TARGETING THE ROLE OF SUBTILISIN-LIKE PROTEASE 2 FOR INHIBITION OF ERYTHROCYTE INVASION BY THE MALARIA PARASITE, *PLASMODIUM*

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Abstract

Malaria is a mosquito-harbored infectious disease causing approximately half a million deaths every year around the world. Out of the five *Plasmodium* species that infect humans, *P. falciparum* is the deadliest. Despite the relative success in decreasing malaria-related deaths through various efforts, emergence of parasite resistance against antimalarials remains a major challenge. This is mainly because the parasite develops resistance before new effective drugs can become available. In addition, there is no approved vaccine for malaria that will prevent the infection in most groups affected. The protection offered by the malaria vaccine candidate, RTS,S, currently on phase III clinical trials, is less than 40% in children when used along with bed nets and other malaria prevention recommendations. Additional vaccine candidates are needed to provide better protection against malaria. The characterization of molecular targets allows the development of inhibitors against the parasite via rational design, helping to advance the development of vaccine and treatment.

Subtilisin-like protease 2 (SUB2) is the only *Plasmodium* subtilisin playing a direct role during invasion of the red blood cell (RBC), a critical step in malaria parasite development during the asexual, symptom-causing stages. SUB2 merozoite surface sheddase (MeSh) activity is essential for parasite survival and RBC invasion. A SUB2-specic inhibitor will lead to impairment of invasion. Additionally, SUB2 is secreted onto the surface of the parasite to access its substrates, staying exposed to the antibodies in the blood, making it a merozoite surface antigen itself and a

candidate for antibody-mediated inhibition. This makes SUB2 both a potential drug target and a vaccine candidate.

At the present, our understanding of SUB2 biochemistry and biophysical properties is limited and now studies have tested this subtilisin as a vaccine candidate. In this dissertation, we show that antibody-mediated inhibition results in decreased parasite infection in a proof-of-principle experiment with mice. We have also attempted to characterize the two SUB2 peptides utilized in immunization experiments by using a self-assembling protein nanoparticle on a different, but related, experiment using a mouse model of malaria. Finally, we develop an expression system for active SUB2 as well as a SUB2-specific protease assay with native SUB2 substrates.

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“Little by little, one travels far.”

J.R.R.T.

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Abbreviations

BCIP	5-bromo-4-chloro-3-indolyphosphate
βME	β-mercaptoethanol
cDNA	complementary deoxyribonucleic acid
CFP	cyan fluorescent protein
DDT	dichlorodiphenyltrichloroethane
diH_2O	deionized water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxynucleotide
HEPES	4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid
HIV-1	human immunodeficiency virus type 1
IPTG	isopropyl β-D-1-thiogalactopyranoside
KLH	keyhole limpet hemocyanin
kDa	kiloDalton
M	molar
MERS	middle east respiratory syndrome
MeSh	merozoite surface sheddase
ml	milliliter
mM	milimolar
MPa	megapascal
NBT	nitro-blue tetrazolium

PCR	polymerase chain reaction
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RPM	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
SAPN	self-assembling protein nanoparticles
SARS	severe acute respiratory syndrome
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBST	tris-buffered saline and tween 20
Tris	tris(hydroxymethyl)aminomethane
µg	microgram
µl	microliter
µM	micromolar
WHO	World Health Organization
YFP	yellow fluorescent protein

Chapter 1

Introduction

Malaria

Malaria is an infectious disease caused by obligate intracellular parasites of the genus *Plasmodium*. Five *Plasmodium* species have been identified to infect humans and *P. falciparum* is known to be the deadliest[1]. Given the appropriate resources and knowledge, malaria is a preventable and treatable disease. However, at least half a million deaths are attributed to malaria worldwide every year [1] .

In theory, successful control measurements and treatment of an infectious disease can be achieved by studying the pathogen responsible of it. Since the late 1880s the causative agent of malaria was known to be a protozoan organism and its mode of transmission was known by the early 1900s [2]. By understanding the causative agent of a disease and its mode of transmission, it is possible to start working on a strategy to deal with this illness. As an example, a mosquito can be killed with insecticides (e.g. DDT) and the vector populations can effectively be reduced when the water sources where they develop are removed [3]. Likewise, the more we study the pathogen itself, the more information we gather in order to target specific mechanisms for its intervention. At the present, we can take advantage of new methods and technologies to design molecules against the parasite upon the characterization of chosen molecular targets [4, 5]. Similar approaches can also be employed to characterize antigens for the development of new vaccines for the prevention of malaria [6].

Numerous factors contribute to the difficulty in controlling malaria in some regions. Some of these contributing factors are [1]: the emergence of drug-resistant

strains of the parasite, insecticide resistance of the mosquito vectors, environmental conditions that favor the spread of mosquitoes, quality and availability of antimalarial drugs, the political and economical stability of the affected regions, resources, cultural and religious beliefs, among others. In this case, to control malaria in the regions where it is still endemic, it is critical to have all the necessary components. The ultimate goal is to eradicate malaria worldwide, as it was possible to accomplish in some regions of the world several decades ago [7, 8].

A crucial part of this plan is to have effective antimalarial treatment available. One of the major challenges that we face in our fight against malaria is that the parasite quickly develops resistance against malaria pharmacotherapy. In the late 1950s, chloroquine resistance was already discovered. More recently, at the beginning of 2015, artemisinin resistance was reported in the Myanmar-Indian border. Resistance to this frontline treatment against malaria was also reported in Cambodia, Laos, Thailand, Vietnam and Myanmar. As a result, artemisinin-based combination therapies can rapidly become less effective in the regions where it is needed the most. This scenario is particularly alarming, as parasite resistance can potentially spread in sub-Saharan Africa, where most of *P. falciparum* malaria deaths are reported [1].

Malaria prevention and other control measurements

To compensate for parasite resistance to existing antimalarials, new compounds have been developed [1]. Still, not all compounds are recommended for general use unless there is strong evidence of effectiveness and safety. For that

reason, the more compounds we have available, the better the chances we have to find those that are effective and safe to use. We will also continue learning about which classes of compounds act better on the parasite and identify those that are safer for humans. Moreover, taking into consideration that malaria is treated differently depending on the severity of the disease (e.g. uncomplicated vs. severe malaria), the individual affected (e.g. children, pregnant women, travelers with no immunity) and the malaria species that is being treated for, we definitely need to have in hand a variety of compounds with different mechanisms of action. This will allow us to treat every malaria case properly, reducing the amounts of deaths. Appropriate use of antimalarials could impact the development of drug resistance in the parasite by reducing the occurrence of these resistant strains. As a consequence, effective drugs will remain functional for a longer period of time. Again, this highlights the importance to continue developing novel compounds against malaria.

Rational drug design [9] promises to aid in hastening the development of effective and safe antimalarials. This methodology consists in developing compounds for a known and specific molecular target, ideally, one that has a critical function in an indispensable biological process of the parasite. This approach utilizes structural information in combination with biochemical, computational and biophysical methods to identify and develop new molecules. In this way, compounds whose molecular targets are known and have been previously characterized and extensively studied, in general, can be developed.

Vaccines are among the vital components in the fight against malaria and they can also be developed based on a specific target [1]. RTS,S/AS01 [10] is

currently in Phase III trials in seven countries in sub-Saharan Africa. This is one of the first antimalarial vaccines that have been developed, and it has been evaluated as a component of other preventive measurements such as, artemisinin-based treatment, and insecticide nets.

The malaria parasite

Malaria is an infectious disease caused by unicellular eukaryotic organisms of the genus *Plasmodium*. These obligate intracellular parasites belong to the phylum *Apicomplexa*. This group is characterized by containing a plastid-like organelle: the apicoplast and a complex apical structure utilized in host cell invasion. Over 100 species of *Plasmodium* parasites have been identified. They are known to infect several vertebrate species including reptiles, birds and mammals. Identifying the species of malaria that affect different vertebrates is important for species conservation, agriculture, ecology, economy and medicine. It is known that malaria infection in humans causes more than half a million deaths every year[1].

Five *Plasmodium* species are known to infect humans: *P. falciparum, P. vivax, P. ovale (two species) and P. malariae* [1]. Non-human primate malaria species can also infect humans, which is the case for *P. knowlesi*. All human and non-human *Plasmodium* species require a mosquito and a vertebrate host to complete all the stages of their life cycle (Figure 1.1). For the purpose of our studies, we are mainly interested in *P. falciparum* because it is the human malaria species that causes most

deaths worldwide [1] . We also study *P. berghei*, a mouse malaria species, in order to perform malaria infection experiments *in vivo* with a model vertebrate[11].

Sexual development: mosquito stages

The malaria parasite undergoes sexual development in the mosquito host [12]. For this reason, the mosquito is considered its definitive host. Out of the thousands mosquito groups, only *Anopheles* mosquitoes are known to transmit malaria. Transmission to the human host occurs when an infected mosquito delivers saliva with sporozoites during a blood meal.

A mosquito becomes infected after feeding on an infected human that has early sexual stages of the parasite circulating in the blood [13, 14]. A few male and female gametocytes are ingested, mixed in the blood meal. Once inside the mosquito's gut, these mature into gametes and fertilization occurs, leading to the development of a motile zygote: the ookinete [13]. Once an ookinete is formed, it has to invade the epithelial cells in the mosquito to move across the midgut until it has access to the basal lamina, where it stays immobilized as an oocyst [12]. At this point in the life cycle, the number of parasites present in the host is greatly reduced. Out of the billions of parasites circulating in the human blood, less than 10 ookinetes usually develop into oocysts in the wild. For this reason, the ookinete is considered a bottleneck [15, 16] point during sexual development, which makes this stage an attractive target for small molecule or antibody-mediated intervention in the mosquito.

Once the oocyst develops, the parasite undergoes multiple replications to increase in numbers and it specializes into an immature form of the infectious stage to humans: the sporozoite. Sporozoites are then released into the mosquito's hemocoel and "circulatory system" so they can migrate and invade the salivary glands to completely mature and become infectious to humans [17]. Once in the salivary glands, the parasites are ready to be transmitted during a bite. This is the stage that is targeted by the current malaria RTS,S vaccine [18] after they enter the human. Priming the human immune system to recognize and interfere with the sporozoites before they reach the liver and continue their life cycle is a valid vaccine strategy.

Sexual development is important for recombination of genetic material, which results in species stability. By this mean, the parasite also acquires and maintains genetic changes that confer resistance to drugs. Targeting mosquito stages for transmission control strategies [19] can prevent the spread of new drug-resistant strains, besides the fact that it will reduce the number of cases of malaria. Bottleneck stages are an attractive target for chemotherapeutic intervention because the number of parasites is already reduced.

Liver stage

After being deposited in the human skin through a mosquito bite, the sporozoites have to find their way to the liver in order to continue their life cycle [20]. The parasites are delivered to the liver via the blood stream after they find the capillaries in the skin. Once in the liver, the parasite transverses and invades the

hepatocytes until they start to transform and develop [20]. During development in the liver [21], the parasite undergoes several rounds of replication to increase in numbers and transforms into a merosome [20], containing merozoites. These merozoites are equipped with molecular components that allow the parasite to invade the circulating red blood cells and develop within them [22]. Fully developed merozoites are released from the liver into the blood stream, where they have access to another target host cell: the erythrocyte [22].

In the liver, the parasite is refuged from the immune system within the hepatocytes [16], leading to no signs of infection. Two human malaria species (*P. vivax and P. ovale*) can remain dormant at this stage [23, 24] for several weeks, without being released into the blood. The *Plasmodium* liver stage also represents a bottleneck in the life cycle since the number of parasites is also reduced from almost a hundred sporozoites that are injected by the mosquito, to a few merosomes that develop finally in the hepatocytes. The liver stage is a target for preventing malaria infection in the blood once the parasite is transmitted to the human host.

Asexual cycle: blood stages in the human host

For the necessary stages, this obligate intracellular parasite has the molecular components for recognizing, invading and developing within a host cell [25, 26]. The merozoite is a highly specialized polar cell that contains all the components necessary for red blood cell invasion at its surface and inside specialized organelles that are discharged through the apical end of the cell during the process of invasion [27]. But first, the free merozoite has to encounter a red

blood cell and become attached to it. Then RBC invasion takes place until the parasite has completely entered the cell without breaking the RBC membrane. As a result of the invasion process, the parasite ends up surrounded by the outside of the erythrocyte membrane previously exposed to the extracellular space, which is subsequently modified by the parasite during the development [22].

There are four forms of the parasite during the RBC cycle [28]: the merozoite, the ring, the trophozoite and the schizont. Of these, only a merozoite is an extracellular form, for a short period of time between egress from the RBC and RBC invasion. The other forms consist of other developmental stages within the RBC. The ring stage develops after the invasion of the RBC is completed. At this stage, the parasite is transformed and begins to become metabolically active. After this, the trophozoite starts to develop, increases in size and becomes highly metabolically active. During this stage, the parasite starts to feed on the very abundant hemoglobin protein in the RBC [29, 30]. Metabolic activity of the parasite is greatly increased such that several distinct cellular processes are taking place. The host cell is being modified by the parasite, *P. falciparum*, and the surface of the erythrocyte is modified so that this host cell can attach to the endothelium in the circulatory system and stay sequestered during the rest of the parasite development [31-33]. Thus, only early stages of the parasite circulate in the blood.

Later asexual stages, however, stay circulating in the blood for the other human malaria species. The stage referred to as the schizont begins with an increase of genomic material due to genomic DNA replication. After the entire genomic content of the parasite is replicated several times, the daughter cells begin to form

and the formation of new merozoites takes place. These new merozoites will then egress from this infected erythrocyte in order to invade uninfected RBCs that are circulating in the blood [22]. Then, the parasite will start development within the RBC once again, perpetuating this asexual cycle. These intraerythrocytic stages will continue to develop until the parasite is eliminated by the host's immune system or until it kills the host. *P. falciparum* is the human malaria species that is responsible for more deaths as a consequence of complications that develop during asexual stages of the parasite.

The parasites in the RBC are largely hidden from the human immune system [34]. The malaria symptoms arise only when the merozoites are free in the blood and can be detected by the immune system for a short period of time. During this window, the parasite is exposed to all the components of the human blood, including antibodies that can have access to the proteins at the surface of the merozoites [35-38]. This offers an opportunity for interfering with the parasite before it infects another RBC. The rapid and diverse metabolic processes taking place at this stage make it an optimal target for antimalarial drugs.

Red blood cell invasion

Invasion of a host cell is essential for the survival of an obligate intracellular parasite such as *Plasmodium*. Entering a host cell guarantees a plentiful food source and refuge from the immune system while ensuring appropriate development needed to continue the life cycle [22, 26, 34, 39]. Red blood cell invasion occurs in highly regulated steps. First, the parasite attaches to the surface of the erythrocyte

in a non-specific orientation. Presumably, this initial contact results from the accidental encounter of a free merozoite and a circulating RBC. This attachment is mediated by adhesins at the surface of the merozoite that engage receptors on the erythrocyte membrane [22]. Secondly, the parasite reorients itself so that the apical domain is facing towards the surface of the RBC. Subsequently, the proteins involved in the process of invasion are discharged from secretory organelles in a sequential manner to assure proper control of this process [22].

Disruption of any important step in this process would be expected to result in unsuccessful invasion and parasite death. For instance, surface ligand processing for maturation is necessary for successful invasion. Two well-studied surface adhesins are merozoite surface protein 1 (MSP1) [40-42] and apical membrane antigen 1 (AMA1) [43, 44]. These are prime targets for vaccine and drug development. These proteins coat the surface of the merozoite and interact with their receptor partners at the surface of the erythrocyte. This attachment is resolved by the removal of the ectodomain of these ligands by a single cleavage event at a flexible juxtamembrane site performed by a sheddase [25, 45]. This surface sheddase, subtilisin-like protease 2, carries out the key maturation step for these ligands. The catalytic activity of this protease is essential for RBC invasion. As with any critical step of a biological process, inhibition of SUB2 activity would render this host invasion step unsuccessful, stopping the life cycle of the parasite at this point.

In the case where SUB2 activity is carried out as usual, the process of invasion would continue uninterrupted to completion once the parasite becomes

completely enclosed in the RBC membrane. During this process, the outer membrane of the RBC forms the parasitophorous vacuole inside the now infected erythrocyte. This complex process of invasion is also supported by other components such as an actin-myosin motor, ions and other multifunctional proteins, providing several targets for inhibition. For the purpose of this dissertation, however, we will focus on SUB2, the second identified *Plasmodium* subtilisin-like protease, which has been established to perform a critical role in invasion and is therefore essential to parasite survival.

Gametocytes in the blood: and so the life cycle continues

Male and female gametocytes [46] develop during the blood stages of the parasite [14]. These are early forms of sexual stages and only can continue their development within the mosquito. Micro- and macrogametes are ingested in the blood meal mixture when a mosquito feeds. Subsequently, these gametes will mature inside the mosquito's digestive system and fuse to form the ookinete [13]. If there is no interference with these developmental stages, the malaria parasite will develop further inside the mosquito, thereby allowing the infection cycle to be completed. And, once again, the infectious form of *Plasmodium*, the sporozoites, will be transmitted to another human during the next blood meal.

Conclusion

In general, the complex life cycle of the malaria parasite offers multiple points for intervention. The asexual cycle in the red blood cells is of special interest

because it results in malaria symptoms in humans. Severe malaria cases, especially those caused by *P. falciparum*, can be fatal, mostly to children and pregnant women. Thus, characterization of molecular targets during this stage can help to develop new treatment and vaccines. Other stages are also of interest for malaria prevention and transmission control.

Proteases at the service of pathogens

All proteases share a common characteristic: they all catalyze a reaction that results in the breaking a peptide bond of a given substrate [47]. The biochemical environment at the active site determines the specific reaction mechanism, in this case, amino acid residues, metal ions, water and other small molecules that may be involved in the biochemistry of a specific protease [48]. As can be expected, there are many other factors that determine substrate specificity, substrate access, and the active or inactive form of the protease, among others. All of these factors allow controlling and specifying the impact of a protease on a given cellular function. In this way, proteases can perform a vast variety of roles. They function as activators or inhibitors of protein function,; they are involved in maturation of ligands and receptors, in the release signaling molecules, and have essential roles in cell death, in the pathogenesis of parasites and perform other vital functions in other organisms [49-56].

Proteases as key targets for treatment of infectious diseases

Infectious agents often require the function of proteases for pathogenesis [57-59], survival and development within the host. In some cases, it is protease produced by the parasite itself, in other cases; the parasite uses the host's proteases to carry out a function for them. An example of the latter case is the use of host serine proteases to cleave the influenza virus hemagglutinin (HA) to permit the virus to gain entry into the epithelial cells in the respiratory tract [60, 61]; targeting the activity of this protease would be expected (or is known to?) halt virus propagation. A similar strategy is used by other viruses, including Ebola virus, the SARS-coronavirus and the MERS-coronavirus [62-67]. Thus, the development of specific protease inhibitors is a promising strategy to prevent the spread of these viruses within the host. Still other viruses code for proteases that can be used as targets, one notable success story involves development of antiretroviral therapeutics against HIV-1 infection, which are based in part on protease inhibitors that target HIV-1 aspartic protease [68-71].

The involvement of proteases in pathogenesis is not only observed with human viruses but also with other disease-causing agents, such as bacteria and protozoan parasites. As an example, *Ralstonia pickettii*, causes severe bacterial infections to individuals with poor health and is usually acquired in hospitals [72]. A metalloprotease was found to contribute to the cytotoxic activity. As another example, *Entamoeba histolytica*, is a protozoan parasite that uses the action of secreted cysteine proteases to adhere to the host cells [73].

Protease inhibitors are often used as chemotherapy against a variety of parasitic organisms. It is important to point out that these inhibitors are all intended to be specific for the target protease, even though, in some cases, the same inhibitor or its derivatives are effective on related proteases encoded by different viruses or pathogens.

