Chapter 4

Biochemical characterisation of the interactions between SELP and *Plasmodium* MSP7s

4.1 Introduction

Following the identification and validation of an *in vitro* interaction between human P-selectin (SELP) and *Plasmodium falciparum* merozoite surface protein 7 (PfMSP7), this chapter describes further biochemical characterisation of this interaction, and expands this characterisation to encompass the broader families of each protein.

4.1.1 The *Plasmodium* MSP7 family

4.1.1.1 MSP7s in a genomic context

The *Plasmodium MSP7* and *MSP7*-related protein (*MSRP*) genes have been defined by homology to the *PfMSP7* gene, first identified as the origin of the 22kDa MSP7 protein fragment found on the surface of the *P. falciparum* merozoite[215, 227, 257, 327]. These multi-gene families comprise variable numbers of protein-coding genes located very close to one another on one chromosome of each *Plasmodium* species with an available genome sequence. The genomes of rodent parasites *P. berghei*, *P.chabaudi* and *P. yoelii* contain three MSP7 family protein-coding genes, the genome of *P. falciparum* contains at least six and that of *P. vivax* contains eleven annotated *MSP7* genes (Figure 4.1)[108, 137, 158, 227]. The persistence of this large multi-gene family across the *Plasmodium* genus suggests that its members play an

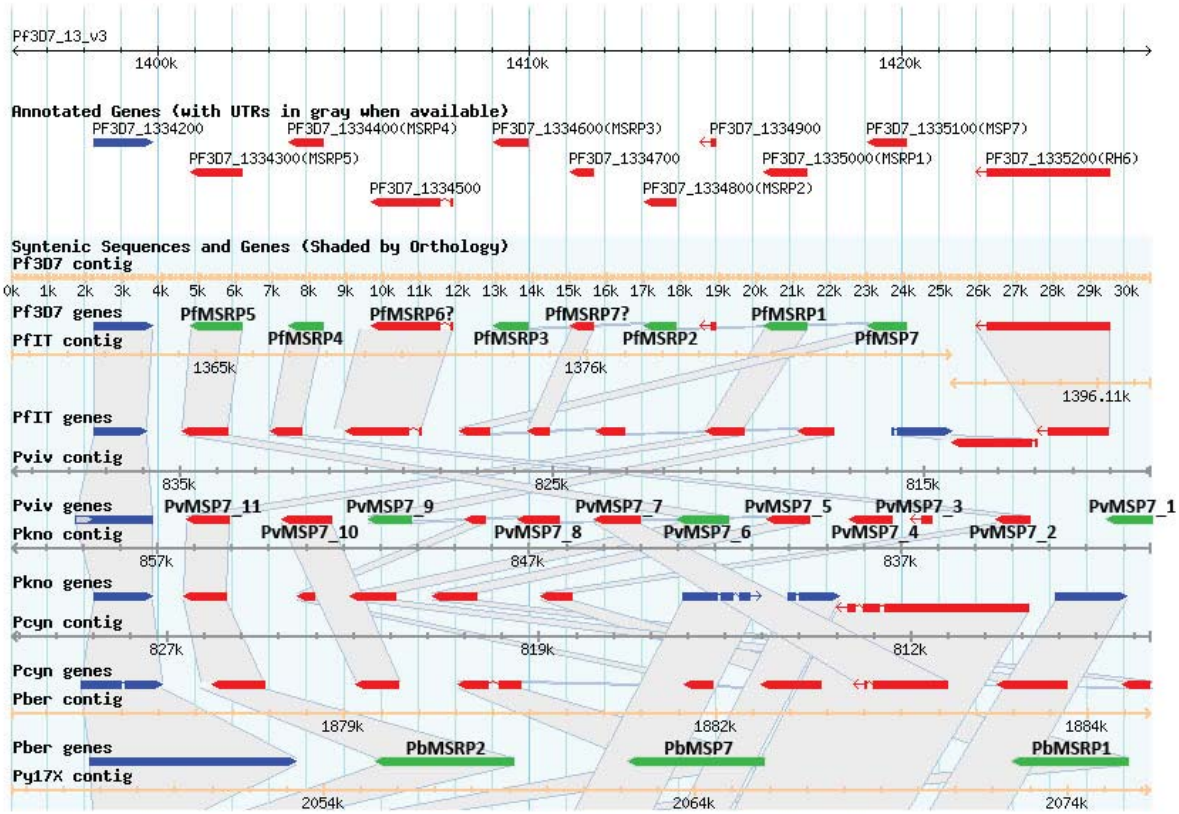

Figure 4.1: ***MSP7* gene organisation and nomencalture in different *Plasmodium* species**
Screenshot from PlasmoDB of the genomic locus containing the *MSP7* family. The *MSP7* genes in *P. falciparum*, *P. vivax* and *P. berghei* are annotated with the nomenclature used to refer to them in the text of this thesis. This nomenclature reflects each gene's description in PlasmoDB at the time of writing with the exceptions of *P. vivax MSP7*s and *P. falciparum*'s putative *MSRP6* and *MSRP7* genes, which are identified as part of this family in Garzón-Opsina *et al.* (2010)[108] and named as such in Heiber *et al.* (2013)[137]. The above labelling of the *P. vivax MSP7*s follows that used in Kadekoppala & Holder's 2010 review of the MSP7 gene family[158]. MSP7 proteins expressed as part of this work are coloured in green.

important biological role. There is currently no evidence at the DNA level to suggest functional divergence between *MSP7* paralogues, indicating that they might be redundant. The expansion in the number of *MSP7* genes in some species relative to others is therefore not fully understood, although it has been suggested that the number of family members has been fixed in each species at the optimal number or gene 'dosage' for the niche they each occupy[108]. Gene duplication events are thought to be responsible for the presence of multiple *MSP7*-family genes in each species. These genes share a similar structure and organisation, though sequence conservation is moderately low; in *P. falciparum* for instance, there is about 38 – 54% overall amino acid similarity between paralogues. Increased amino acid similarity is observed towards the C-termini of the MSP7-family proteins in all species, indicating that this region may be particularly important to the proteins' functionality[158, 227]. There is some evidence to suggest that the 3' end of the *PfMSP7* gene and the central regions of at least two *P. vivax MSP7* genes are under balancing selection, potentially to maintain antigenic diversity as a result of increased immune selection pressure[5, 110, 286, 326]. In contrast, most of the individual *MSP7*-family genes in *P. falciparum* and a number of *MSP7* genes in *P. vivax* are highly conserved, with few SNPs observed between isolates[109, 158, 286, 326]. Taken together the genetic information implies that the different MSP7-family proteins have the potential to carry out similar functions but are differentially exposed to selective pressures.

4.1.1.2 MSP7 protein expression

Transcripts for all MSP7-family proteins have been detected in blood-stage *P. falciparum* parasites, with a general increase in expression seen towards schizogony[160, 216]. However, only PfMSP7, PfMSRP1 and PfMSRP2 proteins have been detected experimentally. These three proteins have been detected by immunoflourescence in trophozoites[215], whilst PfMSP7 and PfMSRP1 have been detected in the detergent-resistant membrane fractions of schizonts[293]. Immunoblotting of trophozoite and schizont lysates detected PfMSP7 and PfMSRP2, but none of the other *P. falciparum* MSRP proteins[160]. Both PfMSP7 and PfMSRP2 have been shown to undergo proteolytic cleavage by subtilisin-like proteases[160, 173, 258, 304] (see Figure 3.1). Whilst the C-terminal fragments of PfMSP7 that are known to be associated with PfMSP1 on the the merozoite surface, the eventual destinations of most of the other PfMSP7 and PfMSRP2 fragments are currently unclear[304]. A 173 amino acid fragment from the N-terminus of PfMSP7 has been expressed with a GFP tag in *P. falciparum* parasites. The fragment could be visualised in the parasitophorous vaculolar space, indicating that this PfMSP7 fragment could be secreted from merozoites[158]. Although PfMSRP2 is thought to be processed in a similar manner as PfMSP7[160, 304] there is some debate as to whether

it associates with PfMSP1 or forms any part of the MSP1 complex[160]. A biochemical purification assay where GST-tagged PfMSP1 was incubated with recombinant PfMSPRP1 and PfMSRP2 indicated that both PfMSP7 family proteins bind to PfMSP1[215], but these interactions have not been shown to occur in parasites[160]. Antibodies against PfMSRP2 as well as N- and C-terminal regions of PfMSP7 have been detected in the serum of individuals living in malaria-endemic areas[158, 258, 349], indicating that these protein fragments are exposed to the immune system[1]. Although their native gene products have not been identified *in vitro* or *in vivo*, GFP-fusions of MSRP5 and putative MSRP6 and MSRP7 proteins have been detected outside of the merozoite, indicating that they are secreted[137].

Comparatively little is known about the destinations of *P. vivax* MSP7 proteins. At least eight are transcribed in late schizonts[32], and the protein with highest amino acid similarity to PfMSP7 (labelled as PvMSP7_2 in Figure 4.1) has been characterised to some extent; like PfMSP7 it is expressed and proteolytically processed, and at least one of the cleavage products is thought to localise to the merozoite surface in schizonts[227]. In rodent parasite *P. yeolii* all three MSP7 family proteins have been detected and are thought to bind PyMSP1. PyMSP7 and PyMSRP2 co-localise with PyMSP1 at the merozoite surface and are thought to induce a low level of protective immunity when administered as vaccines in mouse models[216]. Similarly *P. berghei* MSP7 localises with PbMSP1 at the merozoite surface, though immunoprecipitation experiments indicate that, unlike PfMSP7, PyMSP7 and PvMSP7_2, PbMSP7 does not undergo proteolytic processing[327].

