS and surface plasmon resonance, and the development of flow cytometry and biochemical purification approaches.

High-throughput screening using AVEXIS enabled the identification of an interaction between human SELP and PfMSP7, which I demonstrated in several experimental systems and characterised in detail, identifying the interacting regions of both recombinant proteins. I subsequently tested a range of PfMSP7 homologues and discovered that this SELP-binding property is a conserved feature of multiple members of the MSP7 family, across at least three *Plasmodium* species. Given the known role of SELP in human immunity, I have investigated the hypothesis that MSP7 proteins could have an important immunomodulatory role. The observation that PfMSP7 can block the interaction between SELP and sLe^X, and potentially SELP and leukocytes, *in vitro* provides encouragement that the interaction could modulate the normal *in vivo* interactions between SELP and its sLe^X-containing ligands.

Screening using other approaches did not lead to the identification of any new receptors for *Plasmodium* proteins.

6.2 Implications of SELP/MSP7 interactions

6.2.1 A possible mechanism underlying MSP7-knockout phenotypes

The molecular mechanisms underlying the observed reduction in pathology in PbMSP7-knockout parasites compared to their wild-type counterparts in rodent models are currently unknown. These data regarding the interactions between MSP7 proteins and SELP might contribute to explaining these observations. Gomez *et al.* demonstrated that rodents infected with PbMSP7-knockout parasites were somewhat protected from death and they hypothesised that an immunomodulatory function for PbMSP7 underlay this phenomenon[117]. It is possible that an interaction with Selp that prevents its recruitment and activation of leukocytes (as described in 4.11) could provide a molecular basis for these results.

In addition to the data that suggest an immunomodulatory function for PbMSP7, Spaccapelo *et al.* showed that PbMSP7-knockout parasites induced less cerebral pathology than wild type parasites in experimental cerebral malaria models[313]. This is particularly interesting when combined with the data that show that Selp-knockout mice are completely protected from cerebral malaria[53, 59]. Whilst there may be distinct mechanisms underlying these two observations[1], it is noteworthy that when either binding partner (PbMSP7 or Selp) are lacking from experimental malaria in an ECM-susceptible mouse, cerebral pathology is significantly reduced. In terms of a mechanism by which an interaction between Selp and PbMSP7 could interact to exacerbate ECM symptoms, it is tempting to suggest that PbMSP7 binds to Selp and prevents the adhesion of leukocytes in the mouse brain. However, leukocyte adhesion in the brain is not thought to be affected in infected Selp-knockout mice, which makes this mechanism less likely[53].

Endothelial SELP is thought to preferentially recruit Th1 cells which are associated with the production of pro-inflammatory cytokines, including TNF, IFNγ and lymphotoxin[10, 30], which have all been implicated in exacerbating cerebral malaria[6, 85, 105, 122, 169, 177, 287, 364]. With this is mind, a hypothesis that unifies the data discussed above on the pathology of mice infected with PbMSP7 knockout parasites would be that an interaction between Selp and PbMSP7 prevents the recruitment and activation of Th1 cells, dampening the inflammatory response so as to reduce circulating levels of cytokines that control infection but also exacerbate cerebral pathology.

It is important to note that I did not observe a Selp/PbMSP7 interaction using AVEXIS (Figure 4.6C), which makes it highly speculative to correlate the observed features of infections with

[1] For example increased blood-brain barrier stability in Selp-knockout mice may be responsible for their protection from ECM[155]

PbMSP7-knockout parasites with the parasites' ability to bind Selp. However, the data described in 4.2.2.2 and Figure 4.6C do not eliminate the possibility that PbMSP7 interacts with Selp; the levels of PbMSP7 expression were consistently very low, which made it difficult to ensure that sufficient amounts of sufficient-quality PbMSP7 protein were used in experiments.