Subtilisins: an overview

Serine proteases have been extensively studied and are known to be involved in a broad variety of cellular processes, including protein activation, apoptosis, cell quality control and regulation of signaling pathways [74-79]. Subtilisins are a subclass of serine proteases that are structurally distinct from the chymotrypsin family and their similarity in function is a result of convergent evolution. Mainly, subtilisins drew a lot of attention because of their application in biochemical studies and commercial use [80-82]. Bacterial subtilisins are easy to express in a soluble and active form in bacterial expression systems and they can cleave a variety of substrates, making them suitable for commercial applications, specifically as detergent additives.

However, not all subtilisins are so easily studied by biochemical methods. The *Bacillus amyloliquefaciens* subtilisin, BPN, lacks disulfide bonds, which often make proteins harder to be expressed in a soluble and catalytically active form in bacterial heterologous expression systems [83-86]. These relatively easy-to-

work-with proteases have been characterized at a fast rate. In the case of subtilisins from higher eukaryotes, working out the biochemistry of a protease has proved to be much more difficult.

As an example, in 1999 [40, 87] two independent research groups reported a second subtilisin-like protease encoded in the malaria parasite genome. At the moment, there was a lot of interest in identifying the protease that was responsible for the serine protease activity that was observed to be required during red blood cell invasion by the malaria parasite. This protease was then rapidly targeted for the design of novel antimalarials. Designing an inhibitor for this specific activity could halt host cell invasion during the stage in which this parasite causes potentially lethal symptoms in humans. Since then, however, efforts in developing a heterologous system for the expression of catalytically active protease have not been successful, as reflected in the literature. This indicates that not everything is known about subtilisins and there is still a lot to learn about this group of serine proteases, otherwise we would be able to easily isolate and work out the biochemistry of all subtilisins.

Plasmodium subtilisins

The malaria parasite genome includes the non-redundant single-copy coding sequences of three subtilisin-like proteases [88] (Figure 1.2 and Figure 1.3): subtilisin-like protease 1 (SUB1), subtilisin-like protease 2 (SUB2) and subtilisin-like protease 3 (SUB3). Of these three, SUB1 and SUB2 are known to be essential for parasite survival during blood stages, at least. SUB1 [89-91] plays an important role

during egress [92, 93]. SUB3 is the most recently annotated and it does play a role during asexual stages but it is not essential [94, 95].

SUB2 has been mostly characterized in molecular biology studies during the last fifteen years [40, 45, 96, 97]. Because of the important roles of these proteases, they are considered an appealing target for the design of new antimalarials. On the other hand, SUB2 is also a target for vaccine development since it is exposed to the antibodies in the blood during the process of RBC invasion. This project is focused in the characterization of *Plasmodium* SUB2 with aims to develop novel inhibitors of red blood cell invasion using small molecules and for vaccine development to use host interfering antibodies targeting this specific protease.

Subtilisin-like protease 2

SUB2 is the only type-1 integral membrane subtilisin-like serine protease of the malaria parasite. Unlike SUB1 and SUB3, SUB2 features a C-terminal transmembrane domain and its prodomain is relatively large compared to its *Plasmodium* paralogs and from those found in other subtilisins (Figure 1.2). It also has an N-terminal signal peptide for secretion onto the parasite surface, a conserved serine Asp, His, Ser catalytic triad in its catalytic domain, a juxtamembrane domain and a cytoplasmic tail. SUB2 was first identified during the asexual cycle of the parasite while research groups were looking to identify the serine protease responsible of the merozoite surface sheddase activity observed during RBC invasion.

There is a general interest in trying to elucidate the different roles that SUB2 may play at different stages of the parasite, especially during host cell invasion. Still, it is better known for its role in RBC invasion, after it was determined that SUB2 was responsible for the merozoite surface sheddase activity. It sheds surface adhesins (Figure 1.4) MSP1 and AMA1 [96] at the membrane junction during the process of erythrocyte invasion by juxtamembrane cleavage [98]. It has also been proposed that *Plasmodium* thrombospondin-related apical merozoite protein (PTRAMP) [99] is another SUB2 merozoite substrate. Access to its substrates is achieved upon secretion from the micronemes onto the parasite's surface at the apical end of the parasite. Consequently, SUB2 translocates across the parasite surface to the posterior end in an actin-dependent fashion [45], presumably mediated by its cytoplasmic tail at the N-terminus. Throughout the course of invasion, it stays anchored to the merozoite membrane staying exposed to components of the immune system that are circulating in the blood [97]. Making SUB2 part of the repertoire of surface antigens at the surface of the invading merozoite.

Shedding of surface ligands is not the only known function of SUB2. MSP1 and AMA1 are two substrates for its maturase activity [40, 96], for instance. Other roles are also attributed to SUB2 during other parasite stages as well. For example, in a mouse model of malaria, SUB2 was secreted onto epithelial cells by the *Plasmodium* ookinete during the invasion of the mosquito's midgut. Following secretion, it was found in aggregates associated to actin cytoskeletons [100]. This observation suggests a direct or indirect role in cytoskeletal rearrangement. In addition, at a later stage in the mosquito, SUB2 was found to be present in the

salivary gland sporozoites [101], entertaining the idea of a possible implication in gliding and motility. Still, at present, the better-understood roles of SUB2 are those implicated in the asexual stages, even when these are not fully characterized by *in vitro* studies yet.

So far, we have been able to gather very important information regarding the function of SUB2 during blood stages mostly by molecular biology techniques. Sequence specificity was evaluated in recombinant parasite cultures, by mutating the AMA1 cleavage site sequence. These experiments showed no primary sequence preference for SUB2 cleavage activity. Also, the specific molecular determinants for SUB2 trafficking in the merozoite were solved. This work showed that it is transcribed directly into the endoplasmic reticulum, following by autoprocessing for cleavage of its cognate prodomain, to later get trafficked to the micronemes for secretion during RBC invasion.

A significant advance in SUB2 research could be accomplished by establishing a heterologous system for expressing catalytically active protease for functional studies. Moreover, the development of a SUB2-specific activity assay will provide a tool for further characterization of SUB2 biochemistry and for the development of protease inhibitors specific for its activity. Furthermore, by solving its structure to atomic resolution, we will have in hand accurate insights into the biochemical environment at the active site of SUB2, and specific features of this protease could be revealed as well. This biophysical approach will provide essential information for screening and design of SUB2-specific inhibitors and unveil precise sites for antigen generation. As a result, the understanding of these fundamental

aspects of this merozoite surface sheddase will provide information that would help to better understand the function of SUB2 during other stages.

It is easy to imagine that a pathogen will evolve to produce proteins with functionally distinct roles at different stages. In fact, there is evidence of SUB2 expression during various stages of *Plasmodium* life cycle [97, 100, 101]. The relevance of these needs to be further pursued for validation and the identification of unknown substrates. Following this, the more we understand the role of SUB2 during the various stages of the malaria parasite, the more we will understand the various functions of *Plasmodium* subtilisins. These studies can potentially lead to novel transmission control strategies, multifunctional antimalarial molecules and could lead to the development a more potent vaccine antigen.

Thesis rationale

Malaria is an infectious disease caused by *Plasmodium* parasites. *P. falciparum*, the most deadly species, causes severe infections resulting in more than 500,000 deaths every year. The symptoms of malaria occur as a result of the asexual life cycle that consists of multiple rounds of invasion and egress from the red blood cells. This cycle results in increasing destruction of RBCs and triggers immune responses that cause fever, one of the classical malaria symptoms. By interrupting this cycle, parasites will stop to proliferate and the infection will come to an end. Subtilisin-like protease 2 (SUB2) is a serine protease required for invasion of the

RBC so that the parasite can enter this host cell and develop. This thesis focuses on the characterization of *Plasmodium* subtilisin-like protease 2 as a vaccine candidate for the antibody-mediated intervention of the parasite and target for the development of small molecule inhibitors of red blood cell invasion. In Chapter 2, I present a proof-of-principle set of experiments to test SUB2 as an antigen for vaccine development in a mouse model for malaria infection. Results show that immunization with peptides derived from SUB2 catalytic domain sequences attenuate *Plasmodium* infection in mice and lead to an increase in multiple invasion events. In Chapter 3, I tested a self-assembling protein nanoparticle as an antigen carrier for SUB2 peptide antigens using a malaria mouse model and provide an insightful method for data analysis to assess parasite infection status. In Chapter 4, I provide insights into my efforts in establishing a heterologous system for the expression of active *Plasmodium* SUB2 and to develop a SUB2-specific activity assay. In general, this thesis provides the first attempts in the characterization of SUB2 as a vaccine candidate and important tools for future studies.

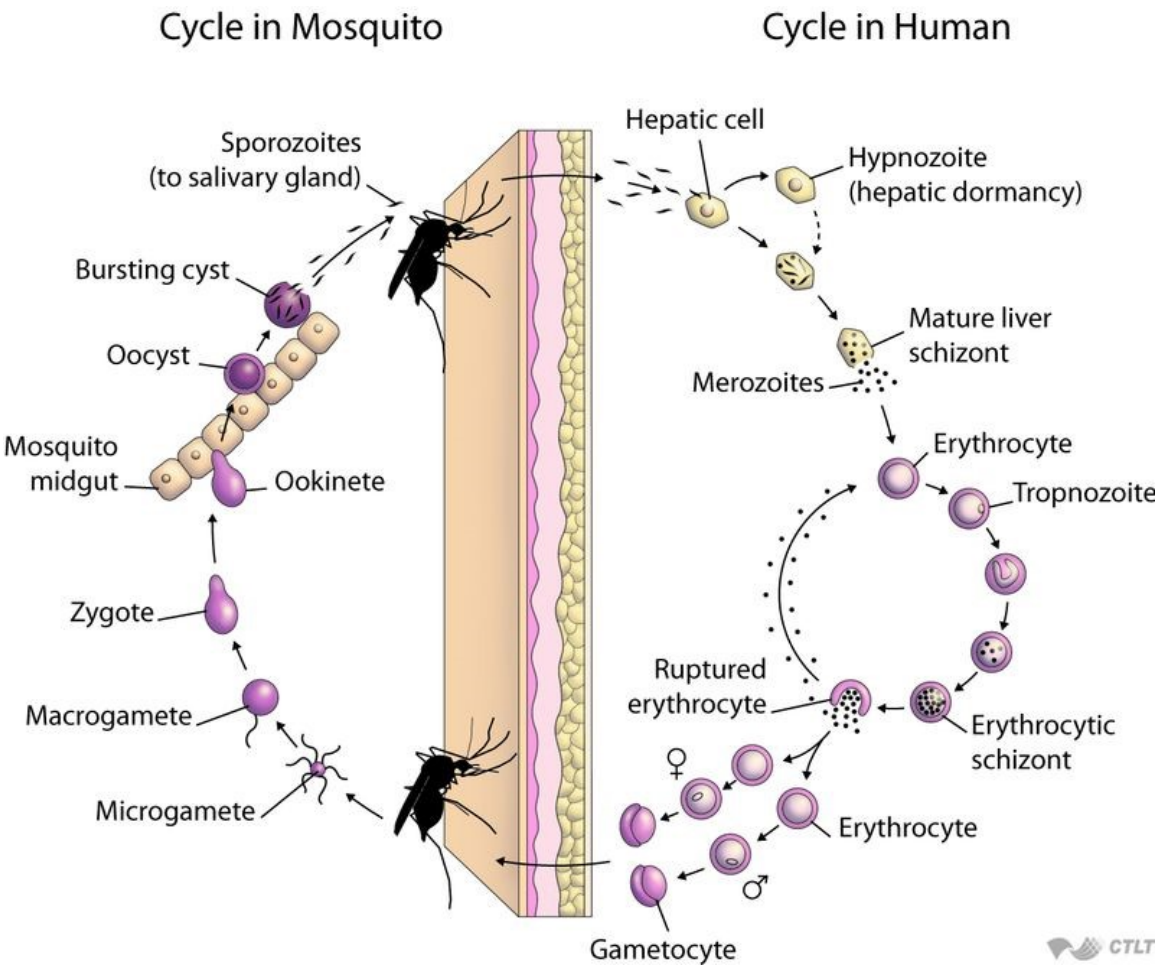

Figure 1.1 The life cycle of the malaria parasite, *Plasmodium*. This figure illustrates all the stages of the malaria parasite life cycle in the human and the mosquito hosts. Sexual development occurs in the mosquito. The mosquito passes on a spore like stage of *Plasmodium*, the sporozoite, to a human during a blood meal. After asymptomatic development in the liver, the parasite transforms into blood-stage parasites that can develop within the RBC, causing malaria symptoms.

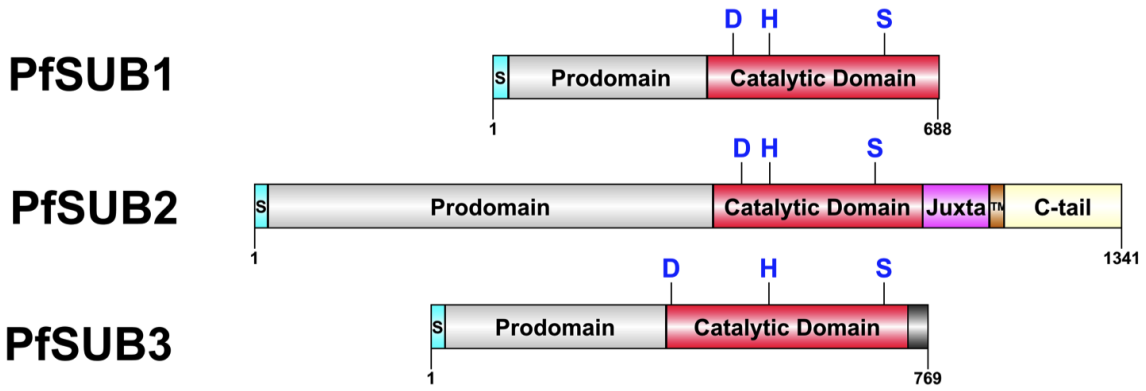

Figure 1.2 The three subtilisin-like proteases of the malaria parasite. Schematic of the three subtilisin-like proteases encoded in the *Plasmodium* genome. These are aligned by the conserved histidine at the active site, showing the differences in the relative distance of the restudies at the active site in the primary sequence of each *Plasmodium* subtilisin. This figure also illustrates the large prodomain (grey and green) present in SUB2 (PDB PF3D7_1136900), compared to SUB1 (PF3D7_0507500) and SUB3 (PF3D7_0507200), its juxtamembrane domain (purple), transmembrane region (black) and cytoplasmic tail (yellow). All three *Plasmodium* subtilisin-like proteases have an N-terminal signal peptide (cyan). Only P. falciparum subtilisins are shown in this figure as an example. This figure was made using the DOG Protein Domain Structure Visualization program [102].

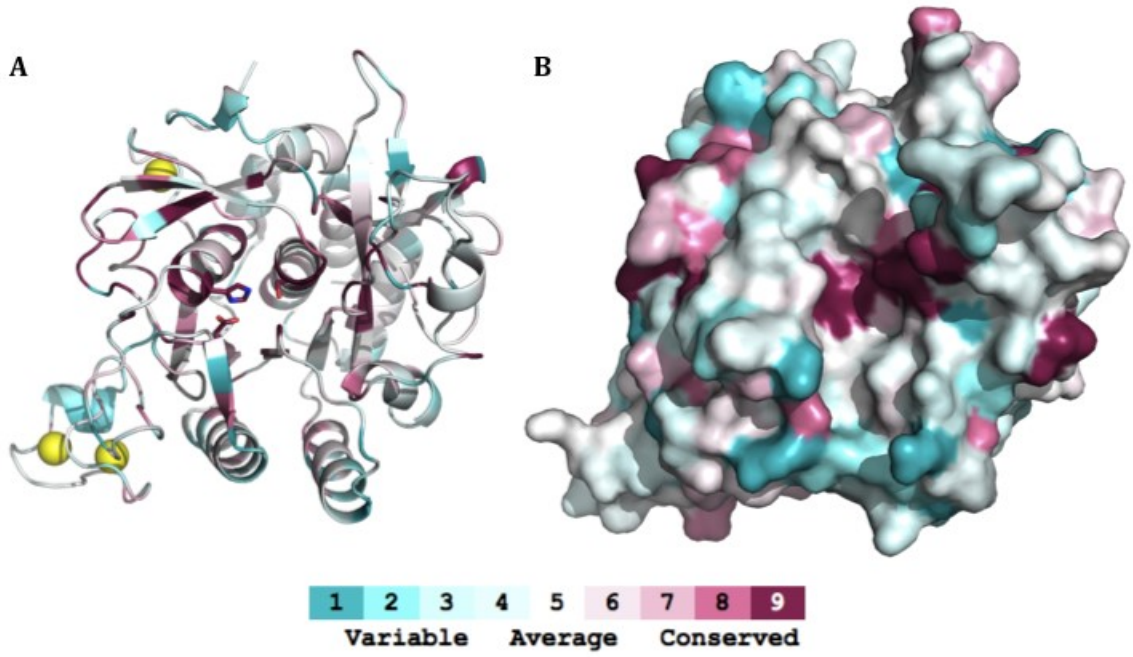

Figure 1.3 Sequence conservation across the three *Plasmodium* subtilisin-like proteases. *Pf*SUB1 structure (PDB 4lvo) was used to map conservation of amino sequence across *Pf*SUB2 paralogues, using the ConSurf Server. A) Cartoon representation showing the canonical topology of a subtilase, the conserved active site residues (sticks) and calcium atoms (yellow) found in the *Pf*SUB1 structure. B) Surface representation showing that the majority of conserved amino acids reside in the active site. Solvent-exposed residues are mostly variable.

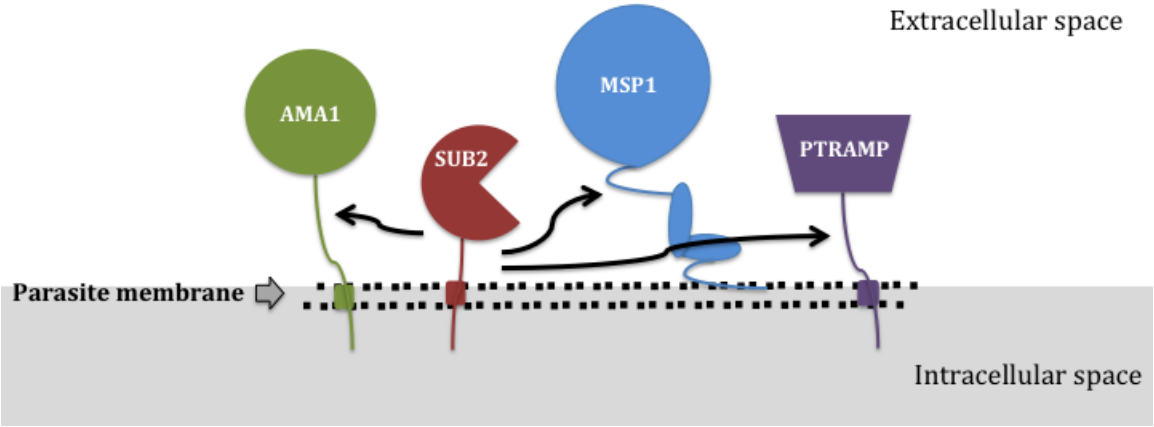

Figure 1.4 SUB2 cleaves its substrates at the surface of the parasite. SUB2 (red) is secreted upon RBC invasion, gaining access to its substrates. Its cleavage site is determined by the distance from the parasite membrane, independent of the sequence (black arrow), releasing the ectodomain of these ligands. From left to right: apical membrane antigen 1 (AMA1) in green, merozoite surface protein 1 (MSP1) in blue and *Plasmodium* thrombospondin-related apical merozoite protein (PTRAMP) in purple.