4.1.1.3 PfMSP7 and PbMSP7 are associated with RBC invasion, but may perform additional functions

The *P. falciparum* MSP1 complex is comprised of four fragments of the PfMSP1 precursor protein and a peripherally-associated fragment of each of PfMSP6 and PfMSP7; the complex forms a major component of the thick, fibrillar coat of the merozoite and has hence been studied intensively as a target for vaccines and therapeutics. The MSP1 complex is known to be required for merozoites to invade erythrocytes. Antibodies against PfMSP7 can block invasion, either by preventing interactions that the MSP1 complex makes with the RBC surface or by preventing the maturation and/or shedding of the MSP1 complex[164, 357]. PfMSP7-knockout parasites still process PfMSP1 and present its peptides on the merozoite surface as normal. Similarly, knockout parasites are still able to survive and invade RBCs in *in vitro* and *in vivo* models, indicating that MSP7 itself is not essential. However, these knockout parasites

[1] Of particular note, naturally-acquired antibodies against PfMSP7 were shown to be protective against severe disease in a cohort of Tanzanian children[273].

do show a slight impairment in their invasion capacity when compared to wild-type. Assays using *in vitro P. falciparum* cultures show a 30% reduction in invasion when PfMSP7 is deleted[159] whilst *P. berghei* deletion mutants grew more slowly *in vivo* and demonstrated a preference for invading reticulocytes[117, 313, 327]. Adding to the evidence that PfMSP7 has an important, though not essential, role in RBC invasion, protein levels are seen up-regulated nearly eight-fold in W2mef strains switching to sialic acid-independent invasion[174]. *Plasmodium* parasites are known to enter RBCs via multiple invasion pathways so it is possible that PfMSP7 is involved in one or more of these. Based on its known localisation to the merozoite surface, it is reasonable to assign PfMSP7's role in invasion to its C-terminal 19 or 22kDa fragment within the MSP1 complex, although peptides corresponding to the N- and C-termini have been reported to bind to the RBC surface, potentially via an interaction with Band 3, and inhibit invasion by up to 50%[106].

Evidence from rodent infections implies that PbMSP7 may play an immunomodulatory role. *PbMSP7*-knockout parasites caused significantly fewer deaths in both a mouse model of chronic infection and an aged rat model. The delay in parasite growth and corresponding slower generation of anaemia in the *PbMSP7*-knockout infected mice was not sufficient to explain these results, leading the authors to suggest that the presence of PbMSP7 impaired the host's ability to respond effectively to the infection[117]. In mouse strains that are susceptible to experimental cerebral malaria (ECM) *PbMSP7*-knockout parasites were unable to induce the ECM phenotypes observed in mice infected with wild-type parasites, critically demonstrating a reduced ability to damage the blood-brain barrier. Again these results could not be explained by the growth impairment of the knockout parasites, indicating that the mechanism by which ECM is induced relies on the presence of the PbMSP7 protein[313]. The significance of the these results to human infections is currently unclear[2] but these data indicate that at least one MSP7 protein may possess functions that do not directly relate to RBC invasion alone.

4.1.1.4 The *P. falciparum* MSRPs have no known function

The presence of multiple MSP7 family proteins in *Plasmodium* species suggests that they might be functionally redundant. However, experiments in *P. falciparum* knockouts do not suggest that MSRPs can provide compensatory functions for PfMSP7's role in RBC invasion. MSRP transcript abundances remain comparable between wild type and PfMSP7 knockout lines, with the exception of PfMSRP5, whose transcription appears to be increased two-fold.

[2] especially given the differences in MSP7 processing and localisation between human- and mouse-infective *Plasmodium* species[215, 216, 227, 258, 327]

The consequence of this up-regulation is unclear, as the protein itself has not been detected at the RBC surface, nor elsewhere in parasite lysates[159]. All *P. falciparum* MSRPs can be individually deleted without any impairment of the parasites' ability to invade RBCs[160]. However, to date there have been no reported incidences of the generation of viable parasites of any species lacking all MSP7 family proteins.

4.1.2 The human selectins

4.1.2.1 The selectin proteins

Lectins are a group of carbohydrate-binding proteins, known for their roles in mediating cellular recognition and attachment events via their interactions with specific sugars. Selectins are classed among the C-type lectins, which were originally characterised by their calcium-dependent binding properties[75]. There are three genes encoding selectin proteins in humans. They are clustered on chromosome one and are thought to have arisen by gene duplication events occurring before the divergence of humans and mice[351]. Each of the proteins are structurally very similar, all type I membrane proteins sharing a conserved N-terminal C-type lectin and EGF-binding like domain then a variable number of short consensus repeats[156] (see Figure 4.2A). E-selectin (SELE) is expressed on endothelial cells whilst L-selectin (SELL) is expressed on the surface of leukocytes[26, 27, 324]. P-selectin (SELP) is localised to the alpha-granules of platelets and the Weibel-Palade bodies of endothelial cells. Upon activation, these bodies fuse with the membrane of their respective cell type, resulting in the rapid translocation of SELP to the surface membrane[209, 317]. All three selectins can be detected as soluble proteins in the bloodstream, a splice variant of SELP lacking a transmembrane domain being the source of much of this circulating protein[76, 237, 296]. Selectins exhibit calcium-dependent binding to a number of extracellular glycoproteins. This binding activity is mediated predominantly by the C-type lectin-like domain which interacts with sLe^X tetrasaccharides[87, 99]. Despite their shared ability to bind sLe^X sugars, the glycoprotein ligand-binding specificity of each selectin differs based on subtle structural differences between the full length proteins[186, 325]. Leukocyte P-selectin glycoprotein ligand 1 (PSGL1) is the best characterised SELP ligand[229, 239, 291, 292]. SELE can also bind PSGL1 but is thought to bind preferentially other leukocyte ligands to mediate rolling interactions[8, 365]. SELL has been shown to interact with PSGL1 with a much lower affinity and has a range of other mucin-like ligands[282, 314]. A low affinity interaction, in the millimolar range, is thought to occur between selectins and isolated sLe^X[268] but the interactions selectins make with their glycoprotein ligands are substantially stronger. It has been hypothesised that an

initial electrostatic interaction occurs between sLe^X and its binding site, following which the selectin molecule makes further contacts with the protein ligand, resulting in a higher affinity interaction[312]. The C-type lectin-like domain has been most heavily implicated in mediating binding behaviour in the selectins, as it is the region with which calcium ions, sLe^X and a range of small-molecule interaction inhibitors bind and it is structurally fairly isolated from the EGF-like domain[113, 123, 133, 312]. However, crystallographic evidence from the structures of SELP's EGF and C-type lectin-like domains in complex with a peptide from PSGL1 suggests that the EGF-like domain may play a role in high-affinity ligand binding[161, 312].

4.1.2.2 SELP-ligand interactions are important in inflammation and blood coagulation

Selectins have a well-characterised role as vascular adhesion molecules, each mediating interactions between leukocytes and endothelial cells[26, 112, 193]. Endothelial selectins and their ligands are necessary for the loose binding, or 'rolling', of leukocytes as they are recruited to vessel walls in the very early stages of inflammation[71, 190, 206, 239]. This is an important process in controlling infection, as evidenced by the recurrent, and often severe, bacterial infections suffered by selectin-deficient mice and patients with Type II Leukocyte Adhesion Deficiency, in which selectin ligands are sub-optimally glycosylated for binding to selectins[38, 89]. More recently it has been discovered that the leukocyte-endothelium interactions mediated by SELP might have a more profound effect on inflammation than by leukocyte recruitment alone. Firstly, it is thought that SELP selectively recruits pro-inflammatory Th1 (T-helper-1) cells in preference to Th2 cells, which can down-regulate inflammation[10, 30]. Secondly, leukocyte PSGL1 binding to endothelial SELP is also thought to trigger signalling pathways that prime cells for enhancing inflammatory responses. For instance this binding promotes phagocytosis in monocytes and leads to an enhanced production of a range of cytokines, including TNFα[83, 352], which is thought to be particularly important for parasite killing in the early stages of malaria[276]. Conversely, it is thought that circulating soluble SELP can prevent these interactions and help to limit the inflammatory response[76, 103, 358].

Roles for SELP in blood coagulation and haemodynamics have also been identified. SELP can induce monocytes to produce tissue factor[51], which both induces signalling in white blood cells and initiates the chain of interactions in the clotting cascade. Since this discovery, SELP has been characterised as a pro-coagulant molecule[7], the interaction between platelet SELP and PSGL1 being important in the formation of thrombi[90].