6.2.2 Implications for vaccine development

Antibodies against PfMSP7 have recently been associated with protective immunity in a cohort of Tanzanian children[273]. This evidence, combined with the abundance of the MSP1 complex on the merozoite surface[115], data suggesting immunisation with *P. yoelii* MSRP2 protects mice, and the documented ability of anti-MSP7 antibodies to impair RBC invasion[164, 357], makes PfMSP7 an attractive vaccine antigen. When designing a vaccine based on any particular protein target, it is informative to determine the precise epitopes that confer protective immunity. More antibodies were detected against PfMSP7$_{22}$ than PfMSP7-N in S. E. Asian serum samples, suggesting that the humoral immune response against the PfMSP7 components that are present on the merozoite surface might be more dominant, though it is not known whether or which of these antibodies provided protection from malaria[349]. Since the work described in this thesis indicates that PfMSP7$_{22}$/PfMSP7$_{19}$ and the N-terminal fragments of PfMSP7 perform distinct functions, it will be important to determine the respective benefits to the host of immune responses against each PfMSP7 fragment. It is likely that antibodies against PfMSP7$_{22}$ can confer protection by preventing merozoite-RBC interactions or by inhibiting the shedding of the MSP1 complex[164, 357], but there is currently insufficient evidence to know whether anti-PfMSP7-N responses would be complementary or deleterious to the host. On one hand, *in vivo* evidence from mice infected with PbMSP7-knockout parasites indicates that the presence of MSP7 enhances pathology in the host[117, 313], such that antibodies that eliminate the function(s) of MSP7 might be expected to be protective. On the other hand, if PfMSP7's N-terminal fragment(s) play an anti-inflammatory role, which might limit immunopathology in the host, it might be advantageous to exclude these regions from protein-based vaccines so as to preserve this potentially beneficial function.

6.3 Summary of potential future experiments

6.3.1 Biochemical details of SELP/MSP7-family protein-protein interactions

Whilst the interaction between recombinant PfMSP7 and SELP has been demonstrated in multiple experiments, there are still some biochemical details of this interaction that remain to be clarified. The following experiments could help to do so:

1. *Further validate the interaction by demonstrating that PfMSP7 binds to native SELP*: Given our concerns about the non-specific interaction behaviour of recombinant SELP, it will be important to verify that the interaction can occur using native SELP. We could do this by re-designing the flow cytometry platelet-binding experiment (Figure 3.12) so as not to use the anti-FLAG antibody, potentially by using recombinant PfMSP7 directly fused to a fluorescent reporter protein.

2. *Determine the molecular basis of PfMSP7 oligomerisation:* We hypothesise that the *in vitro* assembly of PfMSP7 could enhance its binding to SELP. Having confirmed that oligomerisation is a property the SELP-binding N-terminus of PfMSP7 molecule, it would be informative to further narrow down the region(s) involved in oligomerisation using truncated PfMSP7 proteins in size-exclusion chromatography (SEC) experiments and/or by using synthetic peptides to prevent oligomerisation[144]. Techniques such as size exclusion chromatography with multi-angle light scattering (SEC-MALS)[290], X-ray solution scattering (SAXS)[272] or atomic force microscopy[309] could be used to determine the number and conformation of PfMSP7 monomers in each of these complexes, and in complex with SELP. Amino acid analysis of the SELP/PfMSP7 complex could also reveal the stoichiometry of the interaction[288]. This information could help us determine how the complexes form *in vitro* and whether complexes are likely to form *in vivo*.

3. *Accurately determine kinetic parameters of the SELP/PfMPS7 interaction using SPR:* Our initial SPR experiments suggest that larger PfMPS7 complexes bind more strongly to SELP. To separate the increase in SPR signal due to increased complex binding from that due to the increased size of the binding species, we would have to determine binding constants for each species. This would involve producing sufficient quantities of PfMSP7 to saturate binding to immobilised SELP, measuring the binding signal produced at a range of lower concentrations, creating an equilibrium binding curve and

using this to determine the K_D. Given the difficulties experienced in performing these analyses by SPR, it might be necessary to seek an alternative method to calculate biophysical parameters. Free-solution methods such as isothermal titration calorimetry[67] or back-scattering interferometry (discussed in 6.3.3.3) may be appropriate methods.