Chapter 2

Immunization against a merozoite sheddase promotes multiple invasion of red blood cells and attenuates *Plasmodium* infection in mice

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Abstract

Subtilisin-like protease 2 (SUB2) is a conserved serine protease utilized by *Plasmodium* parasites as a surface sheddase required for successful merozoite invasion of host red blood cells and has been implicated in ookinete invasion of the mosquito midgut. To determine if SUB2 is a suitable vaccine target to interfere with malaria parasite development, we examine the effects of SUB2-immunization on the *Plasmodium* life cycle in its vertebrate and invertebrate hosts.

Swiss Webster mice were immunized with SUB2 peptides conjugated to KLH or KLH alone, and then challenged with *P. berghei*. To determine the effects of immunization on parasite development, infected mice were evaluated by blood film and Giemsa staining. In addition, collected immune sera was used to perform passive immunization experiments in non-immunized, *P. berghei*-infected mice to determine the potential role of SUB2 in parasite development in the mosquito.

Following *P. berghei* challenge, SUB2-immunized mice develop a lower parasitemia and show improved survival when compared to control immunized mice. Moreover, SUB2 immunization results in an increase in the number of multiply invaded red blood cells, suggesting that SUB2 antibodies interfere with merozoite invasion. However, passive immunization experiments suggest that SUB2 may not have a major role in ookinete invasion.

By interfering with red blood cell invasion, immunization against SUB2 limits malaria parasite development and confers protection from severe malaria.

Together, these results provide proof-of-principle evidence for future investigation

into the use of SUB2 as a vaccine or drug target to interrupt parasite development in more relevant human malaria models.

Introduction

Obligate intracellular parasites from the genus *Plasmodium* are the agents responsible for malaria, placing an estimated 3.4 billion people at risk of the disease throughout the world [103]. Five species of *Plasmodium* parasites cause human malaria, yet the largest impacts to public health are primarily caused by *Plasmodium falciparum* in sub-Saharan Africa, leading to approximately one million deaths every year [104].

Malaria parasites undergo a complex life cycle in their mosquito and human hosts, which require *Plasmodium* parasites to invade and replicate in multiple cell types and host environments. To accomplish these developmental progressions, *Plasmodium* parasites utilize specific invasion ligands and proteases to facilitate host cell invasion [22, 105]. Merozoite invasion of red blood cells (RBCs) has been studied in the most detail, and involves a large repertoire of surface proteins that contribute to multiple invasion pathways [22]. Similarly, recent evidence suggests that ookinete invasion of the mosquito midgut may also involve multiple surface proteins and invasion pathways [106]. While both merozoite invasion of the RBC and ookinete invasion of the midgut are rapid, these stages have attracted recent attention as targets for a blood stage [107-109] or transmission-blocking vaccines [110-112].

As a shared component of merozoite and ookinete invasion pathways, Subtilisin-like protease 2 (SUB2) is an ideal candidate to interfere with the disease-causing forms of malaria asexual development, as well as development in the

obligate mosquito host. In merozoites, SUB2 accumulates in the parasite micronemes and is secreted onto the merozoite surface upon schizont rupture [45]. There, it is believed that SUB2 interacts with an actin-dependent motor to behave as a sheddase, cleaving surface-bound MSP1 and AMA1 on the parasite membrane [45, 96]. As SUB2 moves to the posterior end of the merozoite during RBC invasion, these substrates are cleaved at a certain distance relative to the membrane with minimal sequence specificity, in contrast to other proteases [45]. While little is known regarding SUB2 function during ookinete invasion, limited evidence would suggest that it is secreted by ookinetes during mosquito midgut invasion [100]. In cells that have undergone ookinete invasion, SUB2 is found in protein aggregates in close association with the actin cytoskeleton and may function to disrupt the host cytoskeletal network to facilitate invasion [100]. While evidence would suggest that SUB2 is an integral component of *Plasmodium* parasite development due to its crucial role in RBC invasion [40, 45], attempts to further define its role in the sexual stages of parasite development have yet to be explored.

Although these invasive stages are transient, both stages likely require SUB2 activity for the processing and shedding of parasite surface ligands. Despite the short window of opportunity to target these stages, naturally acquired immunity predominantly targets proteins involved in merozoite invasion [107, 109]. Included among several merozoite surface antigens or proteins secreted during merozoite invasion, SUB2 was determined to be a strong target candidate to elicit malaria protective immunity [107].

To determine if SUB2 is a viable malaria vaccine candidate targeting both the asexual and sexual life cycles of *Plasmodium*, we designed two synthetic peptides representing solvent exposed regions of the *P. berghei* SUB2 catalytic domain to evaluate the effects of SUB2 immunization in mice. In the present study, we provide evidence that *Pb*SUB2-immunized mice confer protective immunity from developing severe malaria infection by attenuating parasite growth via promoting aberrant merozoite invasion. Our results therefore validate SUB2 as a novel target against malaria infection in a mouse model system.

Materials and Methods

SUB2 homology modeling and visualization

Homology model of *Pb*SUB2 (PlasmoDB code: PBANKA_091170, Gene ID: 3423789) was generated using the I-TASSER Protein Structure and Function Prediction Server using default settings [113]. From all the models predicted by the server, the one with the highest confidence score was used in our study. Models were visualized using PyMol (The PyMoL Molecular Graphics System, Version 1.6.0.0 Schrödinger, LLC).

Mice

Female Swiss Webster mice (~21-24g) were purchased from Harlan and maintained in accordance with the recommendations of the Guide for the Care and

Use of Laboratory Animals of the National Institutes of Health. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University (protocol number MO09H58).

SUB2 immunization

Synthetic SUB2 peptides conjugated to keyhole limpet hemocyanin (KLH) through the cysteine at the N- (Sub2 Peptide #2- CRTSIKIVSKDKKTI) or C-terminus (Sub2 Peptide #1- KYSDRYEMTDELFDC) via a –SH bond were produced by GenScript Corporation (Piscataway, NJ).

Female Swiss Webster mice (~21-24g) were primed with a 50:50 mixture (50 μg/mouse) of both SUB2 peptides in phosphate buffered saline (PBS) or 50 μg of a control KLH carrier in PBS with either complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA) in a 1:1 emulsion and immunized by Intra-peritoneal injection (i.p.). Mice were boosted four times in two week intervals with 50 μg/mouse of peptide in a 1:1 emulsion with IFA via i.p. injection. Serum was collected from each individual mouse prior to priming, as well as the third and fourth boosting immunizations to monitor antibody titers. Two weeks after the final boosting immunization, animals were used for subsequent challenge experiments with *P. berghei* parasites.

***P. berghei* and *P. falciparum* RNA Isolation and cDNA production**

P. berghei ANKA 2.34 total RNA was prepared from blood of an infected Swiss Webster mouse (~10% parasitemia) obtained via cardiac puncture and isolated

using TRIzol Reagent (Invitrogen) according to the manufacturer's specifications. 2 µg of total RNA was used as a template for the production of cDNA using SuperScriptIII (Invitrogen).

Approximately 1 µg of total RNA from asynchronized *P. falciparum* 3D7 parasites was isolated using TRI Reagent (Molecular Research Center, Inc) and treated with DNase I (New England Biolabs) according to the manufacturer's protocol. Synthesis of complementary DNA was performed with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen).

Plasmodium SUB2 cloning

P. berghei SUB2 N476 - N1185 (PlasmoDB code: PBANKA_091170, Gene ID: 3423789) and *P. falciparum* SUB2 N528 - S1135 (PlasmoDB code: PF3D7_1136900, Gene ID: 810927) coding sequences (see Figure 2A) were amplified using cDNA obtained from *P. berghei* ANKA 2.34 or *P. falciparum* 3D7 strains using the respective primers *Pb*SUB2_Fwd: 5' CTCCATG*GCG*AATAATTCAAATGCATTTTTGAGTGTAGAC 3', *Pb*SUB2_Rev: 5' ACGGATCCGTTATCATGCTCATATAAATTATATAAAGC 3', *Pf*SUB2_Fwd: 5' ATCCATG*GCG*AATAATAAAAAAATTTTGTTAAATGTTGAT 3' and *Pf*SUB2_Rev: 5' ACGGATCCACTATCATATTCATACAAATTATATAAGGC 3'. PCR products were amplified using Phusion® High-Fidelity DNA polymerase (New England Biolabs) with an annealing temperature gradient of 52 °C – 70 °C for 30 seconds, followed by extension at 72 ˚C for 2 minutes.

SUB2 PCR products were inserted in frame using *Nco*I and *Bam*HI restriction sites into a modified pRSF-1b vector (Novagen) for expression as an Maltose Binding Protein (MBP)-fusion protein with a C-terminal 6xHis tag for purification and detection purposes as previously described [114]. Positive clones were screened using colony PCR with primers described above and insertion sequences were confirmed by sequencing.

Recombinant protein expression and purification

MBP-SUB2 fusion constructs were transformed into Rosetta 2 (DE3) competent *E. coli* (Novagen) for protein expression. Cells were grown in the presence of 1.5 % glucose and 50 µg/ml Kanamycin in 500 ml 1X Terrific Broth media until OD_{600} of ~ 3.0 and induced with a final concentration of 0.5 mM IPTG. Recombinant proteins were expressed overnight at 20 °C under vigorous shaking at 250 rpm.

Bacteria were harvested by centrifugation at 2,500 RPM for 30 minutes at 4 °C. Bacterial pellets were re-suspended in lysis buffer (25 mM Tris pH 9.0, 100 mM NaCl) and lysis was performed using an Emulsiflex C5 cells disruptor (Avestin Inc.) at 100 MPa. Whole cell lysates were fractionated by centrifugation at 17,000 rpm for 1 hour at 4 °C and the supernatant was applied to an open gravity column (BioRad) containing 1 ml of Amylose resin (New England Biolabs) for affinity capture of the MBP taged fusion protein. Bound protein was washed with lysis buffer and eluted in the presence of 20 mM maltose. Elution samples from the Amylose resin purification steps were applied to an affinity column containing Cobalt-TALON resin (Clontech)

for secondary purification with the 6xHis tag. Bound protein was washed with lysis buffer and eluted with 200 mM imidazole. Elution samples were concentrated using Nanosep Centrifugal Devices (Sigma) with a 10 kDa cutoff.

Western blots

Approximately 1.7 µg of recombinant *Pb*SUB2 and *Pf*SUB2, and ~3 µg MBP (fusion protein only) were separated on a 12 % SDS-PAGE gel. Following electrophoresis, the gel was washed in diH_20 for 10 minutes and equilibrated in 1X transfer buffer (25 mM Tris, 192 mM Glycine, 20 % methanol, 0.0375 % SDS). Proteins were transferred to a PVDF membrane on a Semi-dry transfer cell for 2 hours under constant voltage (25V). After transfer, the membrane was blocked with 5 % milk in 1X TBST for 30 minutes (250 rpm at 37 °C) and washed three times with 1X TBST. Membranes were incubated overnight at 4 °C with serum from SUB2- or KLH- immunized mice at a 1:500 dilution in 1X TBST or with a mouse anti-Maltose Binding Protein antibody (Upstate – Millipore, #05-912) at a 1:10,000 dilution in 1X TBST. After three washes with 1X TBST, membranes were incubated with an alkaline phosphatase-conjugated goat anti-mouse antibody (1:5,000 dilution in 1X TBST). Detection was carried out using NBT/BCIP alkaline phosphatase substrates (Promega).

Plasmodium challenge in SUB2 immunized mice

Following immunization with either the CFA or IFA protocols described above, SUB2 or control KLH mice were infected with ~2x10^2 *P. berghei* mCherry [115] asexual parasites via intra venous (IV) injection as previously performed [116]. To monitor parasite growth, thin smears of tail blood were stained with Giemsa and examined under a microscope to determine parasitemia (% of infected erythrocytes) every day for ten days.

To determine the effects of immunization on mouse survival following the above *Plasmodium* challenge, the survival of immunized mice was monitored for 40 days following the initial infection.

Multiple Invasion Analysis

Ten days after infection with *P. berghei*, Giemsa-stained thin smears from SUB2 or KLH immunized mice with measurable parasitemia were analyzed by light microscopy. Independent of parasitemia, approximately 200 infected RBCs were examined per mouse to determine the number of infected RBCs that contain one or more parasites. The number of single, double, or multiple invasion events are reflected as percentages of the total number of infected RBCs. Mann-Whitney tests were performed using GraphPad Prism to determine significance.

Passive immunization experiments

Swiss Webster mice infected with the mCherry strain of *P. berghei* [115] were examined for similar levels of exflagellation three days after inoculation as

previously described [112]. Mice with matching infections were anesthetized and used for blood feeding control (pre-KLH) or treatment (pre-SUB2) groups of *An. gambiae* mosquitoes for 15 minutes. The anesthetized mice were then taken off the cage and passively immunized (i.v.) with KLH or SUB2 immune sera (final concentration of 2 mg/ml) and allowed to recover for 15 minutes. The passively immunized mice were then fed to sibling groups of *An. gambiae* mosquitoes for an additional 15 minutes to measure any effects on parasite development in the mosquito.

Following feeding, mosquitoes were incubated at 19°C to promote *P. berghei* development. Mosquito midguts were dissected 7 days post-blood meal (PBM), and oocysts numbers were counted using a compound fluorescence microscope. The results of two independent experiments were analyzed by Mann-Whitney using GraphPad Prism to determine significance.

Results

Structural modeling of *P. berghei* SUB2 catalytic domain

A structure model was predicted for the catalytic domain of *Pb*SUB2 by the I-TASSER server and contains a secondary structure topology characteristic of subtilisin-like serine proteases (Figure 2.1A). The amino acid residues that comprise the catalytic triad Asp 705, His 748 and Ser 911 required for catalysis are positioned at the active site of the model (Figure 2.1A). Comparing our predicted model using

the EBI SSM webserver, the closest structural homolog in the Protein Data Bank (PDB) is the subtilase, thermitase (PDB 1twc:E) from *Thermoactinomyces vulgaris.* With an overall root mean square deviation (R.M.S.D) of 1.4 Å for 247 amino acid residues as determined with PDBeFold [117], our predicted structural model for *Pb*SUB2 therefore has a high confidence level, resembling the overall known fold of other subtilases.

Design of *P. berghei* SUB2 peptides

Using proprietary software (GenScript), highly antigenic peptides corresponding to the *Pb*SUB2 catalytic domain were identified. To test these candidate 14 amino acid peptides, the corresponding regions were mapped on a *Pb*SUB2 catalytic domain homology model. Two peptides mapping to opposite flexible solvent exposed regions of *Pb*SUB2 were selected to increase the likelihood that antibodies generated against these peptides would interact with the protease on the surface of merozoites or ookinetes during invasion (Figure 2.1A). Peptide #1 and #2 target unique solvent accessible regions of the catalytic domain of *Pb*SUB2 (Figure 2.1B, left).

The sequence of Peptide #1 is nearly identical (93 %) to the corresponding region of *P. yoelii* SUB2 (Figure 2.1B, right). The two sequences only differ by the amino acid at position Leu 734 in the *P. berghei* sequence and Phe 734 in *P. yoelli*, suggesting a high level of conservation between the rodent malaria species. Less conservation exists between Peptide #1 and the human malaria parasites (*P. falciparum*, *P. vivax*, and *P. knowlesi*), with only 64% similarity (36% ID) to *P.*

falciparum (Figure 2.1B). However, the Peptide #2 sequence alignment reveals more conservation and sequence similarity across *Plasmodium* species. The *P. berghei* and *P. falciparum* SUB2 sequences show 85% similarity (71% ID), while the rodent malaria parasites are completely conserved (Figure 2.1B). Both peptide sequences map to regions of the *Pb*SUB2 catalytic domain (Figure 2.2A).

Mice immunized with SUB2 peptides recognize recombinant *Pb*SUB2

MBP-SUB2 expression constructs containing a short region of the pro-domain and the entirety of the SUB2 catalytic domain (Figure 2.2A) were expressed using a Rosetta2 *E. coli* heterologous system. Recombinant SUB2 was visualized as a single band for *Pb*SUB2, or as two bands for *Pf*SUB2, of approximately 110 kDa full-length protein products (Figure 2.2B). Smaller protein products are likely the result of sample degradation during the purification process or translational truncation products that were observed for both SUB2 constructs (Figure 2.2B). Both full-length and truncated forms of SUB2 were detected using an MBP antibody, confirming the detection of the recombinant MBP-SUB2 fusion protein products (Figure 2.2B). When incubated with immune sera from SUB2-immunized mice, recombinant *Pb*SUB2 is detected in full length and degraded forms while only a faint band corresponding to full length recombinant *Pf*SUB2 protein was detected (Figure 2.2B). Importantly, mice immunized with KLH alone did not recognize either recombinant SUB2 protein (Figure 2.2B).

These results confirm that antibodies were generated in mice immunized with *Pb*SUB2 peptides that can sufficiently recognize recombinant *Pb*SUB2 (Figure

2.2B). Furthermore, immune sera raised against *Pb*SUB2 peptides specifically targets PbSUB2 and does not cross-react with *P. falciparum* SUB2 (Figure 2.2B), suggesting that the conservation in the peptide sequences is inadequate for cross-species protection. However, future immunization experiments are needed to determine the properties of the individual peptides and whether they are capable of cross-species immune recognition of different *Plasmodium* species.

SUB2-immunization attenuates asexual *Plasmodium* development

To monitor the effects of immunization on parasite development, KLH- and SUB2-immunized (IFA or CFA) mice were challenged with ~$2x10^2$ *P. berghei* parasites by intravenous injection and the parasitemia was monitored over the period of ten days. Blood stage infections were detected in 17 of 18 mice, and little variation was seen between mice immunized with the IFA or CFA immunization protocols (Table 2.1). As a result, both immunization experiments were pooled for analysis and summarized in Table 2.1. Compared to control KLH-immunized mice, SUB2-immunized mice showed a slight, but not significant delay in the pre-patency of infection (Table 2.1). However, when the parasitemia was monitored over the period of ten days, asexual growth was significantly attenuated following SUB2-immunization (Figure 2.3A).

In SUB2-immunized mice, parasite growth was reduced by 37, 43, and 56% from days 8-10, effectively reducing parasitemia more than two fold when compared to KLH control mice (Figure 2.3A). In addition, nearly half of the SUB2-immunized mice (4 of 9) had cleared all signs of parasite infection by Day 10 (Figure

2.3B and Table 2.1). None of the KLH-immunized mice infected with *P. berghei* were able to clear the infection over the duration of the experiment (Figure 2.3B and Table 2.1).

SUB2-immunization increases mouse survival after *P. berghei* challenge

Since *P. berghei* asexual development is attenuated in SUB2-immunized mice (Figure 2.3A), we wanted to explore whether SUB2-immunization also protects mice against malaria lethality through the decreased parasite burden.

To measure survival, KLH- and SUB2-immunized mice were monitored for forty days following *P. berghei* challenge (Figure 2.3C). In our experiments, SUB2-immunized mice showed increased survival over control KLH-immunized mice (Figure 2.3C and Table 2.1) On average, SUB2-immunized mice survived for more than one week longer than KLH control mice (Table 2.1), and 7 of 9 mice survived the duration of the experiment (Figure 2.3C). In contrast, only 1 of the 8 infected KLH mice survived the entire forty day period (Figure 2.3C). This would suggest that the attenuated malaria parasite growth seen in SUB2-immunized mice (Figure 2.3A) also translates to an increased survival following *P. berghei* challenge (Figure 2.3C).

SUB2-immunization promotes aberrant red blood cell invasion

Based upon observations measuring the parasitemia of the immunized mice (Figure 2.3A), there appeared to be a noticeable increase in the number of infected RBCs with multiple parasites in SUB2-immunized mice. To quantify these presumed defects in invasion, the percentages of infected RBCs that had one, two, or multiple

(3+) parasites were measured in KLH- and SUB2-immunized mice (Figure 2.4). Validating our previous observation, SUB2-immunized mice had a significant decrease in the number of infected RBCs that had undergone a single invasion event when compared to KLH-control mice (Figure 2.4). We also measured a corresponding increase in the number of double or multiple invasion events (3+) following SUB2 immunization (Figure 2.4).