4.1.2.3 SELP is known to contribute to malarial pathology

As a key part of the inflammatory response, platelets and endothelia are activated which leads to raised levels of soluble and membrane-bound SELP in human and murine malarial hosts. Selectins on the surface of endothelial cells have been implicated in the sequestration of pRBCs. SELP and SELE have been independently identified as receptors for PfEMP1 on the pRBC surface and are thought to help the initial adhesion, or rolling, of pRBCs[241, 302]. These comparatively weak interactions are thought to aid pRBC sequestration by facilitating a stronger interaction between CD36 and PfEMP1[302]. pRBC rolling is reduced in a Selp knockout mouse[53] though no decrease in overall cytoadhesion is observed. Selp-deficient mice have been shown to be substantially less susceptible to ECM compared with wild-type control mice[59]. Complementing this observation, it has also been shown that Selp levels are increased in the brain vessels of mouse strains that are susceptible to ECM, whereas resistant BALB/c strains do not show this accumulation. Tissue specific Selp-knockout mice have been used to isolate endothelial (rather than platelet) Selp as the key contributor to the observed cerebral pathology; the incidence of ECM was decreased in mice deficient in endothelial Selp, whilst those lacking platelet Selp were not protected[59]. *Plasmodium* species' use of SELP as an endothelial receptor may explain these observations, though more recent research suggests that SELP can compromise the stability of the blood-brain barrier in certain situations[155], a state which is thought to precipitate cerebral malaria[233]. More research is needed to determine whether SELP has a pathological role in human malaria, and, if so, whether this is mediated by a direct host-pathogen interaction.

4.2 Results

4.2.1 Characterisation of PfMSP7 binding to selectins

4.2.1.1 The C-type lectin and EGF-like domains of SELP were required to bind PfMSP7

In Chapter 3, I described the identification of an interaction between SELP and PfMSP7. To identify the region of the SELP protein that interacts with PfMSP7, I made a series of biotinylated C-terminally truncated SELP proteins based on the protein's domain structure (Figure 4.2A). I then screened these baits against PfMSP7 prey using AVEXIS. All truncated proteins were able to bind to full length PfMSP7, except the smallest fragment which comprised only the C-type lectin (CTL) domain (Figure 4.2B).

The smallest binding fragment contained the CTL and EGF domains, indicating that both

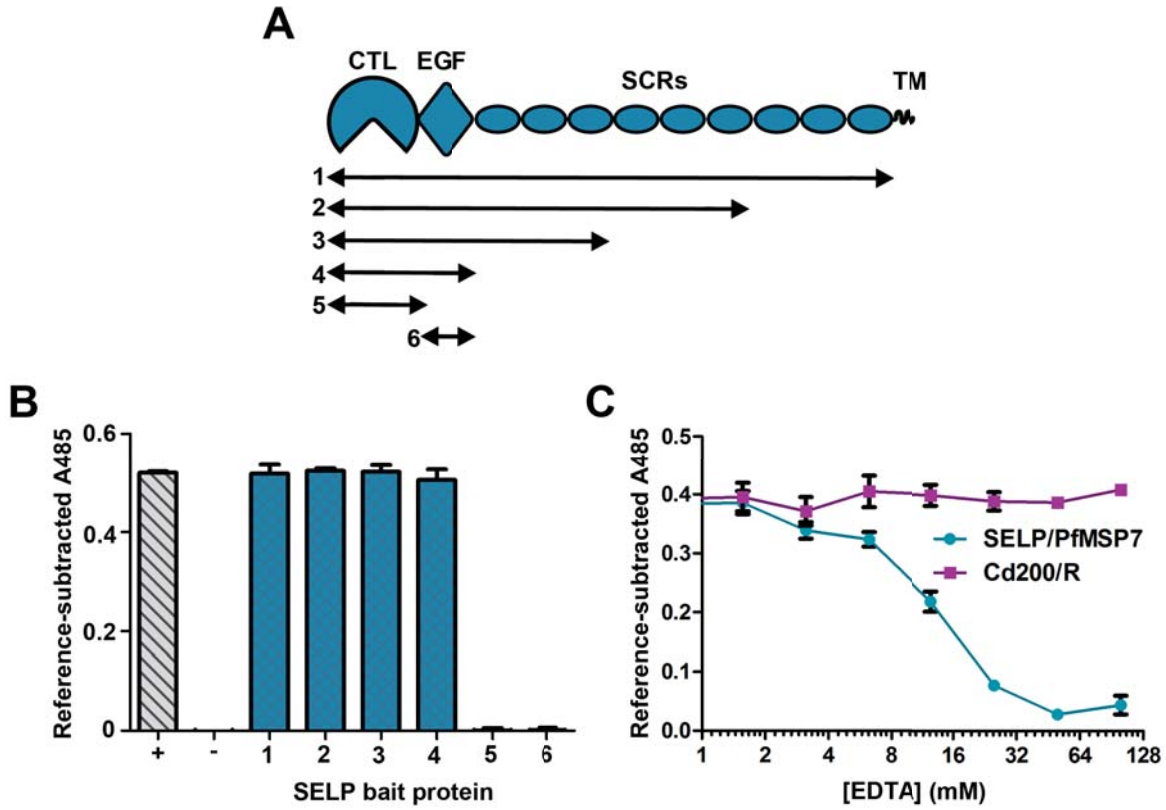

Figure 4.2: **The CTL and EGF domains of SELP were essential for PfMSP7 binding**
A. Schematic representation of the truncated SELP bait proteins used for domain mapping experiments, with C-terminal truncations made after the CTL, EGF, third short consensus repeat (SCR) or sixth SCR domain. The EGF domain, without the CTL domain, was also produced. Domain boundaries were determined using Pfam and are detailed in Table 2.2.
B. AVEXIS signals when truncated SELP baits were screened against PfMSP7 prey. All SELP bait protein fragments containing both the CTL and EGF domains bound PfMSP7 prey. PfMSP7 prey was also screened against OX68 positive control bait (+) rat Cd4 tag region negative control bait (-). Bars represent means +/- SD, *n*=3.
C. AVEXIS signals resulting from the interaction of SELP bait and PfMSP7 prey in the presence of EDTA. The interaction between SELP bait and PfMSP7 prey was blocked completely by around 30mM EDTA. The rat Cd200/Cd200R interaction was unaffected by EDTA. Error bars represent means +/- SD, *n*=3.

domains in combination, or the EGF domain alone, are minimally required for binding. To test whether the EGF domain alone could bind, I expressed the single domain as a bait protein and tested it for binding to PfMSP7 prey (Figure 4.2B). I saw no indication of binding, suggesting that both CTL and EGF domains are minimally required for binding, and that the binding site(s) for PfMSP7 is located within this region. The interactions SELP makes with its known ligands are typically dependent on the coordination of calcium by its CTL domain. To test whether PfMSP7 binding is also calcium-dependent, I incubated the proteins with varying concentrations of EDTA, a divalent cation chelator. By performing the AVEXIS-based binding assay in the presence of EDTA I observed that the interaction could be blocked by about 30mM EDTA, which is in line with published experiments where EDTA has been used to block SELP binding[172] (Figure 4.2C). This indicates that the conformation of the CTL-domain is important for PfMSP7 binding.

4.2.1.2 PfMSP7 bound to SELP and interacted weakly with SELL

SELP is one of the three human selectin proteins. These three selectins have a conserved structure, and a high degree of amino acid similarity within their shared domains (Figure 4.3A) so it is very plausible that PfMSP7 could bind to other selectins besides SELP. To determine whether this occurs, I sub-cloned SELL and SELE ectodomains from cDNA, expressed both as biotinylated bait proteins (Figure 4.3B) and screened them against PfMSP7 prey using AVEXIS (Figure 4.3C). I observed a degree of nitrocefin hydrolysis when SELL bait was used in the screen, indicating that some PfMSP7 prey was bound. Unlike when OX68 (positive control) and SELP baits were used, this nitrocefin hydrolysis did not saturate within one hour. This indicates that SELL can capture PfMSP7, but cannot capture as much of this prey protein as SELP can, implying that the interaction involving SELL is weaker. When I used all three selectins in SPR experiments with a PfMSP7 analyte, I only observed binding in the flow cells containing SELP (Figure 4.3D), which might indicate that PfMSP7/SELL binding phenomenon seen by AVEXIS is not biologically significant.

4.2.1.3 Naturally-occurring SNPs within SELP's binding domain did not affect binding to PfMSP7 without compromising protein function

Sequencing and genotyping have elucidated a number of SNPs occurring in the human *SELP* DNA sequence, and they have been catalogued in the dbSNP database. Using this resource, I identified four non-synonymous SNPs within SELP's CTL and EGF domains (highlighted in Figure 4.3A). Searching the 1000 human genomes data revealed that all of these variants are