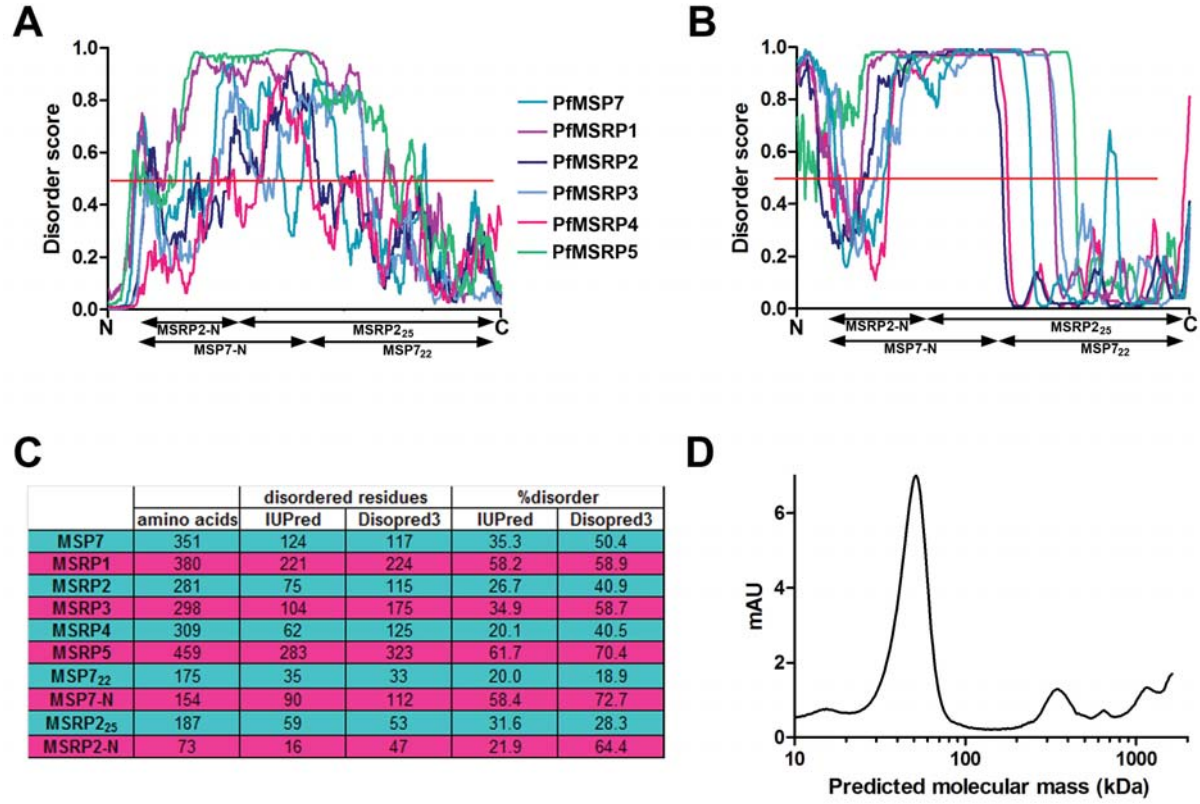
4. *Systematically characterise the oligomerisation and binding behaviour of the MSRPs:* AVEXIS data indicate that SELP-binding is not limited to PfMSP7. We could back up these data by observing the interactions by SPR or in the HEK-cell based flow cytometry assay (described in 2.6.2, 3.2.4 & Figure 2.4). It would be interesting to use SEC to determine whether the PfMSRPs also self-associate and whether this is necessary to promote their binding to selectins. If disordered regions in PfMSP7 are responsible for its oligomerisation, we might anticipate that the *P. falciparum* MSRP proteins would display similar behaviour, as all are predicated to be disordered, particularly at their N-termini (Figure 6.1A & B). Of the SELP-binding PfMSRP proteins, PfMSRP2 appears to be the least disordered (Figure 6.1A, B & C), and thus perhaps the most amenable to analysis by SPR. Early indications are that PfMSRP2 binding to SELP may indeed be easier to characterise, as an SEC-elution profile of purified material gave a monodisperse peak, with little evidence of significant oligomerisation (Figure 6.1D).