Based upon these data and the important functional role of SUB2 in RBC invasion [40, 45], we conclude that SUB2-immunization interferes with merozoite invasion. Although it is not completely understood how SUB2-immunization might influence the production of these aberrant invasion events, previous studies using antibodies to merozoite surface proteins similarly report phenotypes promoting multiple invasion [118, 119].

SUB2 immune sera does not interfere with ookinete invasion in passively immunized mice

One previous study has reported that SUB2 is expressed by ookinetes, implicating that SUB2 may be secreted into the cytoplasm of ookinete-invaded cells as the parasite traverses the midgut epithelium [111]. Immunofluorescence staining identified SUB2 protein aggregates in close proximity to the actin cytoskeleton that suggest SUB2 may play an important role in cytoskeleton modifications during the process of ookinete invasion [100].

To address the role of SUB2 in ookinete midgut invasion and the potential role that SUB2 immune sera could also inhibit ookinete invasion, we performed

passive immunization assays to determine the effects on parasite development in the mosquito. As expected, passive immunization with the control KLH immune sera did not significantly alter *Plasmodium* oocyst numbers (Figure 2.5). Similarly, passive immunization with SUB2 immune sera did not significantly alter oocyst numbers (Figure 2.5), suggesting that SUB2 may either not be required for ookinete invasion of the mosquito midgut or that our immune sera was present in sub-optimal levels needed to inhibit ookinete invasion. These research questions highlight the need for further investigation into the role of SUB2 during the mosquito stages of *Plasmodium* development.

Discussion

Although more than 40% of the world's population is at risk of malaria transmission, only limited resources exist to readily combat *Plasmodium* parasites. Current drug therapies face the ever-increasing risk of resistance [120], and while multiple approaches have thus far been employed to create a malaria vaccine, they have had only mixed results in clinical trials [121]. As a result, new strategies to reduce malaria transmission are desperately needed.

Plasmodium species utilize many different proteases during their complex life cycle in the human and mosquito hosts, and serve as optimal targets to interfere with malaria transmission. Previous reports have demonstrated the required role of a *Plasmodium* subtilase (SUB2) for asexual development through its role as a

sheddase required for merozoite invasion [40, 45]. Additional studies have also implicated SUB2 in ookinete invasion [100], thus making SUB2 an attractive target to interfere with parasite development in both its human and mosquito hosts.

Using a rodent model, we address the potential of targeting SUB2 by immunizing mice against specific SUB2 derived peptides. When compared to control KLH-immunized mice, SUB2-immunization resulted in a slight delay in pre-patency, decreased parasitemia when monitored over a ten day period, and increased survival following infection. Similar results were obtained independent of the method of immunization, suggesting that the effects of immunization are primarily that of the SUB2 antigens and not from non-specific effects mediated by the CFA. Together, these data would suggest that SUB2-immunization greatly impairs parasite growth, likely by interfering with the efficacy of merozoite invasion.

In support of this idea, we detected an increase in the number of multiply invaded RBCs following SUB2-immunization, suggesting that merozoite invasion is significantly altered. Similar effects have been seen in other studies using antibodies targeting merozoite proteins, where it was proposed that multiple invasions are the result of merozoite agglutination [118, 119]. According to this hypothesis, the invasion of some merozoites may be completely blocked, while incomplete inhibition may result in multiple parasites that have been cross-linked by SUB2 antibodies that undergo invasion together as a complex or dissociate once the RBC surface has been recognized. Due to the short time frame in which merozoites

undergo release and invasion into new RBCs, the concentration and rate of antibody binding may be critical factors in invasion inhibition.

Very little information exists regarding the viability of infected RBCs that have undergone multiple invasion events. It has been hypothesized that nutritional and structural limitations following multiple invasion may reduce the production of viable merozoites [118], thus raising the possibility that these infected RBCS may be a "dead-end" for the parasite. As a result, the higher incidence of multiple invasions may have a significant contribution to the decreased parasitemia and increased survival in the SUB2-immunized mice within our study.

While the increased survival of SUB2-immunized mice would suggest that SUB2 immunization can confer protection to the severe forms of malaria infection, we did not attempt to differentiate the potential reasons for morbidity in our *Plasmodium* infected mice. Visible neurological symptoms of cerebral malaria (as defined by [122]) were not observed, leading us to believe that infection-induced mortality was due to other malaria-related causes.

Based upon previous studies implicating SUB2 in ookinete invasion [100], we tested the ability of our SUB2 antibodies in passive immunization assays to examine the role of SUB2 on parasite development in the mosquito. Although we did not detect any differences in oocyst development, it still remains unclear what role SUB2 may have during the process of ookinete invasion. Given the limited amount of immune sera produced, only one concentration was tested in the passive immunization experiments and these may have been suboptimal concentrations to inhibit ookinete invasion. Alternatively, the production of SUB2 by ookinetes may

not be integral to ookinete motility within the mosquito midgut and may not be a viable target to interfere with malaria transmission. As a result, the role of SUB2 in *Plasmodium* ookinetes requires future study.

In summary, our experiments indicate that immunization against a merozoite sheddase can interfere with *Plasmodium* development in mice. While these results are still preliminary using a rodent malaria model, our data provide strong evidence for future investigation into the use of SUB2 as a vaccine or drug target to interrupt parasite development in more relevant human malaria models. In support of this idea, epidemiological studies in Papua New Guinea indicate a strong correlation between the detection of SUB2 antibodies and naturally acquired protective immunity [107] [7]. Similar studies with blood samples from field isolates of African populations to determine the role of SUB2 in naturally acquired immunity could provide further verification for this promising approach as a vaccine candidate. As a result, future experiments will address challenges to increase the efficacy of our approach to inhibit SUB2 function using monoclonal antibodies or small molecules inhibitors to interrupt merozoite invasion.

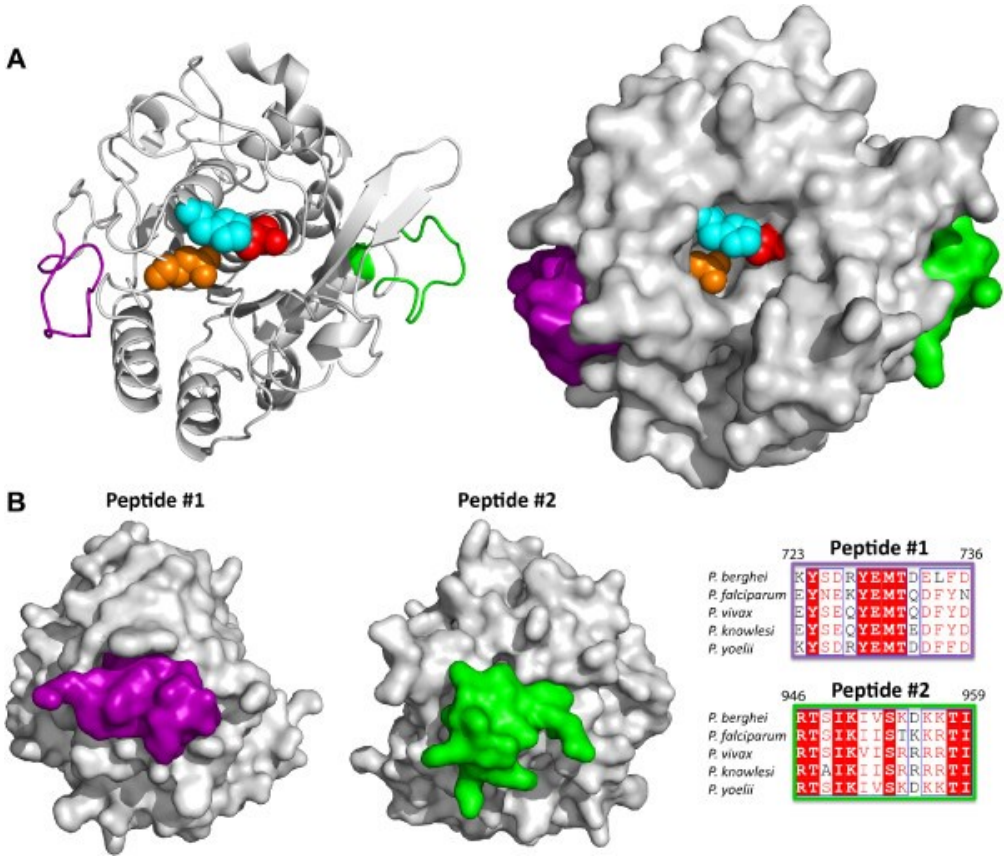


Figure 2.1 *Pb* SUB2 homology models identify peptide targets for immunization. (A) Cartoon (left) or surface representation (right) homology model of the *Pb*SUB2 catalytic domain (residues L672-L971). Regions corresponding to Peptide #1 (purple) and Peptide #2 (green) were used for immunization experiments. Catalytic residues Asp705, His748 and S911 in the active site pocket are shown as orange, cyan and red spheres, respectively. **(B)** Lateral view of Peptide #1 (purple) and Peptide #2 (green) in the *Pb*SUB2 surface representation model reveals that each peptide corresponds to solvent exposed areas (left). Sequence alignments of both peptide sequences with corresponding regions of *P. falciparum*, *P. vivax*, *P. knowlesi,* and *P. yoelii* SUB2 (right) the first and last amino acid position are shown.

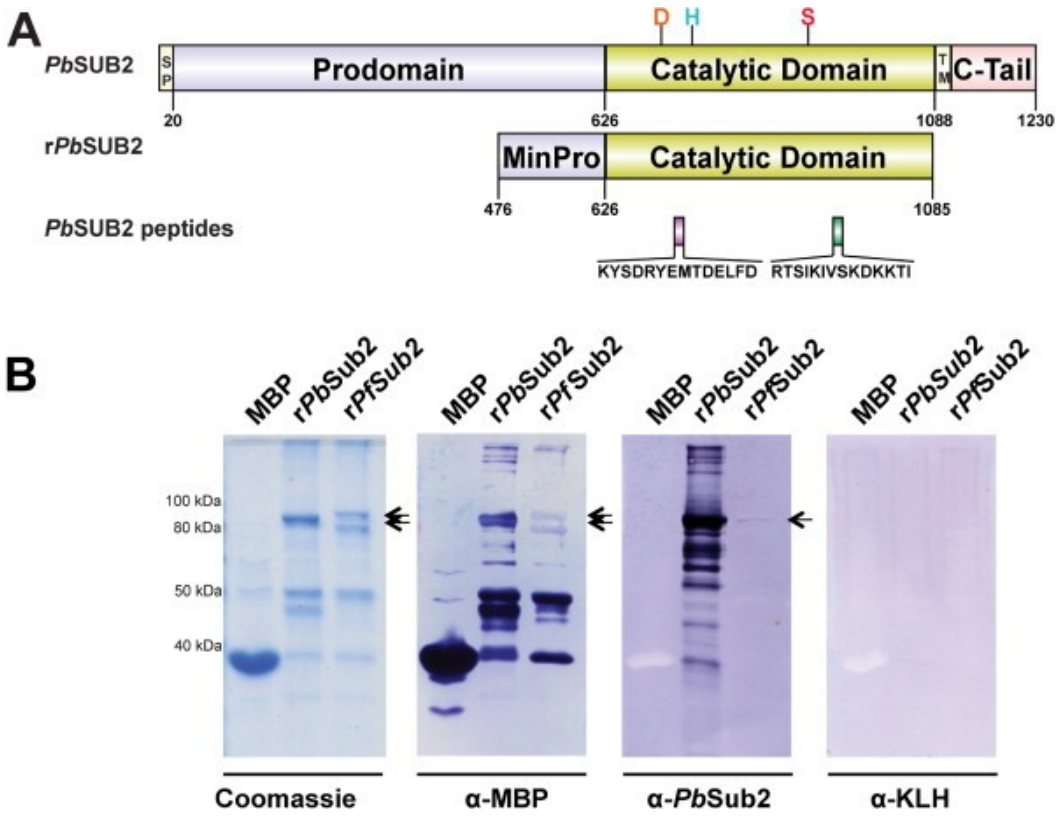


Figure 2.2 Production of recombinant SUB2 and recognition using Sub2 immune sera. (A) Domains of endogenous *Pb*SUB2 (top): signal peptide, pro-domain, catalytic domain with catalytic residues Asp (orange), His (cyan) and Ser (red), transmembrane domain and cytoplasmic tail. Residues at the beginning and end of each domain are shown. Representation of recombinant *Pb*SUB2 (middle) containing a minimal inhibitory domain and the full catalytic domain. Below, *Pb*SUB2 Peptides #1 (purple) and #2 (green) are aligned to endogenous *Pb*SUB2 and r*Pb*SUB2 with peptide sequences. **(B)** Recombinant proteins maltose binding protein (MBP), *Pb*SUB2 or *Pf*SUB2 MBP-fusion proteins SDS-PAGE stained with Coomassie and Western Blot results. Arrows denote full length *Pb*SUB2 and *Pf*SUB2 recombinant products. Approximate sizes in kilodaltons (kDa) are displayed on the left.

Experiment	Adjuvant	Antigen	# Mice	Infected	Pre-patency	Clearance*	Mean survival §
1	IFA	KLH	6	5/6	6.2	0/5	27.3
		SUB2	6	6/6	6.7	4/6	34.6
2	CFA	KLH	3	3/3	6	0/3	31.3
		SUB2	3	3/3	6.7	0/3	40+
Total		*KLH*	*9*	*8/9*	*6.1*	*0/9*	*28.6*
		SUB2	*9*	*9/9*	*6.7*	*4/9*	*36.4*

*Mice with detected parasitemia that had cleared the parasite infection (measured at Day 10).

§Average number of days mice survived following *P. berghei* challenge.

Table 2.1 Summary of immunization experiments

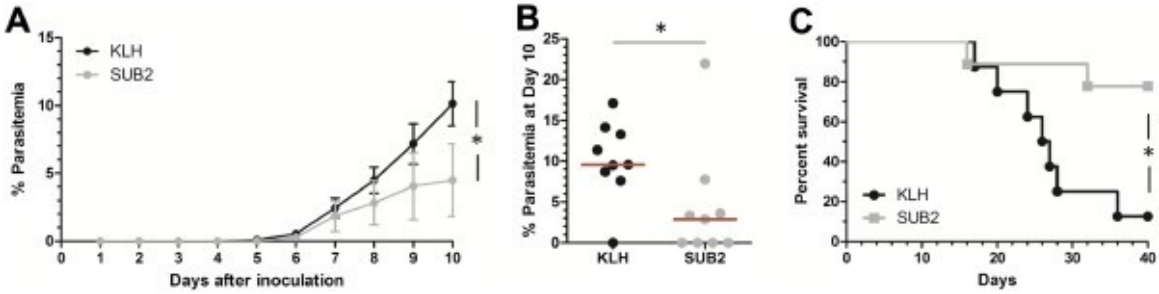


Figure 2.3 SUB2 immunization reduces the intensity of *Plasmodium berghei* infection and increases mouse survival. The parasitaemia of KLH- or SUB2-immunized mice was determined over the period of ten days after infection with *P. berghei* parasites **(A)**. Each point represents the mean parasitaemia (n = 9) with error bars displaying standard errors of the mean and the asterisk denoting significance ($P = 0.0042$). The scatter plot displays the parasitaemia at day 10, with each point representing the parasitaemia of individual KLH- or SUB2-immunized mice **(B)**. The red bar represents the median of each experiment with the asterisk denoting significance ($P < 0.05$). The survival of KLH- and SUB2-immunized mice was monitored over the course of forty days following *P. berghei* challenge **(C)**. The number of surviving mice for each treatment over the duration of the experiment is displayed as a percentage of the total number of infected mice at a given time point. Statistical differences are marked by an asterisk ($P = 0.0082$).

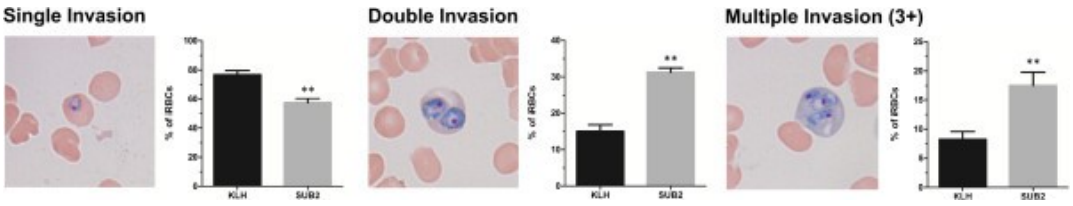


Figure 2.4 SUB2-immunization promotes multiple invasion of red blood cells. Representative images of single, double, or multiple invasion (3+) events in *P. berghei*-infected red blood cells are depicted with their corresponding percentages in KLH- (black) or SUB2- (grey) immunized mice at ten days post-infection. The percentage of each invasion phenotype is displayed as the mean and standard error. Asterisks denote significant differences between KLH-and SUB2-immunized mice ($P < 0.01$).

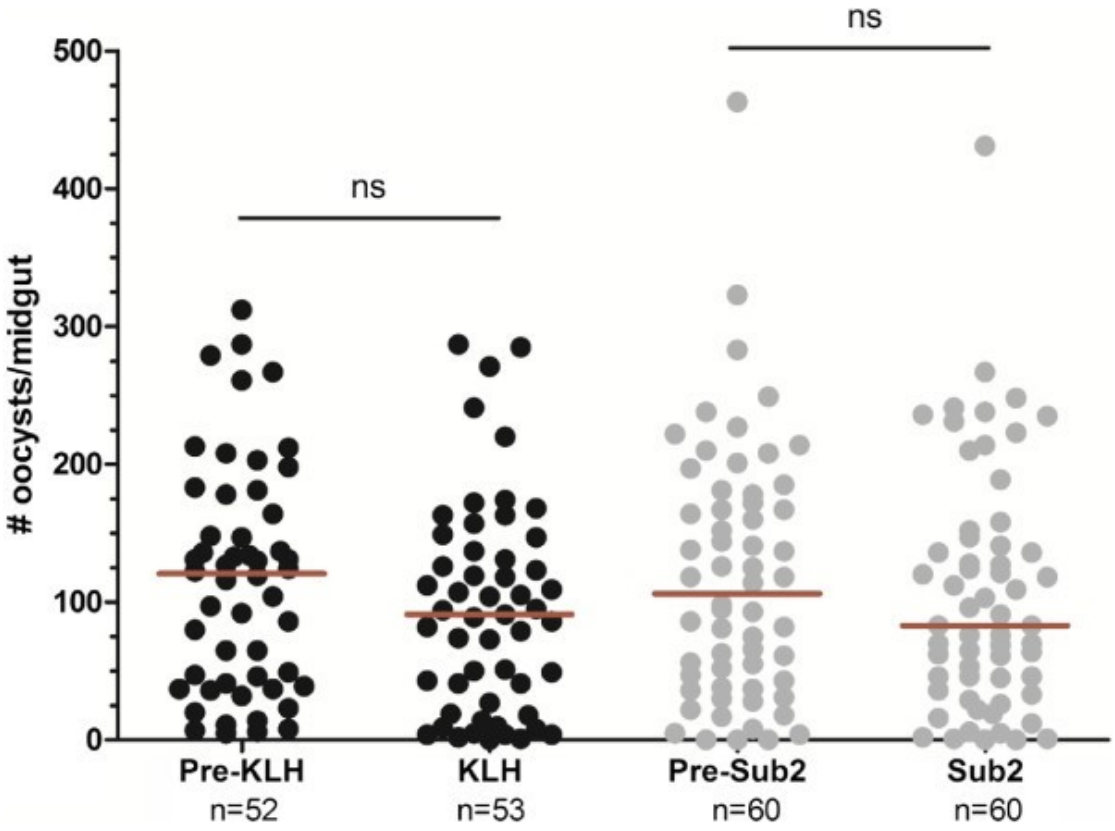


Figure 2.5 Passive immunization with SUB2 immune sera does not influence parasite growth in the mosquito. Oocyst numbers were measured to determine the effects of passive immunization to control KLH- or SUB2-immune sera. *Plasmodium berghei*-infected mice were fed to mosquitoes and oocyst numbers were determined for each experimental group before passive immunization (pre-KLH or pre-SUB2), or following passive immunization (KLH or SUB2). The total number (n) of mosquito midguts examined is displayed under each experimental group. The red bar denotes the median of each experiment. No significant (ns) differences were identified for either experimental group following passive immunization.