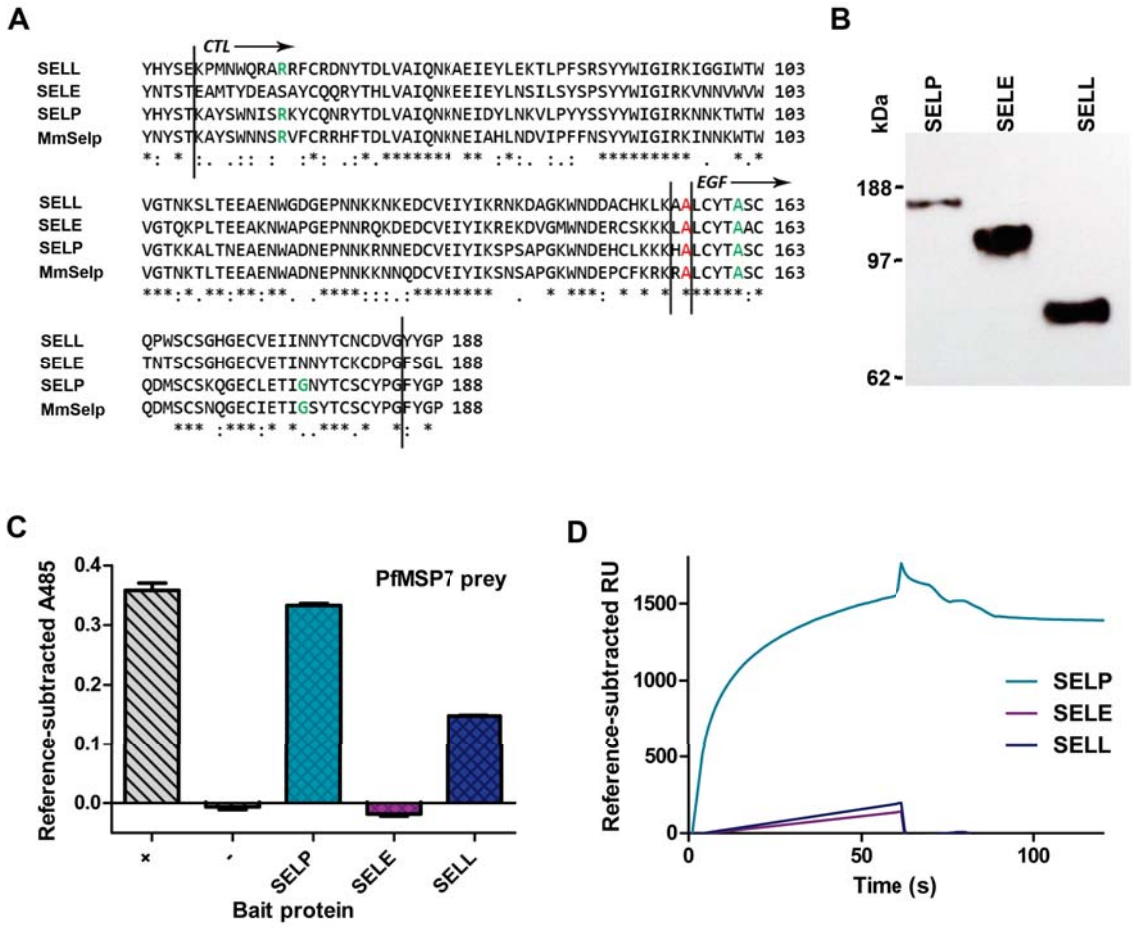

Figure 4.3: **Limited evidence for SELL and SELE binding to PfMPS7**
A. T-COFFEE amino acid sequence alignment of CTL and EGF domains of SELL, SELL, SELP and mouse Selp. Vertical lines indicate the boundaries of each domain as delineated by Pfam. Residues highlighted in red or green are those that are recorded in dbSNP as known variants in human SELP.
B. Selectin baits were successfully expressed and biotinylated. Single bands of expected sizes were observed by Western blotting using streptavidin-HRP.
C. PfMSP7 prey bound to SELP and SELL baits in AVEXIS assays. OX68 was the positive control bait (+) and the rat Cd4 tag region was the negative control bait (-). Bars represent means +/- SD, *n*=3.
D. PfMSP7 analyte did not bind SELL or SELL in SPR experiments.

very rare in all populations. I created expression constructs using site-directed mutagenesis and produced all four variants as bait proteins (Figure 4.4A, B). I then screened these variant proteins for binding to PfMSP7 prey. All variants bound indistinguishably from the wild-type SELP reference, with the exception of the A156T variant (Figure 4.4C). This variant did not bind the anti-SELP monoclonal antibody that was able to block the SELP-PfMSP7 interaction (Figure 4.4D). These results indicate either that A156, which lies at the boundary between the CTL and EGF-like domains, is an important part of the binding region for both the antibody and PfMSP7, or that mutation of this residue significantly affects the folding or conformation of the SELP protein. This particular residue is conserved in the protein sequences of all three human selectins and mouse Selp (Figure 4.4A, highlighted in red), perhaps suggesting that it has an important role in the correct functioning of the protein. To determine whether the A156 mutant was still a functional protein, I tested whether the mutant SELP prey protein could bind to immobilised sLe^X (Figure 4.4E). No sLe^X-binding activity could be detected using this mutant, thus indicating that the variant probably does not fold to form a protein that can carry out the normal *in vivo* binding roles of SELP.

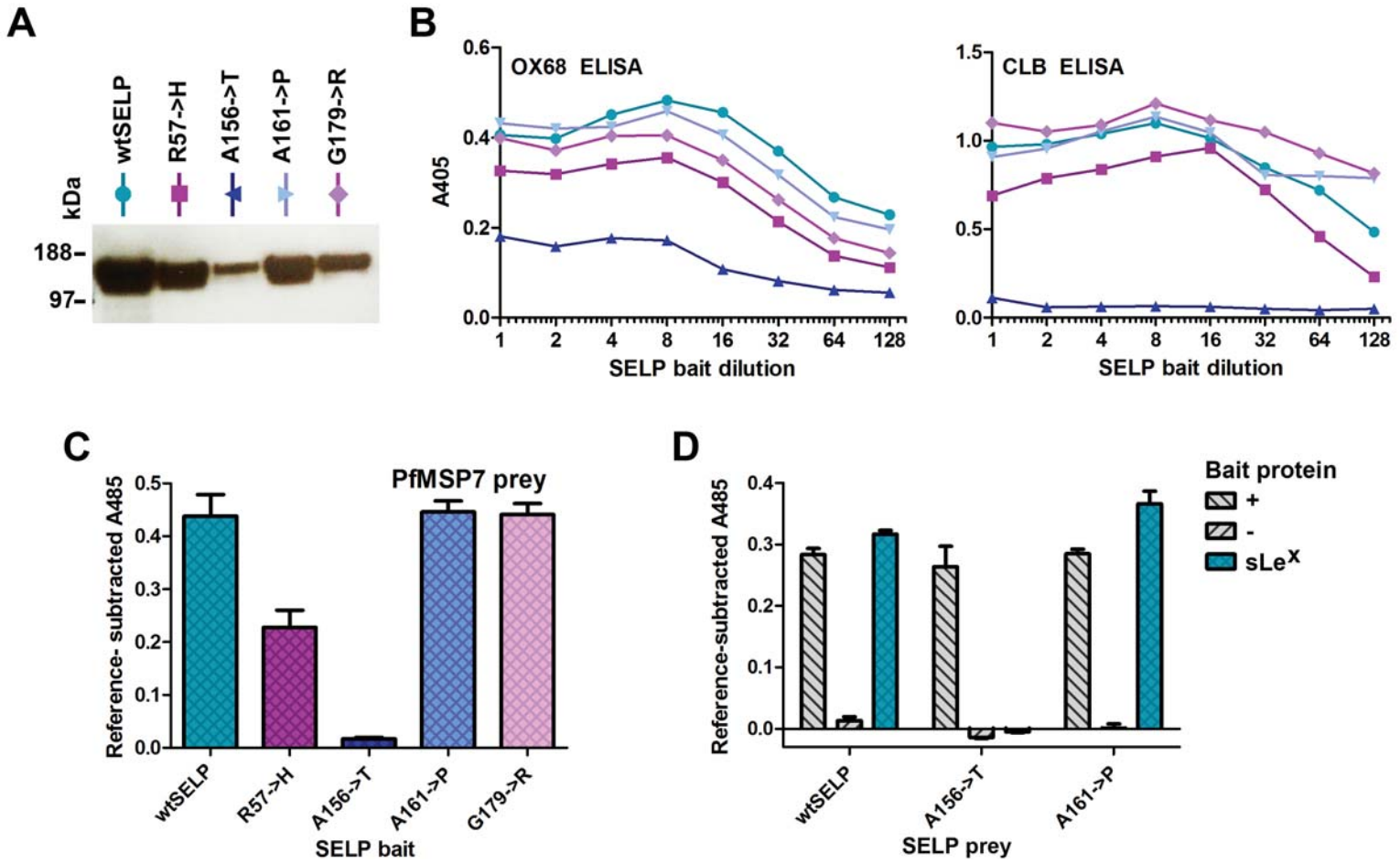

Figure 4.4: **Functional SELP variants retained binding to PfMSP7**

A. All variant baits were detected by Western blotting with streptavidin-HRP. The A156 variant had a noticeably lower expression level.

B. SELP variants were detected by ELISA. All proteins could be detected using OX68, an antibody that binds to the Cd4 tag. The A156T variants could not be detected by CLB-thromb/6, a mAB believed to bind at the interface between the C-type lectin and EGF-like domains of SELP[294].

C. All SELP baits, except the A156T variant, bound to PfMSP7 by AVEXIS.

D. A156T mutant SELP prey is unable to bind to sLe^X bait. Control baits were OX68 (+) and the rat Cd4 tag region (-). Bars represent means +/- SD, n=3.

4.2.2 Characterisation of SELP binding to *Plasmodium* MSP7-proteins

4.2.2.1 The N-terminus of PfMSP7 bound to SELP

To determine which fragment of the full-length PfMSP7 precursor protein binds to SELP, I expressed the merozoite surface-resident C-terminal fragments ($PfMSP7_{19}$ and $PfMSP7_{22}$) and the remaining N-terminal region of the protein (PfMSP7-N) as prey proteins to screen against full-length SELP (Figure 4.5A). PfMSP7-N but neither of the C-terminal PfMSP7 fragments bound to SELP. This suggests that the functions of the N- and C-termini are distinct; $PfMSP7_{22}$'s role in the MSP1 complex is separate from PfMSP7-N's SELP-binding function. Interestingly, all protein fragments showed some binding to PfMSP1 prey. $MSP7_{19}$ and $MSP7_{22}$ are thought to bind PfMSP1 within the MSP1 complex, but it is unclear whether the N-terminus of PfMSP7 associates with PfMSP1 outside of the merozoite cell, or solely during intracellular protein maturation. It is possible that the binding site in PfMSP7-N interacts only within the parasite with the unprocessed form of PfMSP1, since the N-terminus of PfMSP7 is not detected in the MSP1 complex.