6.3.2 Determination of the function of the SELP/MSP7 interaction

Functional assays will be required to determine the potential *in vivo* role of the PfMSP7-SELP interaction. The following assays could be used to test our hypotheses as to the function of the interaction (introduced in 4.3):

1. *Leukocyte rolling assays*: Our initial attempts to use PfMSP7 in rolling assays[74] did not provide us with conclusive data to support or reject the hypothesis that PfMSP7 can modulate the rolling of leukocytes on endothelial cells. Having demonstrated that PSGL1-expressing THP1 cells can adhere to a SELP coated surface, and that PfMSP7 can be used to block this interaction, we hope to revisit and optimise these assays, as they represent a closer proxy for *in vivo* leukocyte-endothelial cell interactions than our existing plate-based assays.

2. *Inflammation assays:* We could test the hypothesis that PfMSP7 can behave as an anti-inflammatory molecule using Selp-dependent mouse model of inflammation. In one model system, inflammation is measured by counting blood and peritoneal neutrophils following injection of thioglycollate into the peritoneal cavity[206]. If PfMSP7-N can



		disordered residues		%disorder	
	amino acids	IUPred	Disopred3	IUPred	Disopred3
MSP7	351	124	117	35.3	50.4
MSRP1	380	221	224	58.2	58.9
MSRP2	281	75	115	26.7	40.9
MSRP3	298	104	175	34.9	58.7
MSRP4	309	62	125	20.1	40.5
MSRP5	459	283	323	61.7	70.4
MSP7$_{22}$	175	35	33	20.0	18.9
MSP7-N	154	90	112	58.4	72.7
MSRP2$_{25}$	187	59	53	31.6	28.3
MSRP2-N	73	16	47	21.9	64.4

Figure 6.1: **Predicted disorder is common to all *P. falciparum* MSP7-family proteins but lower in PfMSRP2**

A. IUPred prediction of disordered residues in the *P. falciparum* MSP7 family. Regions corresponding to PfMSP7$_{22}$, PfMSP7-N, the 25kDa C-terminal region of PfMSRP2 downstream of a validated SUB1 cleavage site (PfMSRP2$_{25}$) and the remaining portion (excluding the signal peptide) of its N-terminus (PfMSRP2-N) are indicated.

B. Disopred3 prediction of disordered residues in the *P. falciparum* MSP7-family

C. Summary of number and proportion of disordered residues predicted in *P. falciparum* MSP7-family, proteins.

D. SEC analysis of a single PfMSRP2 protein preparation gave a monodisperse peak. This might mean that SPR analyses using PfMSRP2 stand to be more successful than those using PfMSP7 (described in Chapter 3).

behave as an anti-inflammatory molecule, we might expect to see a reduced influx of neutrophils in mice that have been injected with PfMSP7 prior to thioglycollate introduction. This would constitute very convincing evidence that PfMSP7 has an immunomodulatory role

3. *Other adhesion assays:* To investigate whether PfMSP7 can affect other SELP-mediated adhesion events in disease it would be possible to introduce PfMSP7 into sequestration assays[101, 367] and RBC clumping assays[259]. We could test the hypothesis that PfMPS7 might modulate haemostasis by adding PfMSP7 into an appropriate assay to measure thrombus formation[281].

6.3.3 Expansion and improvement of screening for interactions occurring between *Plasmodium* proteins and human receptors

AVEXIS has been successful in identifying a number of host-pathogen interactions, including those between SELP and MSP7 proteins, that could contribute to our understanding of malarial pathology at the molecular level[19, 64]. However, this approach cannot be used exhaustively to screen all potential host receptors against our recombinant *P. falciparum* proteins; for instance there are many host proteins that we cannot produce recombinantly at sufficient levels, or with the appropriate post-translational modifications[2]. This makes it advantageous to screen for interactions using native sources of human receptor proteins, for instance in serum or on the surface of intact cells. Using receptors on the surface of intact cells has the particular advantage of conserving the native environment of the receptor proteins such that interactions involving protein complexes or proteins that traverse the membrane multiple times, can be studied.