Chapter 3

Characterization of SUB2-derived peptides using self-assembling protein nanoparticles

Abstract

Subtilisin-like protease 2 (SUB2) is a conserved essential serine protease that functions as a merozoite surface sheddase (MeSh) during red blood cell invasion by *Plasmodium* parasite merozoites. To execute this role, SUB2 is secreted onto the surface of the parasite getting exposed to the antibodies circulating in the blood, kept anchored to the parasite membrane. As a parasite surface protein, SUB2 is part of the assortment of surface antigens and can be targeted for antibody-mediated inhibition. In a proof-of-principle experiment, immunization against this *Plasmodium* subtilisin resulted in attenuation of malaria infection in mice.

To further characterize and optimize antibody-mediated inhibition of *Plasmodium* blood stages by targeting SUB2, a self-assembly protein nanoparticle (SAPN) alone or presenting either SUB2-Peptide1 or SUB2Peptide1+Peptide2 was developed. The SAPNs were used to immunize BALB/c mice; similarly to what is described in Chapter 2 but with important differences. Besides the use of SAPN for antigen delivery, the additional main differences are: an adjuvant was not used for SAPNs immunizations only two, instead of four boosts were performed and no carrier protein was employed in this experiment.

Additionally, on this follow-up approach, a specific number of mCherry *P. berghei* ANKA infected *A. stephensi* female mosquitoes were used for natural inoculation-challenge of each mouse individually. The amount of mosquitoes containing blood after the challenge was also determined. This step was recorded on video for verification of feeding events on each mouse. Any mouse with no blood-

positive mosquitoes was removed from the malaria infection analyses. The progress of the infection was determined by quantifying the percentage of parasitized cells using an ImageStream instrument and the IDEAS software. Additionally, visual confirmation by blood film and Giemsa staining were performed. Immune sera from each mouse were collected prior the challenge and with last surviving mice at the end of the experiment for immunoblotting confirmation for antibody production.

Results revealed no significant difference in the progress and severity of the parasite infection among the three groups of infected mice from which two or more mosquitoes fed, in general. However, In the case of a mouse with only one blood-positive mosquito, no detectable levels of parasitized cells were found with the ImageStream analysis. Only one control mouse showed a significantly higher level of parasitemia compared to the other groups. The mean parasitemia of SAPN-SUB2Peptide1 immunized mice was slightly lower than SAPN control and SAPN-Peptide1+Peptide2 combination. The difference between the groups is not significant with P value 0.5097 (Kruskal-Wallis test).

These results indicate that SAPN-mediated immunization is not sufficient for protection against malaria with targeting SUB2 under the conditions tested in this experiments. Repeating this experiment with some modifications may improve the results and permit us to characterize the effect in protection by each SUB2 peptide. It is possible that a SAPN-based vaccine would have to be optimized for each antigen of choice for optimal protection. Nonetheless, immunoblotting shows that recombinant SAPN harboring either SUB2 Peptide 1 or the combination of Peptide 1 and Peptide 2 is recognized by anti-SUB2 antibodies generated upon immunization

with the same KLH-conjugated SUB2 peptides from previous proof-of-principle experiment (Chapter 2). This indicates that using SAPN for displaying these peptides does not affect how they interact with antibodies. Furthermore, results show that these antibodies can detect both SAPN-SUB2Peptide1 and the SAPN-SUB1Peptide1+Peptide2, but a stronger signal was observed for SAPN-SUB2Peptide1, suggesting that antibodies present in the mouse sera of mice immunized with KHL-conjugated peptides have a higher presence of anti-Peptide1 antibodies which would result in better neutralization. Further studies are required to confirm this assumption.

Introduction

Malaria is a potentially fatal disease caused by parasites of the genus *Plasmodium*. Disease burden and deaths have decreased during the past decade[1] due to a combination of malaria prevention and control measurements. Still, approximately 500,000 deaths are attributed to malaria every year[1]. One of the factors that challenge the efforts of controlling malaria is the emergence of drug resistant strains and the lack of an effective vaccine. Thus, there is a need of novel molecular drug targets and vaccine candidates to overcome parasite drug-resistance and improve the efficacy of a malaria vaccine.

We previously showed that immunization against *Plasmodium* subtilisin-like protease 2 (SUB2) attenuates malaria infection [123] when mice were immunized with two SUB2 peptides. It is uncertain, however, if both peptides are necessary to provide protection or if either is sufficient. Characterization of individual SUB2 peptides protection may lead to a better understanding of the requirements of SUB2-targeted protection and antibody-mediated inhibition of red blood cell invasion.

Protection from malaria has been studied using self–assembling protein nanoparticles (SAPN). SAPN fused to RTS,S target circumsporozoite protein (CSP) was shown to induce an immune response generating antibodies that were effective in inhibition *P. falciparum* sporozoite invasion in cultures [124]. These particles function as a repetitive antigen display [125-127] technology that can be used for presenting antigens to the immune system. SAPN are expressed and purified as a

fusion protein of the target of interest [126]. A detailed protocol for expression and purification has been developed [126], allowing us to quickly advance into studies of characterization of new vaccine targets and the optimization of their use in a mouse model of malaria.

Chapter 2 discusses the effects protection when mice are immunized against SUB2 using two different peptides. This proof-of-principle experimented resulted in the attenuation of malaria parasite infection in SUB2-immunized mice compared to controls. However, the antigenicity of each individual peptide was unclear. In addition, protection did not lead to clearance of the infection in every case, requiring follow-up to pursue optimal results.

To understand the effect in protection by each individual peptide targeting SUB2 surface-exposed catalytic domain SAPN harboring SUB2 peptides were used to immunize mice in the absence of an adjuvant or a carrier protein. In this experiment, three SAPNs were used: SAPN-SUB2Peptide1, SAPN-SUB2Peptide1+2 and SAPN only to compare the protection by the different SAPNs and determine if immunization with a single peptide is sufficient. Following immunizations, mice were challenged with mCherry *P. berghei* parasites via natural inoculation with *Anopheles stephensi* mosquitoes. The challenge was video recorded for verification of results and mosquitoes were evaluated to confirm feeding on the mice. The percentage of parasites circulating in the blood was determined by Image Stream analysis. Mouse survival was monitored as an indicator of the severity of the infection. Antibody production by each mouse was verified by immunoblotting.

Results show that the different SAPNs were not sufficient to elicit an immune response that would result in the production of antibodies without the use of an adjuvant or a carrier protein. However, when tested with mouse sera from KLH immunized mice (Chapter 2), both SAPN-SUB2Peptide1 and SAPN-SUB2Peptide1&2 were recognized with the antibodies in these samples. These results demonstrate that antibodies against *Pb*SUB2 peptides from the previous experiments recognize SUB2 peptides displayed by the SAPN.

Methods and Materials

Expression, purification and assembly of SAPNs

All the three SAPNs (Figure 3.1) were expressed and purified as described on McCoy M.E. et.al, 2013 [126]. Final protein concentrations were 0.0268 mg/ml of SAPN only, 0.007 mg/ml of SAPN-Peptide1 and 0.233 mg/ml of SAPN-Peptide1+Peptide2 combination.

Mice

Female BALB/c mice (~20g) were purchased from Harlan. These mice were maintained according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins

University (protocol number MO09H58). The groups of mice that were used are described in Figure 3.2.

Immunizations

Approximately 20 μg of SAPN alone, SAPN-Peptide1+Peptide2 combination or SAPN-Peptide1, were used to prime female BALB/c mice (~20g) without any adjuvants. In two-week intervals, all mice were boosted two times with 20 μg of SAPN alone, SAPN-Peptide1+Peptide2 combination or SAPN-Peptide1. Before priming, serum from each mouse was collected.

***P. berghei* challenge by natural inoculation with mosquitoes**

BALB/c female mice were each inoculated with 10 *A. stephensi* mosquitoes infected with mCherry *P. berghei* parasites (Figure 3.3A). The infected starved female mosquitoes were added into a cardboard cup with a net covering the top and the bottom. 23 cups were placed on top of a clear shield and a sedated mouse was placed on top of each cup for 30 minutes. A camera was placed at the bottom of the clear shield to record the feeding of the mosquitoes (Figure 3.3B). After feeding was completed the mosquitoes were collected and saved in microcentrifuge tubes for storage at -20 °C. Figure 3.3 details the set up of the mosquito feeding experiment.

Parasitemia

Approximately 20 μl of blood from each mouse were collected into a microcentrifuge tube containing 200 μl of heparin solution and kept on ice. After

centrifugation at 5,000 rpm for 5 minutes, the heparin solution was removed by aspiration. Next, blood cells were fixed in 100 µl of 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 1 hour. After incubation, PFA solution was removed after centrifugation and the fixed cells were washed with PBS. Following the wash step, cells were resuspended in 100 µl PBS and stored at 4 °C.

The percentage of parasitized red blood cells in the blood samples of the mice was determined with Amnis ImageStream system and analyzed with IDEAS software following the specifications in the user's manual. First, cells that were best in focus were gated. Second, single cells were gated based on the bright field Area versus the Aspect Ratio of the cells that were on focus from step 1. Third, the subpopulations were assigned based on the Hoechst DNA dye and mCherry signals. Positive cells for both Hoechst and mCherry were considered parasitized.

Giemsa-stained blood film samples were also performed and stored for verification purposes.

Expression and purification of recombinant *Pb*SUB2

Recombinant *Pb*SUB2 was expressed and purified as described in Chapter 2. In this case, with the Cobalt-TALON affinity purification step was omitted. Protein was eluted with 20 mM maltose in 13 mM HEPES pH 7.5, 250 mM NaCl, 0.5 mM βME. 6 ml of pooled elution samples had a final protein concentration of 0.75 mg/ml measured with a NanoDrop Spectrophotometer.

Immunoblotting

Blot #1. ~18 µg of r*Pb*SUB2 were added to a nitrocellulose membrane (Bio-Rad) that was cut and placed inside a 24-well plate. Samples were blocked overnight with 5 % bovine serum albumin (BSA) in 1X TBST at 4 °C. Blocking was followed by three washes with 1X TBST at 90 RPM for 10 minutes each. As the primary antibody, 1:200 of mouse sera was used and incubated for 1 hour at 37 °C shaking at 50 RPM. Then, all samples were washed three times to remove excess antibody using 1X TBST. An alkaline phosphatase (AP)-conjugated rabbit anti-mouse antibody was used as secondary antibody. Colorimetric detection of the secondary antibody was done with BCIP and NBT AP substrates (Promega).

Blot #2. 1 mg of SAPN alone, SAPN-Peptide1+Peptide2 combination or SAPN-Peptide1 was added to a nitrocellulose membrane as described above. Blocking, washes, antibody incubation and detection were performed as described above.

Results

***P. berghei* challenge with mosquitoes**

To verify that *P. berghei* infected *A. stephensi* mosquitoes were feeding on the mice, this inoculation step was recorded on video and the mosquitoes used in the experiment were stored for analysis. The video shows that all mice were bitten by at least one mosquito except for mouse Peptide 1 #3. Furthermore, no mosquitoes were observed to feed on this mouse on the movie and no blood was present in any

of the mosquitoes intended to inoculate this mouse. There was also no blood found in the tube were the mosquitoes were stored. On average, 8.3 mosquitoes fed on each SAPN control mice, 6.3 on the SAPN-Peptide1+Peptide2 combination, 4.6 SAPN-Peptide1 mice (5.6 without including mouse Peptide 1 #3) and 5.3 on the naïve mice. The number of mosquitoes that fed on each mouse is shown in Table 1.

Plasmodium infection progresses similarly in all groups infected of mice

The protective effects of immunizing with SAPN, SAPN-SUB2Peptide1 and SAPN-SUB2Peptide1+Peptide2 were examined by analysis of mouse blood samples on an ImageStream (Figure 3.4). Samples were taken every day from day 5 after challenge with *P. berghei* to monitor the percentage of parasitized cells through the course of 23 days. Blood stage parasites were detected in all mice that were bitten by mosquitoes, except for mouse Peptide1 #2 that never showed detectable levels of parasitemia. However, analysis of a blood smear sample of this mouse revealed a red blood cell with a ring-like structure inside (Figure 3.5). Including this mouse, 22 out of 23 mice were effectively infected with *P. berghei* during the challenge and 1 out of the 22 infected mice did not develop parasitemia to detectable levels. Average parasitemia values at day 10 post-infection (Figure 3.4B) were ~5 for control SAPN mice, ~3 for SAPN-Peptide1 mice and ~4 for SAPN-Peptide1+Peptide2 combination mice.

Mouse survival is not affected upon SAPN-SUB2 immunization compared to control

To determine the effects of SAPN-SUB2 immunization in mouse survival as an indication of the severity of malaria infection, the survival of immunized mice was observed up to 30 days post-infection (Figure 3.6). At day 30, mice Peptide1 #2, Peptide1 #3, Control #3 and Control #5 were subjected to cardiac puncture terminal blood collection for using sera in immunoblotting experiments and additional analyses.

In this immunization experiment, results indicate that there is increased survival associated with SAPN-Peptide1 and SAPN-Peptide1+Peptide2 immunization compared to SAPN-only (Figure 3.6). 100 % of SAPN-Peptide1+Peptide2 mice were dead by day 23 post-infection and all SAPN-Peptide1 mice were dead by day 25 post-infection, except for mouse SAPN-Peptide1 #2 and #3. These two did not have any detectable levels of parasites in the blood (Figure 3.5). However, 33% of control SAPN-only mice survived until day 30 post-infection, when their blood was collected by cardiac puncture. These control mice were very sick and would probably not have lasted for more than 2 additional days.

SAPN alone is not sufficient for efficient generation of anti-SUB2 antibodies under the conditions tested

To confirm that antibodies against *Pb*SUB2 were present in mice sera after immunizations, two different immunoblots were performed. One of these dot blots consisted in utilizing recombinant *Pb*SUB2 for detection of anti-SUB2 antibodies

(Figure 3.7). The other was used to determine if antibodies against the SAPN only, SAPN-Peptide1 or SAPN-Peptide1+2 respectively were generated (Figure 3.8).

Results show no signal with mouse sera against recombinant MBP-fusion *Pb*SUB2 for all mouse sera samples. Positive controls using anti-MBP and mouse sera from KLH-conjugated SUB2 peptides (Chapter 2) confirm that all the other components of the assay were working properly.

When mouse sera used against the same SAPN it was immunized with, with the exception of control mice SAPN-only #4 and #5, the infected mice did not generate antibodies against the different SAPN antigens (Figure 3.8). Therefore, no antibodies against *Pb*SUB2 were generated upon immunizations in this experiment. Nevertheless, when serum from KLH-conjugated mice (Figure 2.2) was used against either SAPN-Peptide1 or SAPN-Peptide1+Peptide2, the results were positive with a stronger signal on the SAPN-Peptide1 sample (Figure 3.8). Demonstrating that anti-SUB2 antibodies can interact with SUB2 peptides displayed using a SAPN.

Together, these results strongly suggest that there was no difference between SAPN control and SAPN-SUB2 mice because no antibodies were generated against the antigen whatsoever; thus, there were no protective antibodies targeting SUB2. In contrast, the samples using mouse serum from the KLH-SUB2 peptides experiments validates SAPN-SUB2 as an antigen for anti-SUB2 antibody generation.

Discussion

Despite the global efforts in controlling malaria, there is still a lot of work to do for successful malaria control and to contemplate the ultimate goal of malaria eradication. Complete elimination of malaria will prevent about 500,000 deaths caused by this infectious disease every year[1]. To succeed in fighting malaria, we need novel effective drugs and vaccines.

A rational approach by targeted design of inhibitors provides the advantage of knowing the basics of the molecular target by which the approach works. Instead of working with an unknown target that requires identification and further characterization. In the present study, subtilisin-like protease 2 (SUB2) is targeted for antibody-mediated inhibition of invasion of the erythrocyte by the malaria parasite (antibody mediated protein-protein interaction inhibition). As an antigen-delivery mechanism, a self-assembling protein nanoparticle (SAPN) [126] is employed for displaying of various copies of SUB2 peptides that were previously shown to reduce malaria infection in a mouse model of malaria infection. SAPN was previously used for studying the protective immune mechanism induced by circumsporozoite (CSP) protein[124, 128], a key component in the current vaccine candidate for malaria, RTS,S [10].

Here, in a similar approach to the one described in Chapter 2, mice were immunized with a SAPN-fusion protein (Figure 3.1) displaying SUB2 Peptide 1 or SUB2 Peptide 1 (Figure 2.1) and Peptide 2 combination (SAPN-Peptide1 and SAPN-Peptide1+Peptide2 respectively) or SAPN alone as a control to further examine

antibody-mediated inhibition of parasite development within the blood. Another purpose of this immunization-based experiment was to set the basis of SUB2-targeted immunization to determine the optimal procedure to achieve protective immune response for the clearance or prevention of malaria infection. Other components were also modified to assess in the analysis of the data.

An important modification to the experiments with the KLH-conjugated peptides is that, in the current experiment, inoculation of mice with *P. berghei* was done via mosquito feeding. This is the natural course of malaria transmission to the vertebrate host. We were also interested in evaluated SUB2 immunization for, not only treatment but also prevention of infection prior to liver stages or before the infection establishes within the blood.

In general, results of this experiment remain inconclusive since, under the conditions tested, the use of SAPN for immunization with SUB2 peptides was not effective in eliciting an immune response leading antibody generation (Figure 3.7). There are several possible explanations to why the results were not as predicted. One of them is that the use of an adjuvant may be required for generating antibodies against SUB2 using these two peptides as antigens. It is also possible that KLH was playing an important role as a carrier protein in previous experiments Another possibility is that a higher amount of protein or additional boosts were required for the desired results. It could also be that a combination of more than one of the above is required for effective protection using SUB2 peptides #1 and #2. This all could be because these antigens are too small (~2 kDa) to elicit an immune response alone

since SAPN was not providing additional antigenicity in our experiments. However, cannot reach a definite conclusion based on our data.

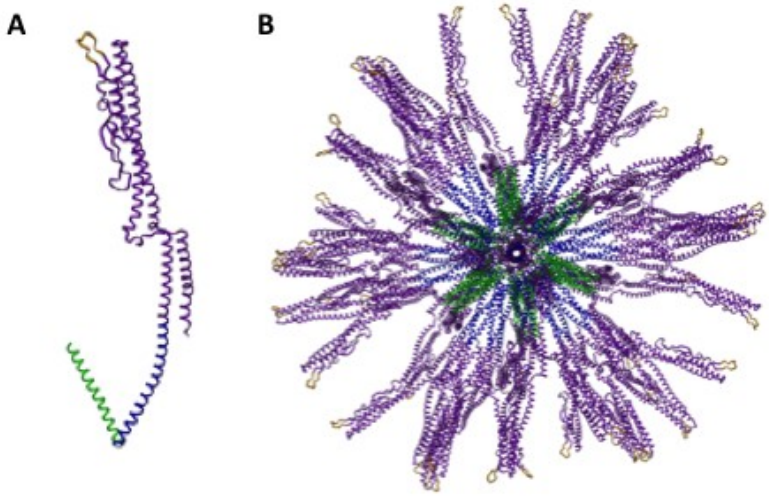
Additional to the data shown above, live images were obtained using an IVIS Series Pre-clinical In Vivo Imaging System (PerkinElmer) at approximately 19 hours, 38 hours, 8 days and 15 days post-challenge in attempts to detect mCherry signal during liver stages. This information would help to assess if these immunizations were targeting the sporozoites before they reached the liver or if the infection was stopped right after the hepatic stage and prior establishing in the blood. Unfortunately, this study was inconclusive due to the high levels of mouse hair autofluorescence that resulted in high background signal and, probably, because of the hepatic stages were not severe enough to emit a detectable mCherry.