4.2.2.2 SELP-binding was a characteristic of multiple members of the *P. falciparum* MSP7 family

MSP7 has at least five paralogues in *P. falciparum*, each with a similar overall structure but divergent sequence[158]. To determine whether these paralogues share a conserved SELP-binding function, I expressed them as prey proteins to screen against SELP bait (Figure 4.6A). PfMSRP2 and PfMSRP5 bound to SELP, though we cannot eliminate the possibility that the other PfMSRP proteins also bind; I could observe PfMSRP1-mediated nitrocefin hydrolysis after several hours of incubation with the substrate, indicating that a small amount of this prey was captured on SELP bait. The PfMSRP3 and PfMSRP4 prey proteins showed very low expression levels, and thus had to be significantly concentrated, which could impact their ability to bind SELP by AVEXIS. By concentrating cell culture supernatants, we concurrently increase the total protein concentration alongside that of the protein of interest. Thus the preys are incubated with the baits in a much more protein-rich environment, which could potentially block specific interactions from occurring as efficiently.

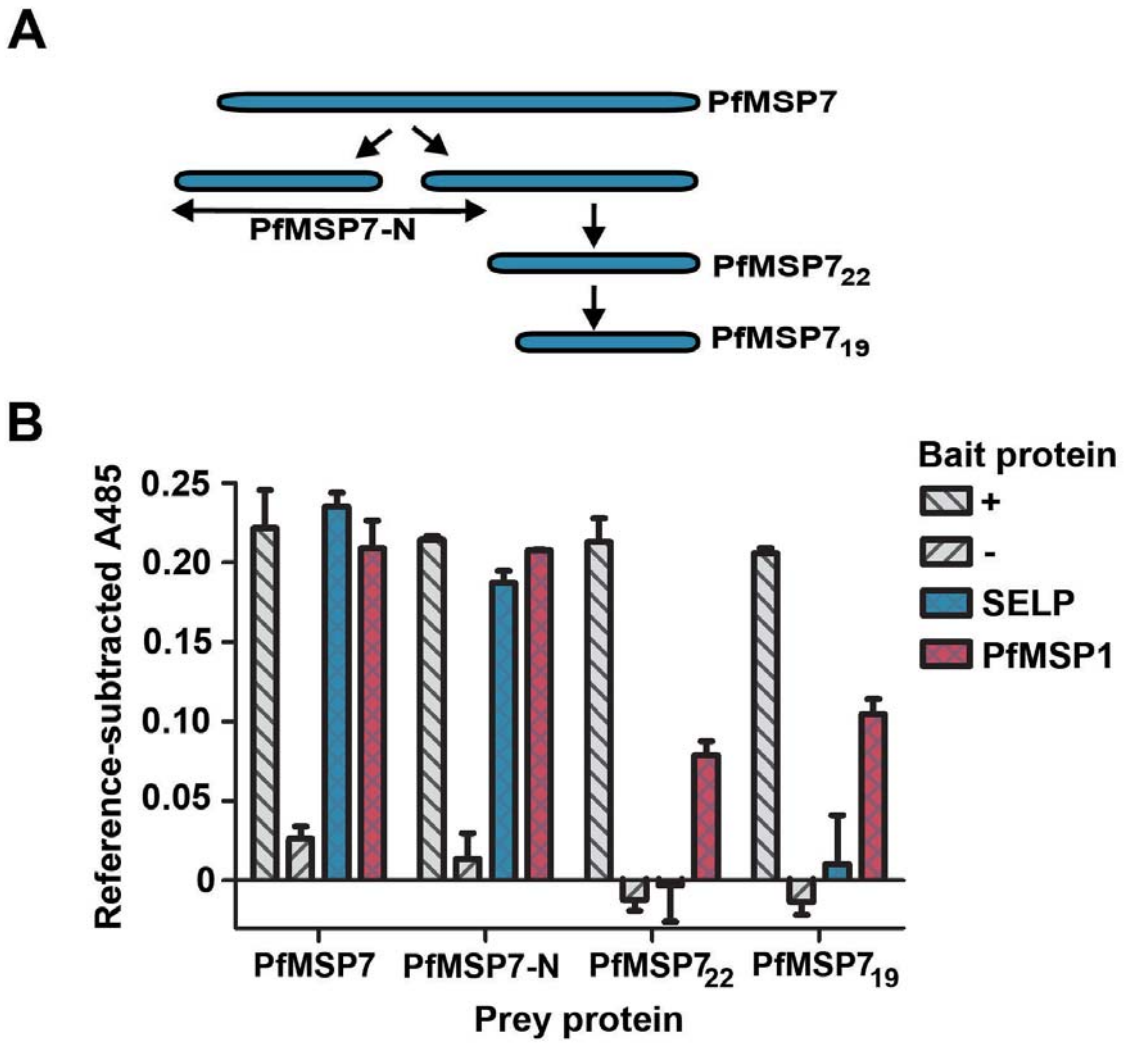

Figure 4.5: **The N-terminus of PfMSP7 bound to SELP**
A. Schematic representation of the truncations made to the full length PfMSP7 precursor. The boundaries of each protein fragment are detailed in Table 2.3. Transitions shown represent the proteolytic processing events believed to occur *in vivo*, detailed in Figure 3.1.
B. SELP bait bound the N-terminal, but not the C-terminal fragments of PfMSP7 by AVEXIS. All PfMSP7 fragment preys bound to PfMSP1 bait. OX68 was the positive control bait (+) and the rat Cd4 tag region was the negative control bait (-). Bars represent means +/- SD, n=3.

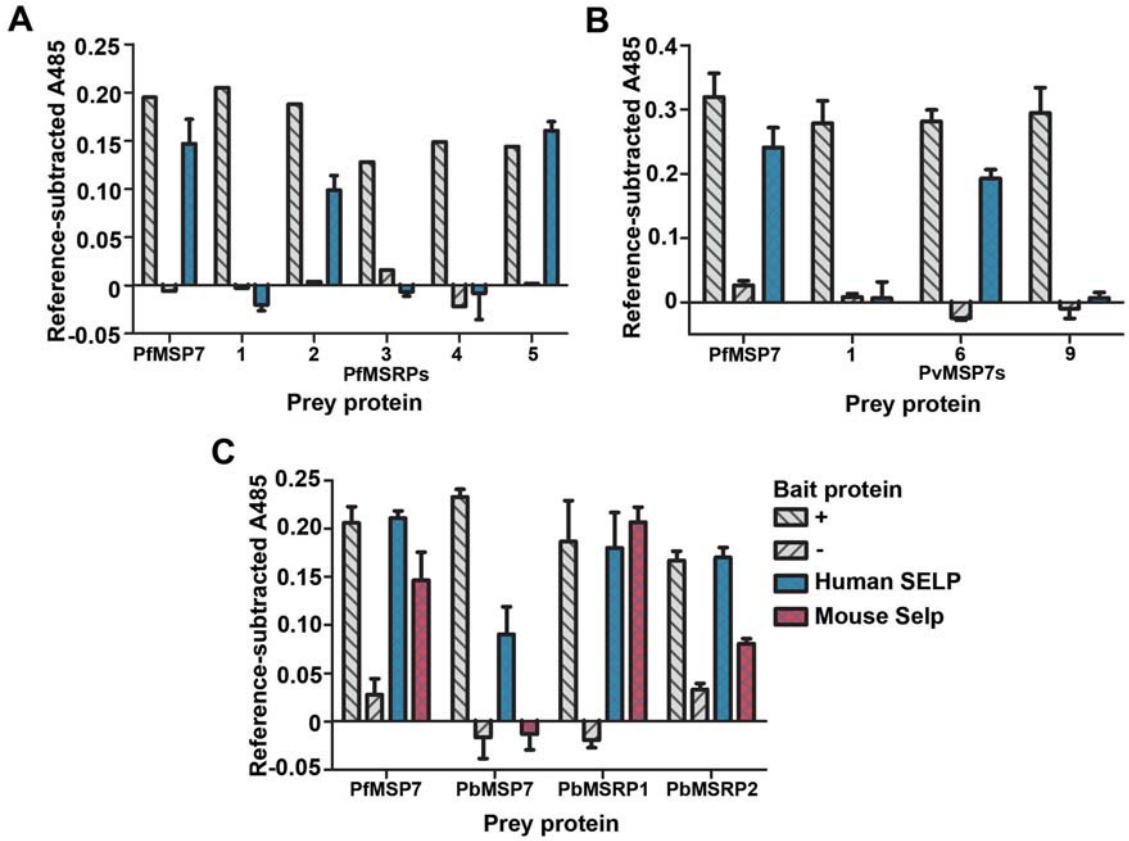

Figure 4.6: **SELP-binding is conserved in multiple *Plasmodium* MSP7s**
A. SELP bait was screened against six *P. falciparum* MSP7 family proteins. PfMSRP2 and PfMSRP5 preys, as well as PfMSP7, were able to interact with the SELP bait. SELP-bound PfMSRP2 prey was able to saturate nitrocefin hydrolysis more rapidly than PfMSP7.
B. Three of the *P. vivax* MSP7 proteins were screened against SELP. PvMSP7_6 (PVX_082675) prey bound to SELP bait.
C. *P. berghei* MSP7-family preys bound to human SELP and mouse Selp bait.
In each experiment, OX68 was used as the positive control bait (+) and the rat Cd4 tag region as the negative control bait (-). Bars represent means +/- SD, *n*=3.