The following approaches could potentially facilitate the identification of novel host-pathogen interactions that could in turn help to elucidate the molecular mechanisms involved in the pathogenesis of malaria:

1. *Screen more proteins for interaction with human serum:* In Chapter 5, I screened 56 *P. falciparum* proteins for interactions with human serum. There are many more serum-exposed *Plasmodium* proteins, including some included in our own existing protein expression libraries; I expressed at least 20 proteins that I did not screen, as their levels

[2]For example, we have been unable to produce recombinant Glycophorin A that is sufficiently sialylated to bind to PfEBA175. Recombinant PSGL1 was also unable to bind to SELP, which could very plausibly mean that HEK293E cells did not reproduce the post-translational addition of $sLe^X$to PSGL1 that occurs in leukocytes *in vivo*.

in cell culture supernatants were insufficient to saturate the protein-binding capacity of the paramagnetic beads. By scaling-up the production of these bait proteins, and enhancing their concentration by purification, it would be possible to screen more *P. falciparum* candidate bait proteins.

2. *Improve the sensitivity of the biochemical purification approach:* Despite screening a large number of proteins, the biochemical co-purification approach did not reveal any novel host-pathogen protein-protein interactions. This may be a consequence of the high stringency, and hence insufficient sensitivity, of this approach. By making the improvements identified in 5.4.2, and in particular by increasing the avidity and stability of interactions whilst decreasing the stringency of the detection method, the power of this method to detect novel interactions could be enhanced.

3. *Develop back-scattering interferometry (BSI) for the discovery and quantification of Plasmodium protein-host interactions*: BSI, described in Figure 6.2A, is a relatively new technique that can be used to measure binding events. In very recent years, the Bornhop laboratory has developed BSI as a highly sensitive method for studying biological interactions in free solution[3]. Various examples of simple protein-protein or protein-small molecule interactions have been studied using BSI[31] and it is also possible to use cell membrane preparations instead of isolated proteins in this system[13], which makes BSI an exciting new tool for studying interactions occurring at the cell surface. In the course of this work, we have been collaborating with the Bornhop laboratory to determine whether BSI can be used to study interactions occurring between our recombinant *P. falciparum* proteins and the RBC surface; preliminary data has indicated that BSI is a very sensitive method that can be used to accurately determine the kinetic parameters of interactions occurring between known *P. falciparum* invasion ligands and their receptors on intact human RBCs (Figure 6.2B). Thus, this method has great potential as a screening tool for the discovery of novel interactions between parasite and RBC surface proteins. Since BSI has also been applied to the detection of molecules in more complex matrices, including serum[175], it might also be possible to use this technique to screen our recombinant *Plasmodium* proteins against human serum; BSI could represent a highly sensitive detection method for serum-binding proteins, whose receptors could be subsequently be identified using biochemical purification and mass spectrometry approaches discussed in Chapter 5.

[3]In its earlier stages BSI used immobilised receptors on the surface of the chip, in a similar manner to SPR