Other data not considered in this study was the multiple invasion events that were observed but not quantified due to the negative results of immunizations. This analysis should be included in future experiments.

Overall, we collected important information on other observations that should be taken into consideration in other experiments of this nature. It is possible that sometimes, important misleading information is inadvertently included in data analysis leading to wrongful conclusions. In our case, we documented information that is not often collected in this study for an in-depth analysis of the data. By recording the inoculation step of the experiment we were able to exclude data from one mouse that was not bitten by mosquitoes (Table 3.1), thus was never infected and could not be used in the parasitemia analysis. In later studies, by improving light conditions of recording, it will also be possible to quantify how many times

each mouse gets bitten by a mosquito or, at least, how many mosquitoes fed on each mouse. This information could help in data interpretation.

In conclusion, it was not possible to determine if SAPN is the best tool of antigen delivery for SUB2-targeted immunization. Nevertheless, we were able to show that antibodies that recognize recombinant SUB2 (Figure 2.2) can detect SAPN molecules harboring a combination of both SUB2 Peptide 1 and Peptide 2 or SAPN displaying Peptide 1 alone (Figure 3.8). This demonstrates that SUB2 peptides are displayed by SAPN in a way that can be recognized by the antibodies circulating in the blood. It does not proof, however, that SAPN-SUB2 alone can elicit an immune response for generating protective antibodies. Still, SAPN remains an attractive technology for future studies for optimization of SUB2 protection in mice. Follow-up studies are required to incorporate modifications to the current protocol.



SAPN antigens	Description
SAPN only/control	Naked SAPN
SAPN-Peptide 1	SUB2 Peptide 1
SAPN-Combination	Co-assembled SAPN-Peptide1+SAPN-Peptide2

Figure 3.1 A SAPN-SUB2 fusion protein was developed for mice immunization. A) Structural model of coiled-coil SAPN-SUB2 subunit. The segment corresponding to the SUB2 peptide is shown in yellow. B) A structural model of the assembled SAPN displaying SUB2 peptides. Bottom: Table listing the three SAPNs utilized in these experiments. The SAPN only particle consisted of the SAPN protein without a SUB2 peptide sequence. SAPN-Peptide1 comprised of the SAPN protein with the SUB2 Peptide 1 sequence encoded in it for presenting to the target immune system. The last, SAPN-Peptide1+Peptide2 or "combination" is a mixed SAPN that includes both, a SAPN-Peptide1 protein and a SAPN-Peptide2 protein that were co-assembled to create a SAPN displaying both peptides simultaneously.

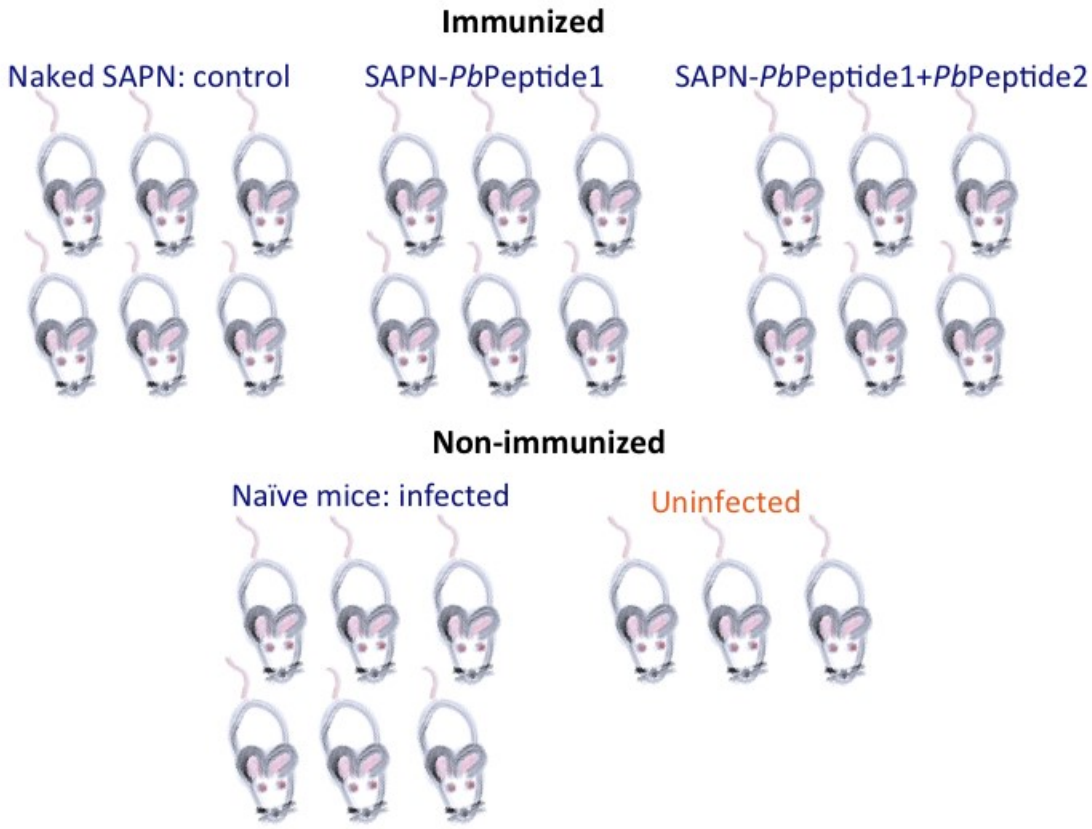


Figure 3.2 Groups of mice employed in this experiment and their purpose. A total of 23 mice were used this immunization experiment that consisted of three groups of 6 mice that were immunized with the different SAPN antigens: SAPN-Peptide1_Peptide2 (combination), SAPN-Peptide1, SAPN only (negative control). Two groups of mice that were not immunized were included in the experiment as additional controls. These consisted of one group of 6 naïve mice; these were challenged with *P. berghei* without being previously manipulated as a positive control of infection. The second group of non-immunized mice included only 3 mice there were never immunized or inoculated with *P. berghei* as a negative control during analyzes.

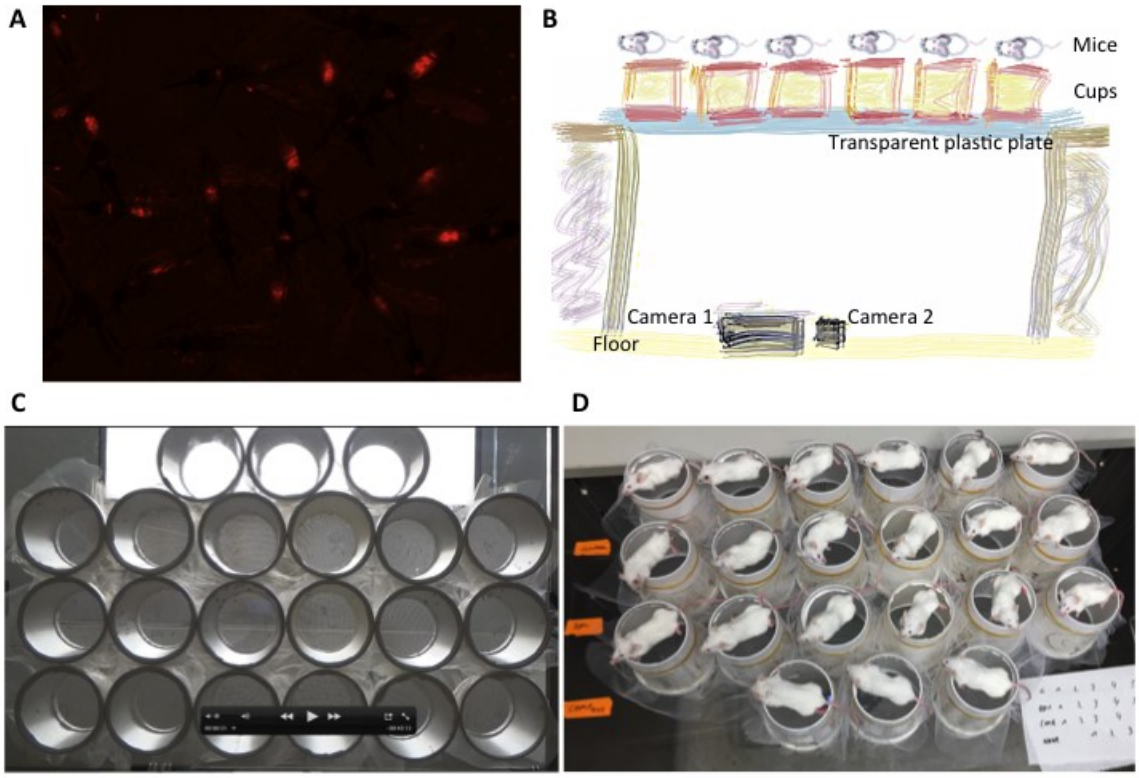


Figure 3.3 *P. berghei* challenge by natural inoculation with mosquitoes. A) Microscopy image of *A. stephensi* mosquitoes approximately 24 hours prior to feeding. The mCherry signal coming from mosquitoes gut or salivary glands demonstrates the mosquitoes are prepared for transmission of the parasites to the mice during a blood meal. B) Experimental set up of the feeding experiment. C) A screenshot of the movie showing the experimental set up from the camera's perspective from the bottom of the plastic plate prior placing the mice on top of the cups. D) Top view showing the array of the anesthetized mice placed on top of the cups containing mosquitoes.

Mouse #	1	2	3	4	5	6
SAPN Control	9	6	9	9	7	10
SAPN-Combination	4	3	6	9	6	10
SAPN-Peptide1	9	1	0	5	7	6

Table 3.1 Number of mosquitoes bites received by each mouse during natural inoculation via mosquito feeding. This table shows how many mosquitoes contained blood after the feeding experiment. The results were determined by observation of each individual mosquito collected after the feeding. It does not reflect how many times a mouse was bitten by mosquitoes but how many mosquitoes ingested blood from the mice. These data also do not distinguish between probing or actual feeding by mosquitoes.

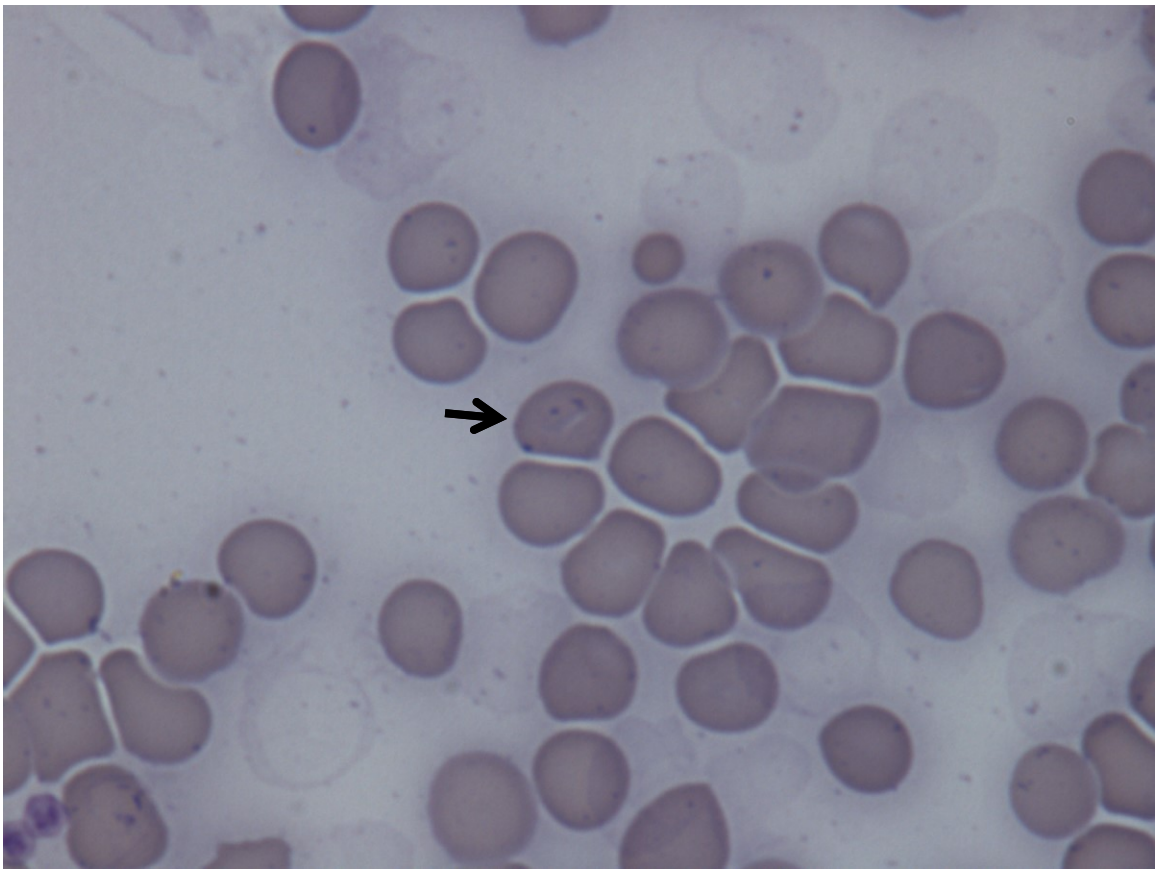


Figure 3.4 Ring stage parasite found on SAPN-Peptide1 mouse #2 blood sample at day 3. A red blood cell with a ring stage parasite (black arrow) found on the blood smear of SAPN-Peptide1 mouse #2 at day 9 post-infection showing that this mouse resulted infected during a bite of one mosquito. Additional analyses are required for confirmation in the future.

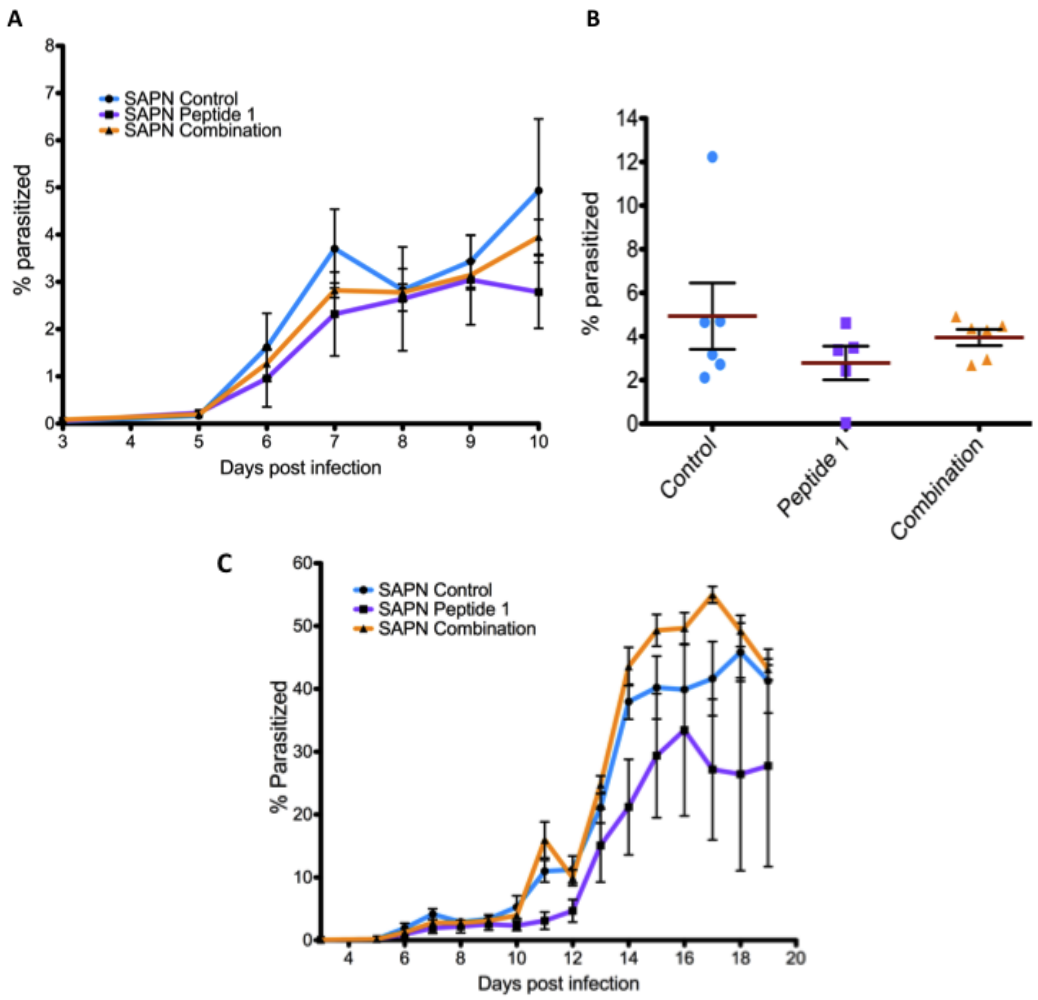


Figure 3.5 Parasitemia of SAPN immunized mice. A) Parasitemia from day 3 to day 10 post-infection. Blue shows data from SAPN control mice, purple corresponds to the data from SAPN-Peptide1 mice and orange indicates data from SAPN-Peptide1+Peptide2 mice. Results show a similar trend between all groups of mice. SAPN-Peptide1 mice have an average of almost half the parasitemia compared to the control. B) Parasitemia of individual mice at day 10 post-infection, data points shown in blue, purple or orange. Mean values of each group are marked with a red bar; the standard deviation values are shown in black. C) Parasitemia up to day 19 post-infection. Hyperparasitemia is observed after day 10 post-infection.