4.2.2.3 The MSP7-SELP interaction was conserved across *Plasmodium* species

To determine whether SELP binding is a conserved feature of *Plasmodium* MSP7s, I expressed three of the eleven *Plasmodium vivax* MSP7s as prey proteins to test by AVEXIS for binding to SELP. PvMSP7_6 prey bound to SELP at sufficient levels to saturate nitrocefin hydrolysis within an hour, whilst no binding was evident when using PvMSP7_1 or PvMSP7_9 preys (Figure 4.6B). I also cloned and expressed the ectodomain of mouse Selp and screened it against the three MSP7 paralogues found in *Plasmodium berghei* (Figure 4.6C). Human SELP and mouse Selp bait bound sufficient PbMSRP1 prey to saturate nitrocefin hydrolysis within one hour, which is a good indication that this *P. berghei* protein interacts with mammalian P-selectin. Similarly, nitrocefin hydrolysis was saturated when I screened PbMSRP2 prey against human SELP. Nitrocefin hydrolysis was also observed, although it was not as rapid, when I used mouse Selp bait. This indicates that PbMSRP2 binds to both selectin baits but possibly does not bind to mouse Selp as strongly as it does to the human protein. However it is also possible that a lower proportion of the recombinant mouse Selp bait is correctly folded and fully functional. PbMSP7 prey expression levels were very low, and the cell culture supernatant was concentrated significantly to optimise and standardise prey activity. I did not observe any nitrocefin hydrolysis when screening PbMSP7 prey against mouse Selp bait, but saw a colour change when using human SELP bait. In summary, I found evidence that *P. berghei* MSRPs bind to selectin but I do not have enough evidence to suggest that PbMSP7 binds.

4.2.3 Investigating the influence of PfMSP7 on known binding interactions of SELP

4.2.3.1 PfMSP7 blocked the interaction between sLe^X and SELP

There is currently no defined biological role ascribed to the N-terminal fragments of PfMSP7. It is likely that PfMSP7's N-terminal fragment(s) are released into the blood stream upon pRBC lysis[158], where they could potentially interact with a myriad of host proteins. One hypothesis concerning the role of PfMSP7's N-terminus is that an interaction with SELP could act to prevent its normal role in the host's anti-parasite immune response. To start to investigate whether PfMSP7 could potentially modulate the normal binding functions of SELP, I tested whether PfMSP7 could block the interaction between immobilised SELP and a sLe^X-alkaline phosphatase conjugate. By incubating immobilised SELP with pentameric PfMSP7 and then introducing the sLe^X reagent, I was able to block the interaction between SELP and sLe^X

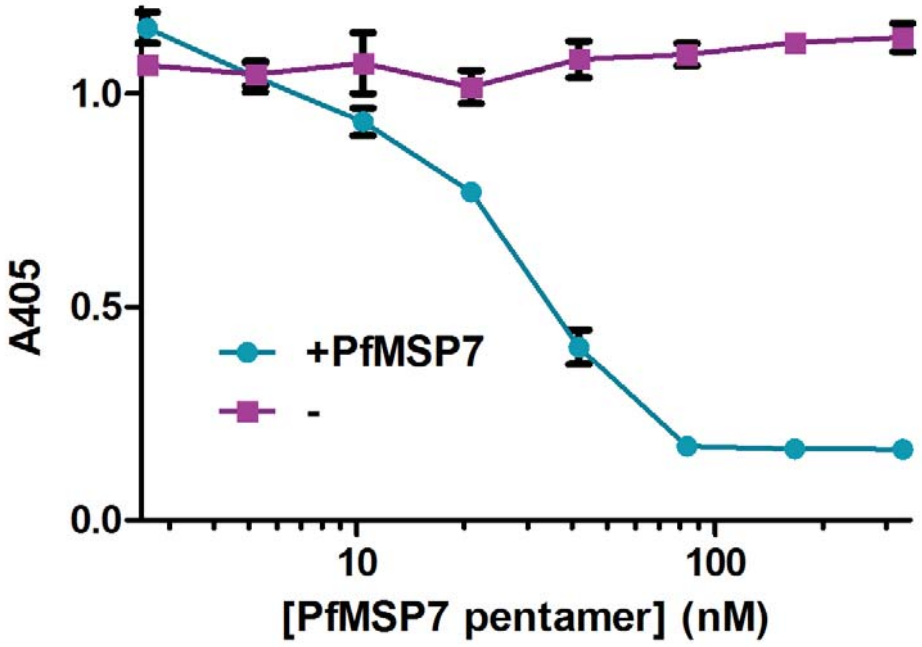

Figure 4.7: **PfMSP7 could block the SELP/sLe^X interaction**
The interaction between SELP bait and a sLe^X-alkaline phosphatase conjugate was detected using absorbance at 405nm produced by the activity of a phosphatase substrate. The interaction was blocked completely by incubating the SELP bait with 0.1µM PfMSP7 pentamers. Bars represent means +/- SD, *n*=3.

(Figure 4.7). This indicated that it is theoretically possible for PfMSP7 to interfere with the interactions that SELP makes with proteins involved in normal immune functionality.

4.2.3.2 PfMSP7 could block cellular adhesion to SELP

To further establish whether it would be theoretically possible for PfMSP7 to prevent the interactions by which leukocytes adhere to endothelium, I developed an assay based on monocytic cells binding a receptor coated-surface (see 2.4.2). I observed that THP1 cells could bind to both SELP and an anti-PSGL1 antibody (but not negative controls) immobilised on the surface of a microtitre plate. This showed that the cells had maintained expression of PSGL1 receptors in culture and also verifies that recombinant SELP is able to bind native ligands such as PSGL1 on leukocytes (Figure 4.8A).

To determine whether PfMSP7 could prevent the SELP-mediated binding of THP1 cells, I

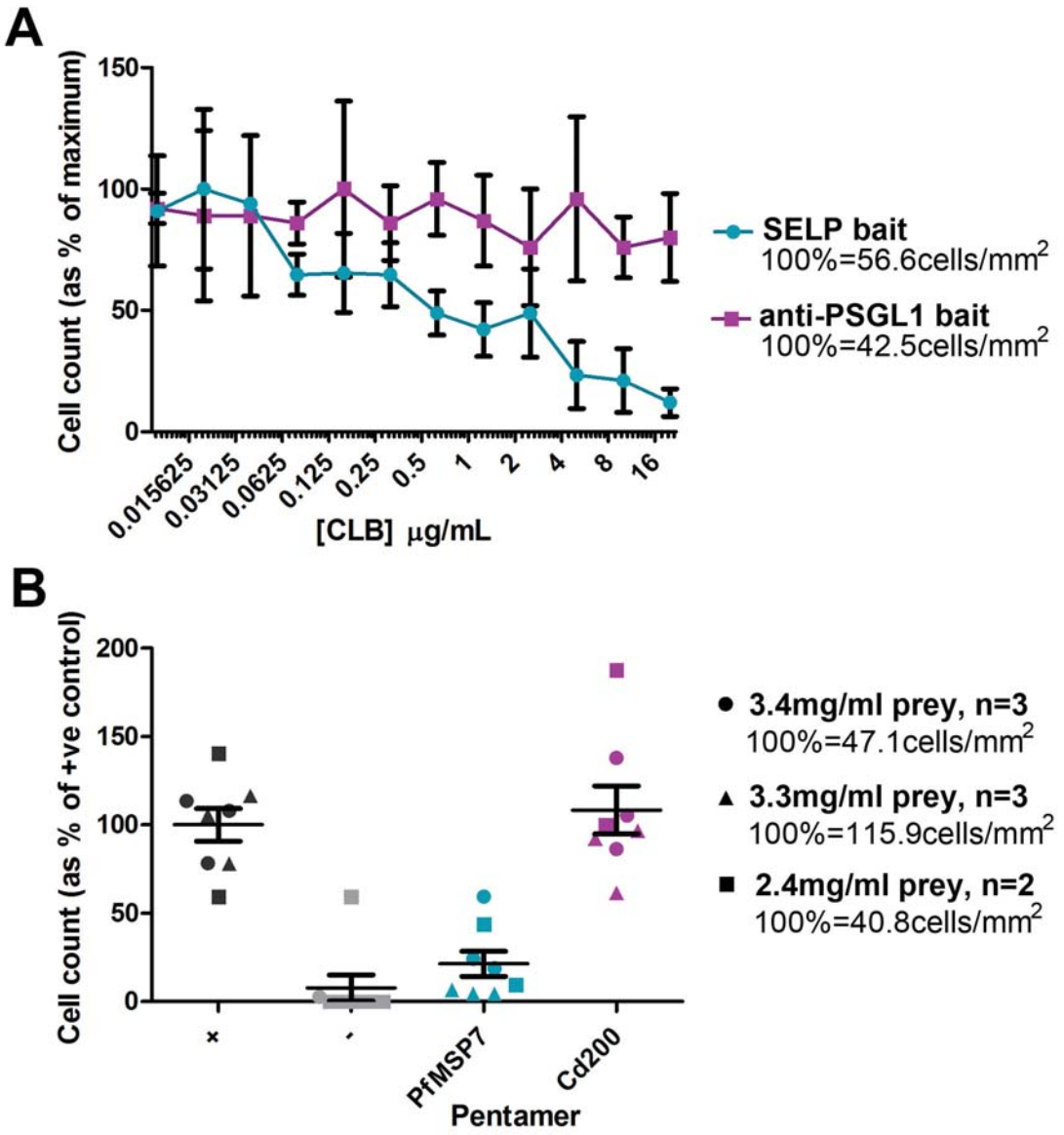

Figure 4.8: **PfMSP7 could block the adhesion of THP1 cells to SELP**
A. THP1 cells adhered specifically to immobilised SELP and anti-PSGL1 antibodies. The interaction between cells and SELP could be blocked by pre-incbating the bait proteins with the CLB-thromb/6 anti-SELP monoclonal antibody. THP1 binding via anti-PSGL1 was unaffected by CLB-thromb/6 antibody. Error bars represent means +/- SD, *n*=3.
B. Pre-incubation of SELP bait with PfMSP7 could prevent the binding of THP1 cells. As a positive control (+), cells were captured using SELP bait which was not subsequently incubated with any other recombinant prey. As a negative control (-) Cd200, to which the cells should not bind, was used as a bait protein. Cd200 pentamer (used instead of PfMSP7) could not block the interaction. This experiment was performed three times, each using an independent PfMSP7 prey preparation, once in duplicate and twice in triplicate. Error bars represent means +/- SD, *n*=8.