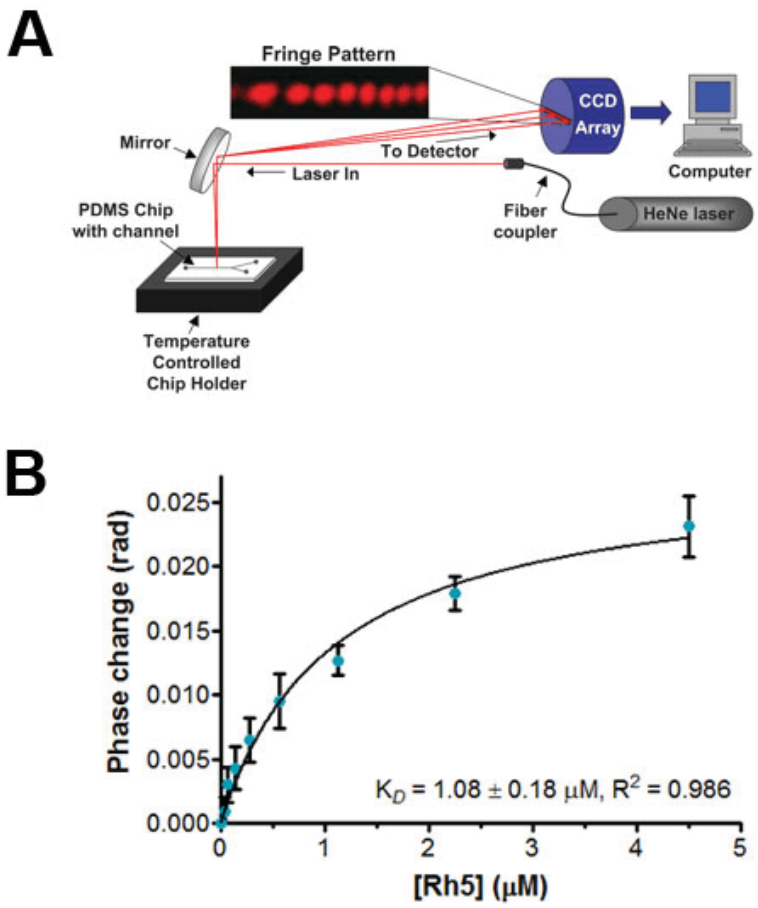


Figure 6.2: **BSI as a method to discover and measure protein-RBC interactions**
A. Schematic representation of the BSI methodology. In brief, a helium-neon laser beam is directed onto a microfluidic chip containing the sample. Photons take different paths through the matrix, being reflected in complex patterns from the walls of the channel such that they are out-of-phase (and thus interfere) upon their exit from the channel. This results in the formation of a visible interferometric fringe pattern. The fringe pattern can be recorded using a CCD camera, then analysed. An increase in the refractive index of the solution will proportionately decrease the speed at which the photons travel through the matrix, meaning that their phase upon exit from the channel will be different. Consequently, the waves interfere with each other differently and the position of the fringe pattern changes. BSI can be used to measure tiny shifts in the position of the fringes and infer a change in the refractive index of the solution. Biomolecular binding events can bring about profound changes to the organisation of molecules in solution, and hence cause refractive index changes. The formation of a new complex in the solution has been shown to induce measurable phase shifts in the fringe pattern detected by BSI, even at very low concentrations of the binding species. Figure from Bornhop *et al.*, (2007)[31]. Reproduced with permission from AAAS.
B. BSI was used to measure the interaction of purified recombinant PfRH5 and BSG receptors on the suface of intact RBCs. In brief, RBCs were incubated with a range of PfRH5 concentrations for one hour and the phase of the interference fringe pattern was measured using the BSI instrument. Phase change was calculated by subtracting this reading from that using negative control RBCs that had been pre-incubated with anti-BSG antibody, to prevent PfRH5 binding. These data allowed the plotting of a saturation binding curve and the estimation of the K_D at around 1µM, which is almost identical to that calculated for the interaction between recombinant PfRH5 and BSG by SPR by Crosnier *et al.*, (2011)[64]. Error bars represent mean +/- SEM, n=7. Data courtesy of Phoonthawee Saetear, Vanderbilt University.

6.4 Concluding remarks

Whilst the main result of this work is the discovery of a set of interactions occurring *in vitro* between human SELP and *Plasmodium* MSP7 proteins, I have also developed and optimised experimental techniques that can be used more broadly for the discovery and characterisation of protein-protein interactions. Going forward, the interactions between SELP and MSP7s require further validation and functional analyses so as to precisely define their role in the pathogenesis of malaria. Concurrently, the further use, development and refinement of interaction detection methodologies such as those discussed in this thesis could lead to the identification of additional host-pathogen interactions. The processes of validation, biochemical characterisation and functional analysis, as described for SELP/MSP7 interactions, could then contribute to our existing knowledge about the molecular details of the interactions *Plasmodium* parasites make with their hosts. These insights could contribute to the rational design of drugs and vaccinations, to combat the global burden of malaria.