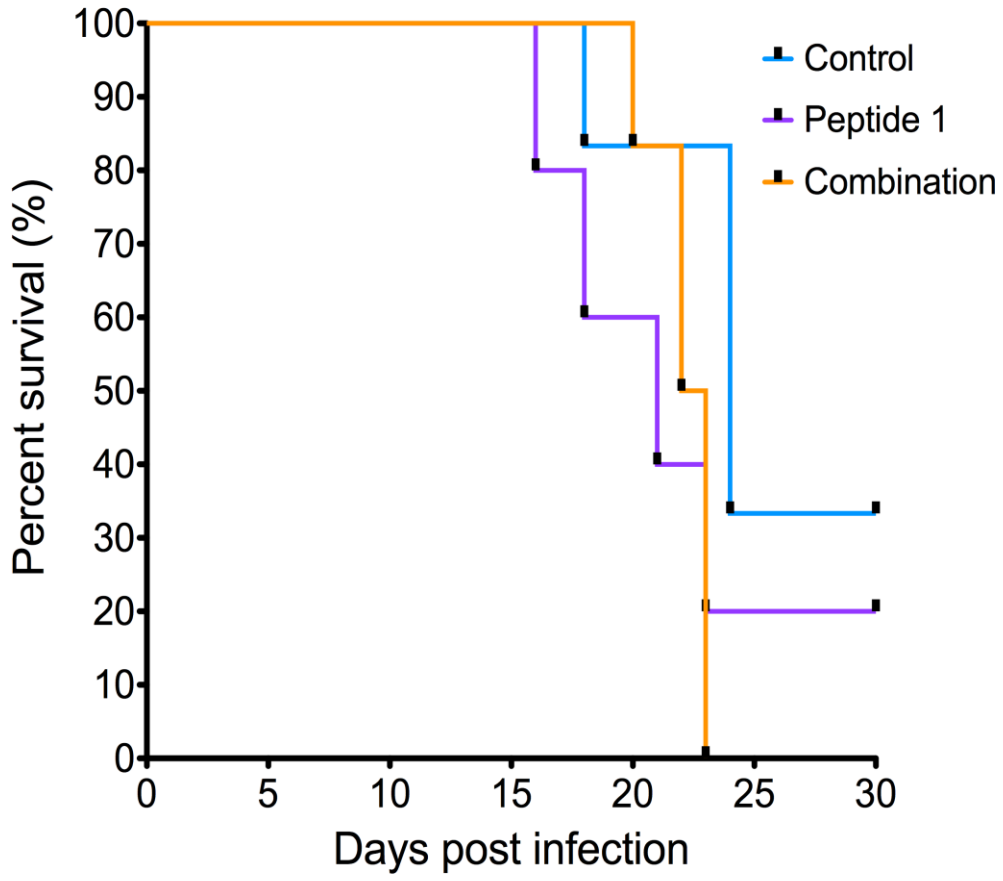
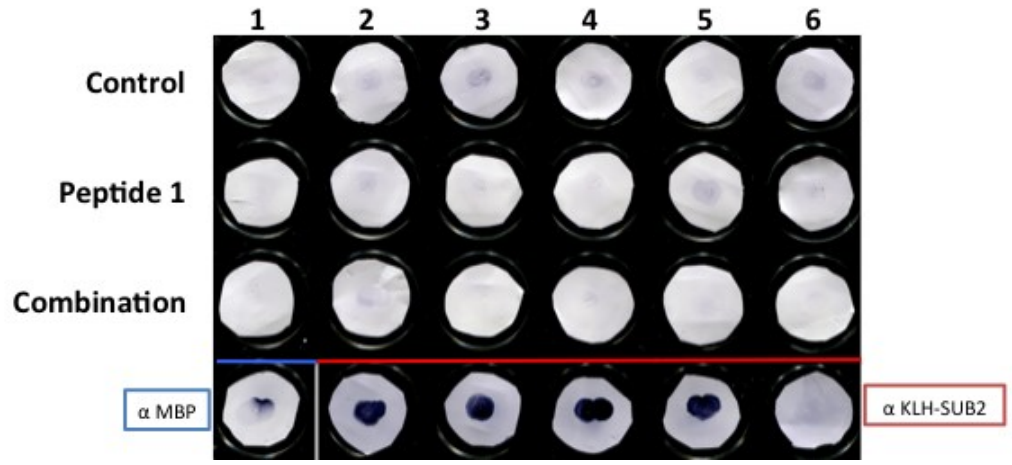


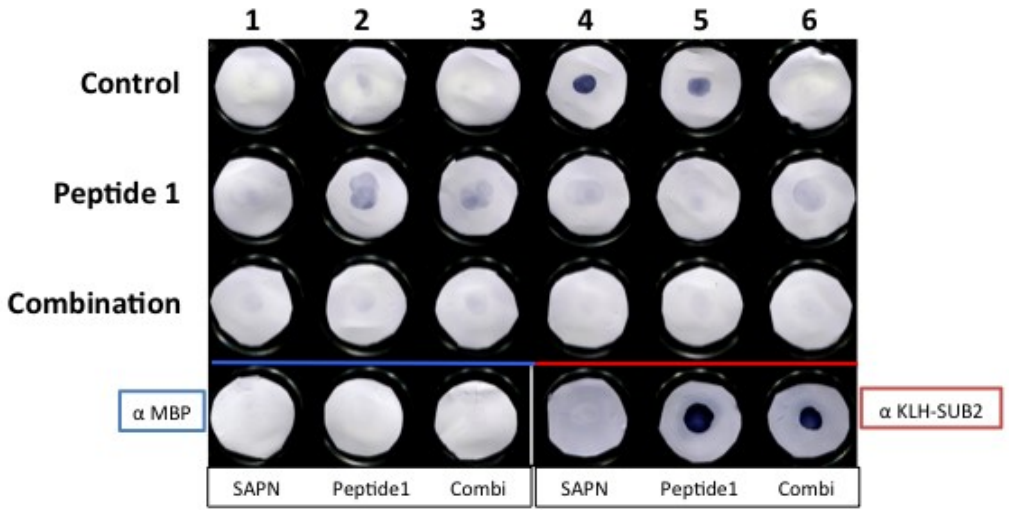
Figure 3.6 Plot of mouse survival. Survival of the mice was monitored over a period of 30 days post-infection. Data are shown in blue, purple and orange for SAPN control, SAPN-Peptide1 and SAPN-Peptide1+Peptide2 respectively. By day 24, all Peptide1+Peptide2 combination mice were dead. At day 30, the blood of two (33 %) SAPN control mice that were still alive but very sick and two (33 %) SAPN-Peptide1 mice that were alive and well, was collected by cardiac puncture.



Experiment #1: r*Pb*SUB2 (MBP-fusion protein, same used for KLH-SUB2 peptides)

Mouse: Control #1 **Protein:** rPbSUB2 **Primary Antibody:** C#1 serum	**Mouse:** Control #2 **Protein:** rPbSUB2 **Primary Antibody:** C#2 serum	**Mouse:** Control #3 **Protein:** rPbSUB2 **Primary Antibody:** C#3 serum	**Mouse:** Control #4 **Protein:** rPbSUB2 **Primary Antibody:** C#4 serum	**Mouse:** Control #5 **Protein:** rPbSUB2 **Primary Antibody:** C#5 serum	**Mouse:** Control #6 **Protein:** rPbSUB2 **Primary Antibody:** C#6 serum
Mouse: Pept1 #1 **Protein:** rPbSUB2 **Primary Antibody:** P#1 serum	**Mouse:** Pept1 #2 **Protein:** rPbSUB2 **Primary Antibody:** P#2 serum	**Mouse:** Pept1 #3 **Protein:** rPbSUB2 **Primary Antibody:** P#3 serum	**Mouse:** Pept1 #4 **Protein:** rPbSUB2 **Primary Antibody:** P#4 serum	**Mouse:** Pept1 #5 **Protein:** rPbSUB2 **Primary Antibody:** P#5 serum	**Mouse:** Pept1 #6 **Protein:** rPbSUB2 **Primary Antibody:** P#6 serum
Mouse: Combi #1 **Protein:** rPbSUB2 **Primary Antibody:** Combi#1 serum	**Mouse:** Combi #2 **Protein:** rPbSUB2 **Primary Antibody:** Combi#2 serum	**Mouse:** Combi #3 **Protein:** rPbSUB2 **Primary Antibody:** Combi#3 serum	**Mouse:** Combi #4 **Protein:** rPbSUB2 **Primary Antibody:** Combi#4 serum	**Mouse:** Combi #5 **Protein:** rPbSUB2 **Primary Antibody:** Combi#5 serum	**Mouse:** Combi #6 **Protein:** rPbSUB2 **Primary Antibody:** Combi#6
Mouse: N/A **Protein:** rPbSUB2 **Primary Antibody:** anti-MBP	**Mouse:** N/A **Protein:** rPbSUB2 **Primary Antibody:** anti-SUB2 mouse	**Mouse:** N/A **Protein:** rPbSUB2 **Primary Antibody:** anti-SUB2 #4	**Mouse:** N/A **Protein:** rPbSUB2 **Primary Antibody:** anti-SUB2 #5	**Mouse:** N/A **Protein:** rPbSUB2 **Primary Antibody:** anti-SUB2 1/2 mouse #1 B4	**Mouse:** N/A **Protein:** rPbSUB2 **Primary Antibody:** anti-SUB2 #1 mouse #4 B4

Figure 3.7 Immunoblotting with recombinant *Pb*SUB2 to confirm production of antibodies against *Pb*SUB2. Top) A dot blot done using a 24-well culture plate to test production of anti-SUB2 antibodies by each individual mouse. The same construct MBP-fusion recombinant protein *Pb*SUB2 tested previously in Chapter 2 was used in this experiment. Results revealed that no anti-SUB2 antibodies were detected for the immunized mice. Mouse serum from KLH-SUB2 experiments (Figure 2.2) was used as a positive control. Bottom) An outline summarizing the details for each sample.



Experiment #2: SAPNs

Mouse: Control #1 **Protein:** SAPN **Primary Antibody:** C#1 serum	**Mouse:** Control #2 **Protein:** SAPN **Primary Antibody:** C#2 serum	**Mouse:** Control #3 **Protein:** SAPN **Primary Antibody:** C#3 serum	**Mouse:** Control #4 **Protein:** SAPN **Primary Antibody:** C#4 serum	**Mouse:** Control #5 **Protein:** SAPN **Primary Antibody:** C#5 serum	**Mouse:** Control #6 **Protein:** SAPN **Primary Antibody:** C#6 serum
Mouse: Pept1 #1 **Protein:** SAPN-P1 **Primary Antibody:** P#1 serum	**Mouse:** Pept1 #2 **Protein:** SAPN-P1 **Primary Antibody:** P#2 serum	**Mouse:** Pept1 #3 **Protein:** SAPN-P1 **Primary Antibody:** P#3 serum	**Mouse:** Pept1 #4 **Protein:** SAPN-P1 **Primary Antibody:** P#4 serum	**Mouse:** Pept1 #5 **Protein:** SAPN-P1 **Primary Antibody:** P#5 serum	**Mouse:** Pept1 #6 **Protein:** SAPN-P1 **Primary Antibody:** P#6 serum
Mouse: Combi #1 **Protein:** SAPN-P1,2 **Primary Antibody:** Combi#1 serum	**Mouse:** Combi #2 **Protein:** SAPN-P1,2 **Primary Antibody:** Combi#2 serum	**Mouse:** Combi #3 **Protein:** SAPN-P1,2 **Primary Antibody:** Combi#3 serum	**Mouse:** Combi #4 **Protein:** SAPN-P1,2 **Primary Antibody:** Combi#4 serum	**Mouse:** Combi #5 **Protein:** SAPN-P1,2 **Primary Antibody:** Combi#5 serum	**Mouse:** Combi #6 **Protein:** SAPN-P1,2 **Primary Antibody:** Combi#6 serum
Mouse: N/A **Protein:** SAPN **Primary Antibody:** anti-MBP	**Mouse:** N/A **Protein:** SAPN-P1 **Primary Antibody:** anti-MBP	**Mouse:** N/A **Protein:** SAPN-P1,2 **Primary Antibody:** anti-MBP	**Mouse:** N/A **Protein:** SAPN **Primary Antibody:** KLH mouse	**Mouse:** N/A **Protein:** SAPN-P1 **Primary Antibody:** KLH mouse	**Mouse:** N/A **Protein:** SAPN-P1,2 **Primary Antibody:** KLH mouse

Figure 3.8 Immunoblotting to confirm antibody production against the different SAPNs used for immunization. Top) A dot blot was done using a 24-well culture plate to test production of anti-SAPN, SAPN-Peptide1 or SAPN-Peptide1+Peptide2 antibodies by each individual mouse. Antibody production was only confirmed for SAPN control mouse #4 and #5. There is a higher-than-background signal for SAPN-Peptide1 mouse #2 and #3. No other positive samples were detected. Although there was no signal using the SAPN control, there was a strong signal for SAPN-Peptide1 and SAPN-Peptide1+Peptide2 combination. SAPN-Peptide1 was the strongest, suggesting a higher preference for this peptide. Bottom) Sample details.

Chapter 4

Development of a SUB2-specific biochemical assay for *in vitro* characterization

Abstract

From the five species of *Plasmodium* parasites that infect humans, *P. falciparum* results in more severe malaria cases leading to deaths. Drug resistance of this species has been reported for all antimalarials currently available, including combination therapy in different malaria endemic regions. As a result, there both, effective vaccines and working drugs against malaria are urgently required for prevention and treatment of this potentially fatal infectious disease.

Calcium-dependent serine protease activity is essential for invasion of the host cell during the asexual developmental stages of the malaria parasite within the red blood cells (RBC). This activity leads to the shedding-off of merozoite surface ligands during invasion of the erythrocyte, catalyzed by subtilisin-like protease 2, the only *Plasmodium* subtilisin directly implicated in RBC invasion. Due to its critical role in parasite development during disease-causing blood stages, SUB2 represents a prime target for vaccine development and design of novel small molecule inhibitors of malaria infection. Yet, expression and purification of SUB2 have not been successfully achieved for *in vitro* biochemical and biophysical analyses.

To develop a *Plasmodium* SUB2-specific protease assay, we attempted to establish catalytically active recombinant protein expression and purification protocol. Various DNA constructs comprising the catalytic domain of *Pf*SUB2 were developed and tested for protein expression in *E. coli*. These constructs include three different catalytically inactive variants were developed by mutating Ser and

His residues at the active site. Protease activity was initially tested using commercially available activity assays with general substrates.

Results demonstrate that many constructs including different domains of *PfSUB2* can be expressed in *E. coli*. Yet, recombinant *Pf*SUB2 (r*Pf*SUB2) is expressed insoluble in the absence of a solubility-adding tag, such as maltose-binding protein (MBP). MBP-SUB2 can be partially purified by affinity purification, but the yield and purity were not sufficient for biophysical analysis, such as protein crystallography. Additionally, it was not possible to conclusively show that r*Pf*SUB2 was catalytically active using commercially available protease assays and in tests with general substrates. AMA1-derived substrates were developed for a SUB2-specific assay. Preliminary studies did yield positive results.

There were indications, however, that r*Pf*SUB2 could have been active during expression in *E. coli*, such as slow cell growth and lysis. Yet, these observations could not be confirmed in these studies. Nonetheless, r*Pf*SUB2 can be successfully employed in immunoblotting analyses, as discussed in Chapter 2 and 3. Follow-up studies are required to establish an alternate heterologous expression system for *Pf*SUB2.

Introduction

Obligate intracellular parasites have evolved to utilize protease activity for unique cellular mechanism leading to pathogenesis [129]. Host cell invasion is key for the survival of these unicellular organisms and the completion of their life cycle within their hosts, resulting in infectious disease symptoms [129]. *Plasmodium*, the causative agent of malaria, requires merozoite surface sheddase activity (MeSh) [45] for a successful invasion of the host cell during their asexual cycle within the vertebrate host.

Inhibition of shedding off the antigens at the merozoite surface may prevent or impair invasion [45], resulting in unsuccessful entry of the parasite into the erythrocyte. By preventing access of an obligate intracellular eukaryote to its target host cell, will have repercussions in the establishment of infection and its capacity to evade the host's immune system. A decreasing number of parasites in the blood could potentially lead to the complete clearing of the infection.

MeSh activity has been attributed to the only *Plasmodium* subtilisin with a sheddase role during invasion: subtilisin-like protease 2 (SUB2)[45]. SUB2 (Figure 4.1) is one of the three *Plasmodium* subtilisins encoded in the malaria parasite genome. It performs an essential and non-redundant role during red blood cell invasion that results in the merozoite surface sheddase activity that was reported before the identification of this second *Plasmodium* subtilisin. To this date, there is substantial understanding of the role of SUB2 upon invasion of the erythrocytes during the asexual stages of the parasite [40, 45, 96, 97, 99, 123] .

However, the absence of a heterologous expression system for *in vitro* studies makes it impossible to develop a SUB2-specific protease assay. Biochemical and biophysical characterization of SUB2 is necessary for the better understanding of the mechanism of action of this subtilisin. These studies will also make possible to use a rational design approach to the development of small-molecule inhibitors or erythrocyte invasion by targeting SUB2.

To establish an expression and purification protocol for *P. falciparum* SUB2 expression in *E. coli*, we have designed various expression constructs. These include constructs with different domains of SUB2, different fusion tags and AMA1-derived [96] substrates for a SUB2-specific activity assay. Protease assays with general substrates casein and gelatin were used to test SUB2 activity. However, it is not clear if SUB2 is expressed catalytically active in *E. coli* under the conditions tested.

Methods and Materials

Plasmodium cDNA

P. berghei ANKA and *P. falciparum* 3D7 isolation of total RNA and production of cDNA were performed as described in Chapter 2.

*Pf*SUB2 and *Pb*SUB2 homology model and visualization for construct design

The predicted structure of *Pb*SUB2 was obtained using the Iterative Threading ASSEmbly Refinement (I-TASSER) server as described in Chapter 2.

*Pf*SUB2 predicted structure (Figure 4.2) was determined in the same manner but using *Pf*SUB2 sequence (PDB PF3D7_1136900, Gene ID: 3423789).

Primary *Pf*SUB2 and *Pb*SUB2 constructs

A primary construct for *P. falciparum* SUB2 N528 - S1135 (PlasmoDB code: PF3D7_1136900, Gene ID: 810927) and *P. berghei* SUB2 N476 - N1185 (PlasmoDB code: PBANKA_091170, Gene ID: 3423789) SUB2 were developed for storage of SUB2 sequence into a cloning vector for subcloning of SUB2 into various constructs for diverse purification and detection purposes. *Pf*SUB2 or *Pb*SUB2 coding sequences were amplified from cDNA obtained from *P. falciparum* 3D7 or *P. berghei* ANKA strains using Phusion® High-Fidelity DNA polymerase (New England Biolabs) and PCR program specifications described in Chapter 2.

Using *NcoI* and XhoI restriction sites, the PCR product was inserted in frame into a pHAT-5 vector (Bailey lab, BMB). Screening of positive clones was performed with the primers mentioned above using colony PCR followed by sequencing confirmation.

Secondary *Pf*SUB2 constructs

Secondary PfSUB2 constructs are those subcloned from the primary construct (Figure 4.3). SUB2 PCR products were inserted in-frame using *Nco*I and *Bam*HI restriction sites into a modified pRSF-1b vector (Novagen). This vector allows the expression of a Maltose Binding Protein (MBP)-fusion protein with an additional C-terminal 6xHis tag added in the primer sequence. These tags were used

for purification and detection purposes as described in Chapter 2. Positive clones were screened by colony PCR and insertion of the sequences was confirmed by sequencing. This protocol is similar to the one described in Chapter 2.

*Pf*SUB2 active site mutants

Catalytically inactive mutants H797G, S960A and H797G-S960A (Figure 4.4) of *Pf*SUB2 were generated using a Site-Directed Mutagenesis Kit (Agilent Technologies) following the specifications of the instruction manual. Mutations were confirmed by sequencing.

AMA1-derived substrates

Native apical membrane antigen 1 (AMA1) sequences cleaved by SUB2 as described in [100], were each cloned into a GKAR 1.2 vector (Jin Zhang Lab, JHU) using the restriction sites for SphI (5') and BglII (3'). This allowed cloning of the different sequences between the yellow fluorescent protein (YFP) and blue fluorescent protein (CFP) on the vector (FIGURE 4.7A).

Primers where designed with overhangs ends simulating the cleavage of restriction enzymes, annealed and ligate into the expression vector. DNA oligos pairs ordered from Integrated DNA Technologies (IDT), Juxtamembrane_site-Forward: cggCGCGCAGAAGTTACGAGCAACAATGAAGTCa and Juxtamembrane_site-Reverse: agatctGACTTCATTGTTGCTCGTAACTTCTGCGCGccgcatgc, Poly_Alanine-Forward: cggaggGCTGCCGCAGCGGCTGCCGCAgaagtta and Poly_Alanine-Reverse: agatctaacttcTGCGGCAGCCGCTGCGGCAGCcctccgcatgc, Proline_Rich-Forward:

cggCCGGCACCCGTACCCCCGAACCCGGAACCTa and Proline_Rich-Reverse: agatctAGGTTCCGGGTTCGGGGGTACGGGTGCCGGccgcatgc, and Intramembrane_Site-Forward: cggAAAATCATTATCGCATCTAGTGCGGCAGTCa and Intramembrane_Site-R: agatctGACTGCCGCACTAGATGCGATAATGATTTTccgcatgc, were annealed following protocol on the IDT website. Each primer pair was combined in a 1:1 molar ratio by mixing 50 µl of 100 µM of the forward primer and 50 µl of 100 µM of the reverse primer with 100 µl of 2X annealing buffer (200 mM potassium acetate, 60 mM HEPES pH 7.5). The mix was incubated for 5 minutes at 94 °C on a heat block. Then, the heat block was turned off and samples were allowed to cool down for about 45 minutes when the temperature of the samples dropped close to room temperature. Primers were stored at – 20 °C until ligated into the GKAR 1.2 vector. Screening was performed to confirm insertion of the different linkers by restriction enzyme digestion and analyzed by agarose gel electrophoresis. Finally, positive clones were confirmed by DNA sequencing.