incubated the SELP receptors with serial dilutions of PfMSP7 prey[3] prior to the addition of cells. I observed a modest, incremental decrease in adhesion with increasing concentrations of PfMSP7 above approximately 2.5μM, but even 20μM PfMSP7 did not completely block the binding of cells. Using the highest concentrations of PfMSP7 possible (around 35μM or 7μM (700pmoles) of pentameric protein) from three independent preparations, I was able to block the interaction from occurring (Figure 4.8B). The requirement for such a high concentration of PfMSP7 pentamer to achieve a blockade of leukocyte binding is surprising, especially since a theoretical maximum of just 20pmoles of SELP protein can be immobilised in a single well. Significantly lower quantities of PfMSP7 (2pmoles of pentamer, effectively 10pmoles of PfMSP7 monomer) were required to block the interaction between SELP and sLe^X-AP (Figure 4.7). This discrepancy might reflect differences in the proportion of functional proteins in the preparations, such that a lower proportion of SELP and/or a higher proportion of the PfMSP7 used in Figure 4.7 participated in binding interactions compared to the preparations used in these experiments using THP1 cells. However the relative difficulty in blocking the interaction between SELP and THP1 cells might lie in part with the strength of the interactions involved; the interaction between individual $sLe^X$and SELP proteins is of relatively low affinity, with estimations ranging between 0.1 and 7.8mM [33, 171, 268], whereas the interaction between SELP and PSGL1 and the interaction between SELP and neutrophils are of substantially higher affinity, with calculated K_Ds of 3-320nM and 70nM respectively [63, 213, 342]. As a consequence it is likely to be more challenging to use PfMSP7 to block the interaction between SELP and THP1 cells than it is to block the interaction between SELP and isolated sLe^X. These *in vitro* data show that it is theoretically possible for PfMSP7 to prevent the SELP-mediated interactions between leukocytes and endothelia, but, given the high affinity of the SELP/PSGL1 interaction, this may require a high local concentration of PfMSP7 proteins.

4.3 Discussion

4.3.1 Further evidence that the PfMSP7-SELP interaction is biologically relevant

The data presented in this chapter further validate that recombinant PfMSP7 and SELP interact. The interacting domains on both proteins can be isolated (Figures 4.2A & 4.5) and the interaction can be replicated using variant SELP proteins, though PfMSP7 is unable to

[3] for 90 minutes, after which unbound protein removed by washing

bind to the potentially mis-folded A156T SELP variant[4] (Figure 4.4). Combined with the evidence that PfMSP7 prey cannot bind to SELP in the presence of EDTA (Figure 4.2B), which changes the conformation of the binding domain, this indicates that PfMSP7 can only bind to functionally active recombinant SELP. AVEXIS data indicate that SELP-binding is a conserved property of MSP7 proteins in multiple parasite species (Figure 4.6); these MSP7 proteins are diverse in sequence yet maintain the ability to bind SELP. Whilst this may not be the sole function of the MSP7 family, the ability of the proteins to bind SELP - an important adhesion molecule and component of the host immune system - may be part of the reason a repertoire has been conserved in *Plasmodium* parasite species. By demonstrating that PfMSP7 is able to block the interaction of SELP with sLe^X and, albeit less potently, with leukocytes (Figures 4.7 & 4.8), I have shown that there is a possible *in vivo* function for SELP/MSP7 interactions, whereby MSP7 prevents the binding of SELP to its human ligands (see 4.3.3).

4.3.2 The SELP-PfMSP7 interaction is unlikely to be involved in RBC invasion

The true test of the importance of this biochemical data will be if we can demonstrate an *in vivo* function for this interaction. Existing knowledge about PfMSP7 has implicated the protein in merozoites' entry into host RBCs[159, 164, 327]. It is unlikely that the interaction between SELP and PfMSP7 is involved in the invasion process for two main reasons. Firstly, SELP is not known to be present in the RBC proteome[262] and was not detected on the cell surface (Figure 4.9). Secondly, our data indicate that it is the N-terminus of PfMSP7 that is involved in SELP binding, and this part of the protein is not detected on the merozoite surface. To further investigate the role and localisation of PfMSP7, I purified His-tagged PfMSP7 to immunise rabbits and produce anti-PfMSP7 polyclonal antibodies. These antibodies bound specifically and sensitively to recombinant PfMSP7, as observed by ELISA and Western blot (Figure 4.10A). However, they were not able to replicate the invasion-blocking effect previously reported for anti-PfMSP7 polyclonal antibodies[164]. The levels of invasion observed when this antibody was added into an RBC invasion assay were 99.5% (+/- 3.345) of that observed when no antibody was added[5]. Despite being able use this antibody to detect very small quantities of recombinant PfMSP7 by Western blotting, I was unable to detect any PfMSP7 in the lysate from Percoll-purifed cultured *P. falciparum* schizonts (Figure 4.10B). This is surprising as PfMSP7 fragments are expected to be abundant on the surface of, and secreted from

[4] It is possible that this variant is a sequencing error in the database.

[5] This assay was performed by Dr Leyla Bustamante and is described in Theron *et al.*, (2010)[330]. The assay was performed in triplicate

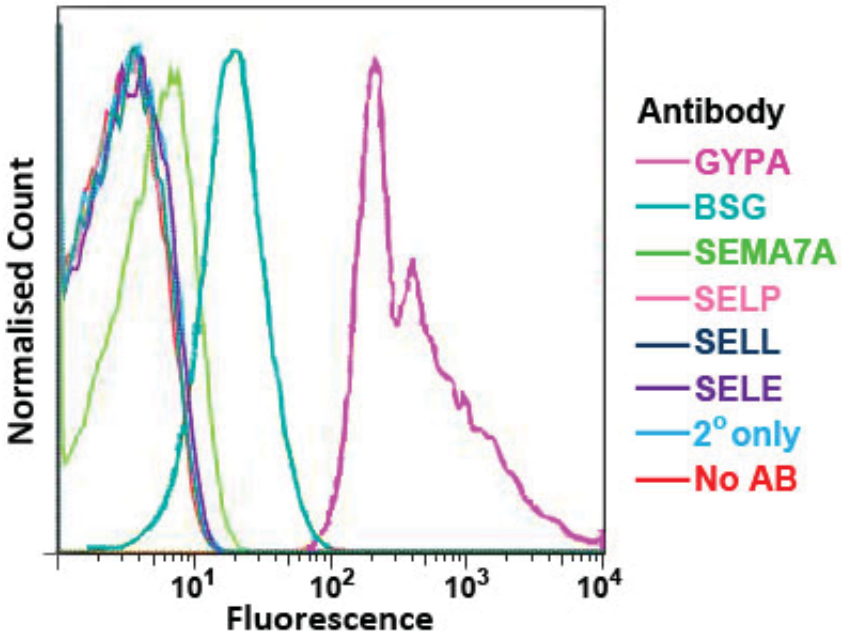

Figure 4.9: **Selectins were not detectable on the RBC surface**
A. Monoclonal antibodies against a range of RBC surface proteins and selectins with an Alexa488-conjugated secondary could not detect selectins on the RBC surface. Positive control proteins Glycophorin A (present at approximately 10^6 copies/cell), Basigin (10^4 copies/cell) and Sema7A (10^3 copies/cell) were all detected. 2000 events were counted.

schizont-stage parasites. However, I was also unable to detect PfEBA175 or PfMSP1 in this lysate using polyclonal antibodies in the same manner. This might indicate that this lysate is not a reliable source of intact merozoite proteins. Whilst our invasion assay results imply that PfMSP7 does not play an important role in RBC invasion, we cannot use these data to eliminate this possibility as we cannot be certain that the antibody is able to bind to PfMSP7 of parasite origin.

4.3.3 SELP-binding may have anti-inflammatory function

The binding of endothelial SELP to leukocyte PSGL1 is thought to enhance inflammation and promote phagocytic activity of immune effector cells. This process is potentially deleterious to the parasite as it results in the deployment of cytokines such as TNFα, which has known parasite-killing activity. An attractive hypothesis concerning the role of the SELP/PfMPS7 interaction is that the N-terminus of PfMSP7 acts as an anti-inflammatory mediator, binding SELP, blocking its interaction with PSGL1 and preventing the downstream enhancement of the anti-parasite immune response (see Figure 4.11). The evidence that PfMSP7 can block

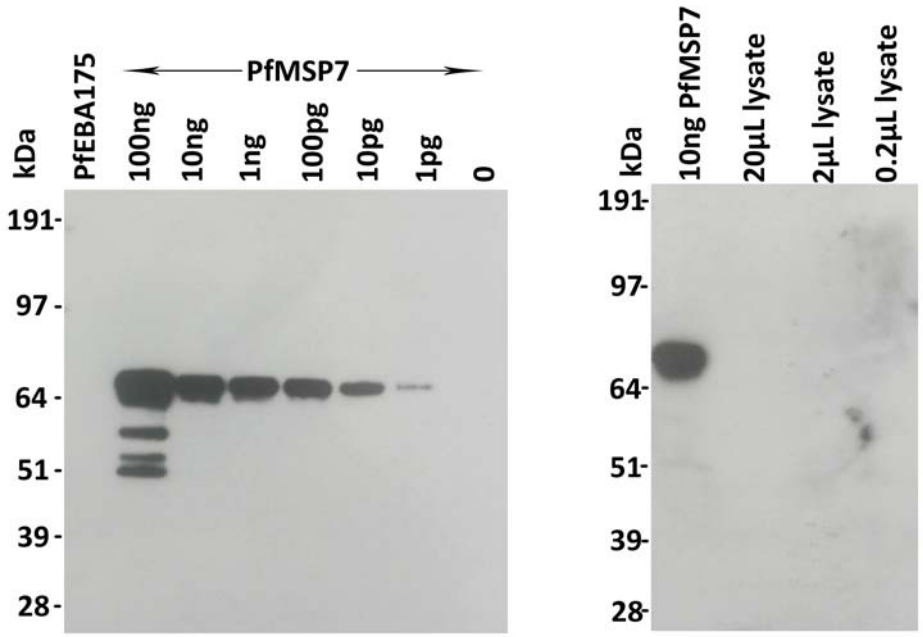

Figure 4.10: **PfMSP7 was not detected schizont lysate**
A. Polyclonal anti-PfMSP7 antibodies were used to detect even picogram quantities of PfMSP7. The antibodies were specific to PfMSP7 as no bands were observed when 100ng PfEBA175 was transferred to the membrane.
B. The same antibodies were not able to detect PfMSP7 fragments in Percoll-purified schizont lysate.

the interaction SELP makes with sLe^X and potentially leukocytes is an encouraging first step towards demonstrating that PfMSP7 has an anti-inflammatory role. We have collaborated with another research group who have developed an assay to measure SELP-dependent leukocyte rolling on endothelial-derived cells[74]. We hypothesised that by incubating the endothelial cells with PfMSP7 we would see a reduction in rolling when flowing leukocytes over the cell monolayer. In preliminary assays we did not observe a reduction in rolling, in fact both the PfMSP7 and Cd200 negative control seemed to increase rolling. The assay conditions are optimised for using antibodies to block protein interactions involved in rolling, and may need to be refined further for using pentameric proteins. I would like to revisit these assays in the future.

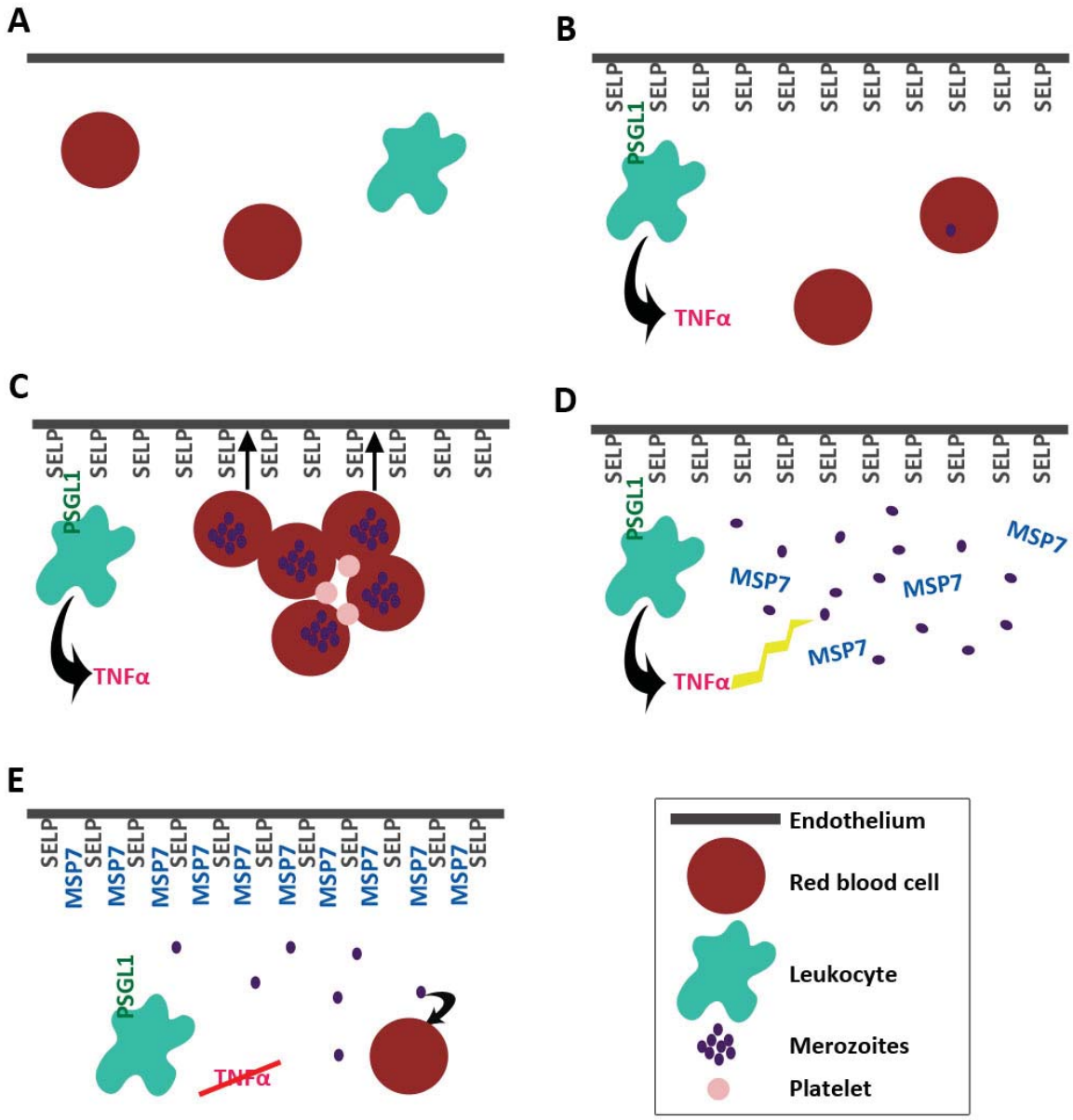

Figure 4.11: **Hypothesised anti-inflammatory activity of PfMSP7**
A. Prior to infection the endothelium is in a resting state.
B. Early in infection, the endothelium is activated and rapidly presents surface SELP. This results in the rolling adhesion of leukocytes via sLe^X-containing ligands such as PSGL1. This interaction can promote cytokine production and phagocytosis in leukocytes.
C. Aggregated pRBCs sequester on the endothelium, where they may be targeted by immune effectors.
D. The pRBCs rupture, releasing merozoites and PfMSP7 fragments into the blood stream. Free merozoites are vulnerable to the host immune response.
E. PfMSP7 N-terminal fragments bind endothelial SELP and prevent endothelium-leukocyte interactions. This dampens the immune response and allows more merozoites to successfully re-invade new RBCs.

4.3.4 Interactions between SELP and MSP7s could modulate a range of other cellular processes, including cytoadhesion.

Inflammation is not the only process in which an interaction between SELP and PfMSP7 could play a part. For instance, SELP is also an important player in blood coagulation[7, 51, 90]. Given the precedent set by blood-borne bacteria for pathogens producing anti-coagulant mediators to avoid being confined by a growing thrombus[318] it is possible that PfMPS7 could perform a role of this kind. The previous implication of Selp and PbMSP7 in the generation and severity of cerebral malaria in mouse models, such that the absence of either gene mitigates ECM, is particularly striking[59, 313]. It is possible that the binding of MSP7 proteins to SELP directly or indirectly contributes to blood-brain barrier damage, and/or affects the accumulation of leukocytes (in ECM) or RBCs in cerebral microvasculature. SELP is amongst the repertoire of endothelial receptors suggested to bind to PfEMP1 on the surface of the pRBC[302, 367]. This interaction is thought to enhance the sequestration of pRBCs but is not essential for the process to occur. By virtue of its potential binding to PfEMP1, SELP has also been implicated in platelet-mediated clumping of pRBCs[259]. It is plausible that MSP7s could affect either of these SELP-mediated adhesive processes, either by preventing them from occurring or by exacerbating them. In the hypothetical case whereby MSP7s enhance, rather than block, these cytoadhesive processes this phenomenon could contribute to explaining the deleterious effect of the presence of PbMSP7 on the outcome of infection for the host. The experimental work required to accurately define the function of the SELP/MSP7 interactions observed *in vitro* will be discussed in 6.3.2.