Expression of recombinant protein

Protein expression of SUB2 constructs was generally done as described in Chapter 2. Rosetta™ 2 competent cells (Novagen) were transformed with expression vectors and grown overnight at 37 °C in a 50 ml starter culture. Protein was expressed in 500 ml cultures of Terrific Broth (TB). However, modifications were applied for other constructs to test optimal expression conditions (Figure 4.5).

Purification of recombinant protein

MBP-SUB2 constructs were purified as described in Chapter 2. Some modifications apply to some other constructs containing different tags for affinity purification (Figure 4.6).

Zymogram gels

Protease activity of rSUB2 was tested using Ready Gel® zymogram precast gels (Bio-Rad) with casein or gelatin and with SDS-PAGE gels made in lab using Bio-Rad manual with 0.1 % gelatin or 0.1 % casein. For electrophoresis, refolding and developing steps, the user's instructions manual from Bio-Rad was followed. Developing step was usually performed overnight at 37 °C for optimal results.

***Pf*SUB2 protease assay with AMA1-derived substrates**

A preliminary protease assay test with *Pf*AMA-1 derived *Pf*SUB2 substrates was performed by incubation of wild type and S960A *Pf*SUB2 inactive mutant. Reactions were carried out in a protease assay buffer (25 mM HEPES pH 8.25, 150 mM NaCl and 5 mM $CaCl_2$) at 37 °C overnight. Results were analyzed by SDS-PAGE electrophoresis and coomassie brilliant blue stain. Some of the reactions included TEV protease for removal of MBP tag during the reaction. Two positive controls of proteolysis were included in the assay: trypsin and a bacterial subtilisin.

Results

Zymogram gels

The activity rSUB2 was tested with in-gel casein and gelatin general substrates by zymography (Figure 4.7), both precast and lab made gels. With these assays there was no *Pf*SUB2 activity detected but the trypsin control was positive.

Protease assay test with GKAR AMA1-derived fluorescent substrates

Cleavage of fluorescent substrate with the poly alanine linker (Poly A) for the *Pf*SUB2-specific protease assay was tested and analyzed by SDS-PAGE (Figure 8). Wild type *Pf*SUB2 activity was compared to S960A *Pf*SUB2 in the presence or absence of TEV protease. Under none of these conditions was there cleavage of the Poly A substrate by *Pf*SUB2. On the other hand, positive controls trypsin and a bacterial subtilisin digested the full ~ 50 kDa Poly A substrate to a ~27 kDa and a 25 kDa band respectively. TEV did not show activity against the Poly A substrate but it did cleave the MBP tag from the full-length recombinant SUB2. Results show the substrate is cleavage by trypsin and by a subtilisin. Thus, rSUB2 is not active under the conditions tested in the assay.

Discussion

Due to its critical role in red blood cell (RBC) invasion and because it is essential for *Plasmodium* survival during asexual stages, subtilisin-like protease 2 (SUB2) is an attractive target for inhibition of parasite development during blood stages. Current malaria treatment is not based on SUB2 or other *Plasmodium* subtilisins. Biochemical and biophysical characterization of this molecular target will establish the basis of SUB2-targeted small-molecule inhibitors of RBC invasion.

Screening and development of small molecule inhibitors require expression and purification of the target protein of *in vitro* and biophysical studies. At the present, though, there are no reports of an effective expression and purification protocol for catalytically active recombinant *Plasmodium* SUB2. It is our purpose to establish an expression and purification protocol using *E. coli* as a heterologous expression system by developing various expression constructs using a structural model of SUB2. We have also attempted to develop a SUB-specific protease assay using native *Pf*SUB2 substrates previously characterized in parasite cultures.

From our data we concluded that it is unclear if *Plasmodium* SUB2 is expressed actively in *E. coli*. There are indications of protein toxicity while using E. coli as an expression system, that suggest SUB2 was expressed active in several occasions. However, we do not have conclusive data that will support these observations. Sequencing of the expression constructs isolated from *E. coli* cultures in which SUB2 was already expressed could reveal mutations in the SUB2 sequence

that might render the protein inactive leading to evidence that the activity of this protease is detrimental to the host.

Various SUB2 constructs can be expressed in *E. coli* using standard expression conditions; still SUB2 constructs containing the catalytic domain are insoluble unless expressed fused to MBP. This is not the case for the minimal domain of SUB2 that is highly soluble and can be expressed in high amounts with only a hexahistidine tag. Insolubility of the protein can indicate it is not correctly folded in *E. coli* or that it requires other buffer conditions or cofactors to aid in solubility. It is also possible that other truncations would lead to more stable and soluble protein since not all the possibilities were tested. Even if SUB2 will remain in the insoluble fraction upon cell lysis after successful expression, a preliminary circular dichroism (CD) experiment showed a signal for secondary structures additional to those found in MBP alone, when an MBP-SUB2 construct was tested. This specific CD experiment suggested that the catalytic domain of SUB2 was folded, at least partially, after being expressed in *E. coli*.

With the small amounts of soluble SUB2 we attempted several experiments such as protein crystallization and surface plasmon resonance that were not successfully carried out. Some crystals were obtained during primary screens with SUB2 protein but there were proved to be salt or they were non-diffracting.

Recombinant SUB2 was mostly useful for immunoblotting as confirmation of antibody generation with excellent results (Figure 2.2). This proves that constructs were well designed and the protein was expressed successfully. We have yet to find the best conditions for testing SUB2 activity. Additionally, trying out other

eukaryotic expression systems might yield better results. It is possible that SUB2 requires more sophisticated cellular machinery for protein expression that can provide additional processing and post-translational modifications.

Testing of additional assay conditions should include *Plasmodium* growth media or blood sera to supplement for reaction components that might be missing for the buffers used in the lab. Since SUB2 is active when exposed to the extracellular environment during invasion, these tests should definitely show if the construct tested is active. In addition, SUB2 purified from the parasite might be a way to go for an *in vitro* assay until a recombinant version expressed in another system is proved to be active.

It is not trivial to accomplish *in vitro* studies of SUB2, however, it is important to pursue its biochemical characterization. *In vitro* studies will also contribute to the understanding of the mechanism of function of SUB2 at the biochemical level. In complement, biophysical characterization of its molecular structure will allow further characterization and set the basis for the development of SUB2-specific inhibitors. These approaches will facilitate the analysis of this subtilisin on an environment isolated from the cell, allowing the use of active site mutants. This would not be possible in the parasite since the activity of SUB2 is essential during asexual stages.

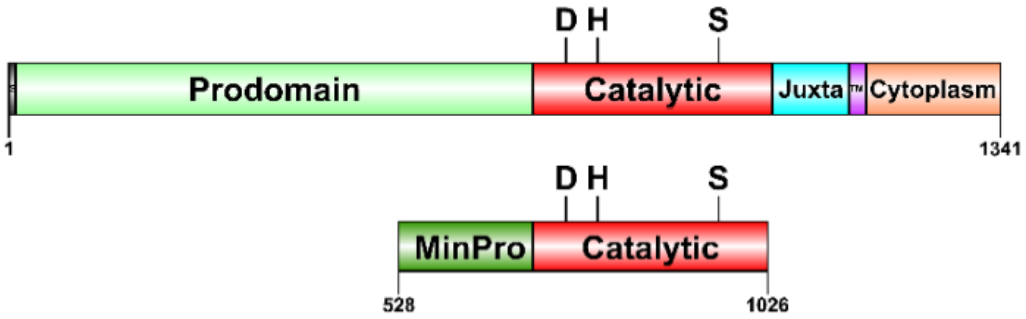


Figure 4.1 Schematic of full length SUB2 compared to the minimal prodomain of SUB2 attached to the catalytic domain. Top) Domains found in full length SUB2: a signal peptide (black), prodomain (green) catalytic domain (red) with activity site residues (Asp, His and Ser), juxtamembrane domain (cyan), transmembrane domain (puple) and cytoplasmic tail (orange). Bottom) minimal prodomain (dark green) and catalytic domain (red). This minimal prodomain was included in some of the construct to aid in protein folding and stability. The juxtamembrane domain of SUB2 was unintentionally present in some of the constructs as a result of excluding the transmembrane domain while maintaining all the conserved regions adjacent to the catalytic domain. This figure was made using the DOG Protein Domain Structure Visualization program [102].

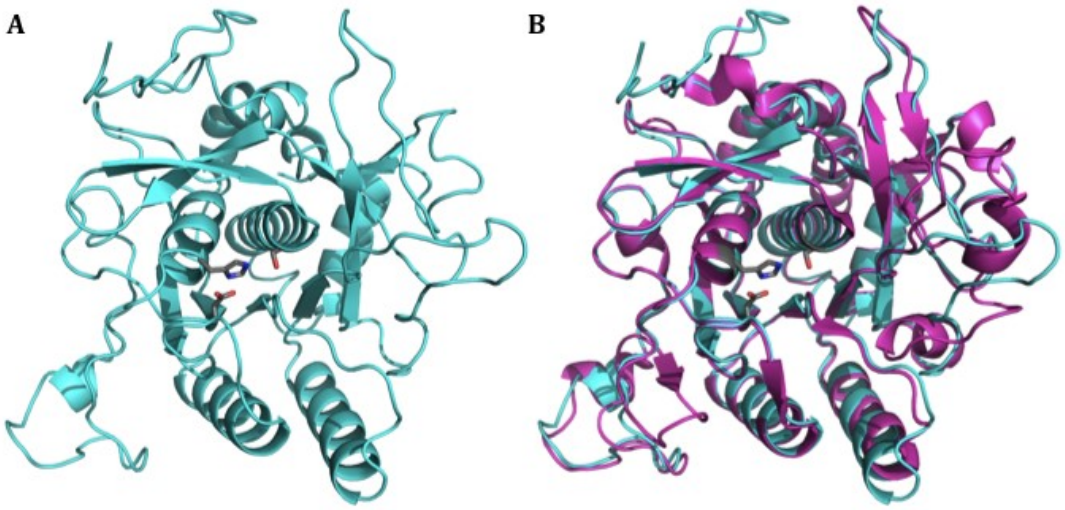


Figure 4.2 A predicted model of SUB2 was utilized as guidance in construct design. A structural model of PfSUB2 was generated using the I-TASSER server. A) Cartoon representation of *Pf*SUB2 catalytic domain. The catalytic triad Asp, Ser and His is shown (sticks). B) PfSUB2 model aligned to *Pf*SUB1 structure (4lvo) showing a fair alignment of the main secondary structures around the catalytic triad, yet, secondary structures distant from the active site are not accurately modeled but they are correctly positioned.

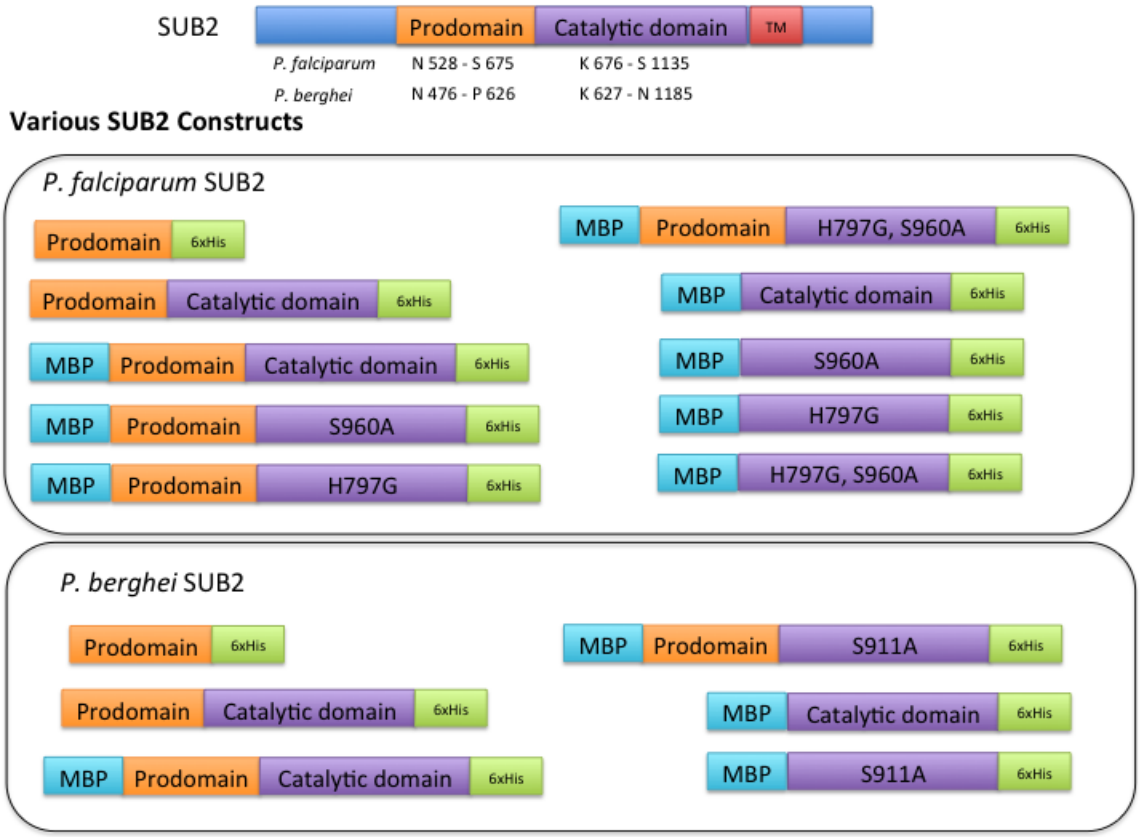


Figure 4.3 Example of *P. falciparum* and *P. berghei* SUB2 expression constructs. This figure shows several SUB2 constructs that were developed for biochemical and biophysical studies. Not all SUB2 constructs that were developed are included in this figure. Here, the prodomain (orange) is referred to the minimal prodomain that is included in these constructs for protein folding and stability purposes. The catalytic domain (purple) also includes the juxtamembrane region, which is also conserved. Maltose binding protein (MBP) was used to enhance solubility of recombinant SUB2.

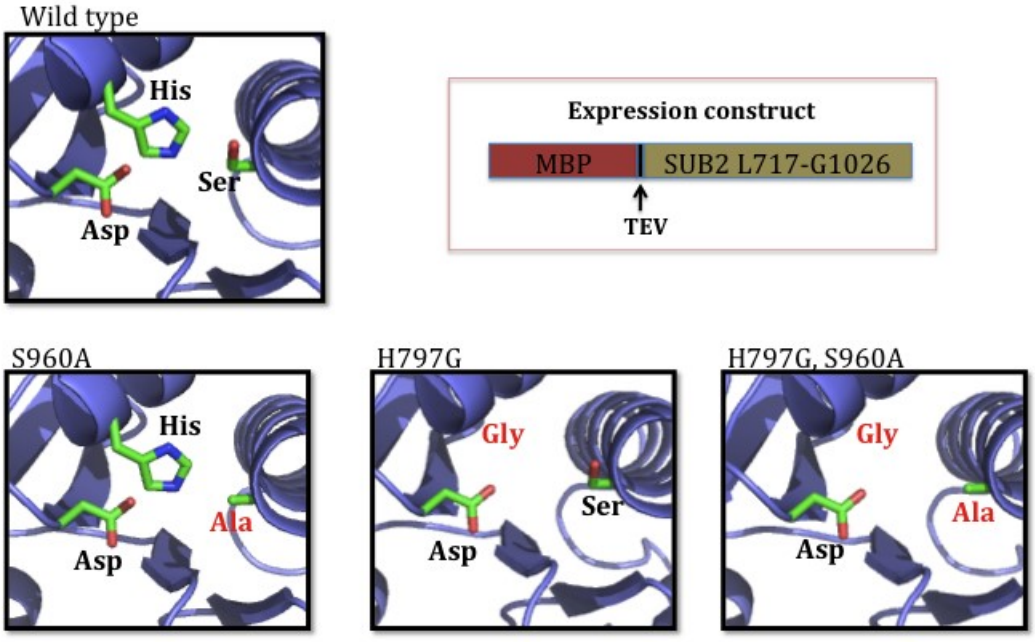


Figure 4.4 Active site mutants of SUB2 were developed for a SUB2-specific protease assay. Top) Left, active site residues (green, sticks representation) of wild type *Pf*SUB2. Right, a schematic of the MBP-fusion construct used for developing the mutants by site-directed mutagenesis. Bottom) active site mutants: S960A inactive mutant, H797G mutant that can be activated by addition of imidazole (chemical-rescue mutant) and H797G, S960A double mutant as a negative control. Mutation is labeled with red fonts. These images were created using a homology model of *Pf*SUB2 and PyMOL.

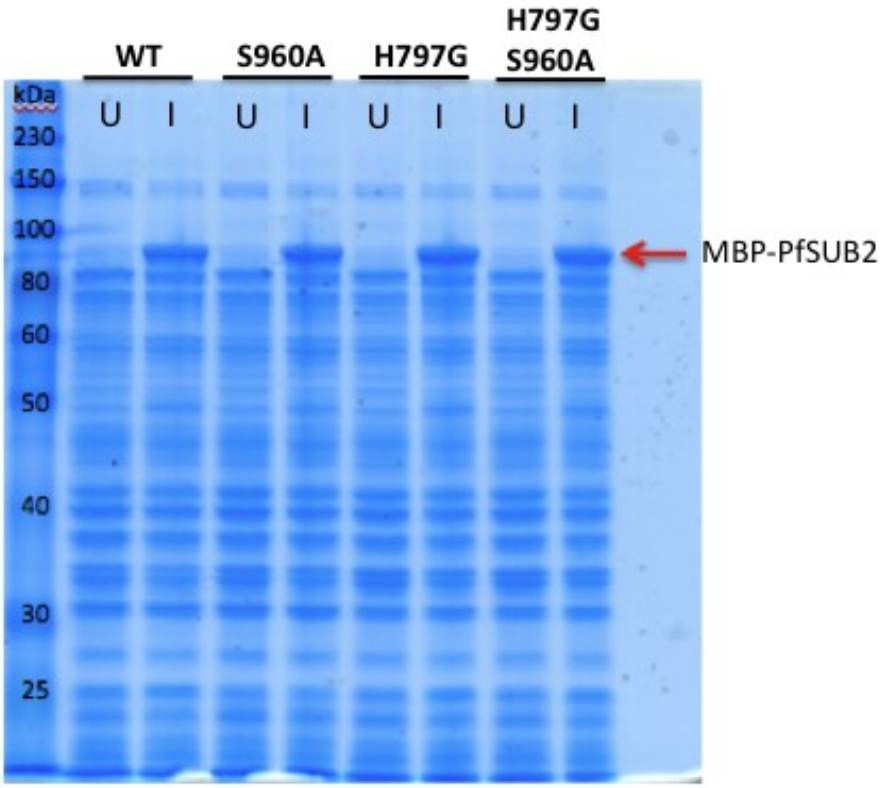


Figure 4.5 Expression of *Pf*SUB2 mutants and wild type. All SUB2 mutants and wild type were expressed in *E. coli*. Uninduced (U) and induced (I) whole cell samples were analyzed by SDS-PAGE stained with coomassie brilliant blue. Full length recombinant *Pf*SUB2 can effectively be expressed in *E. coli* with the constructs that were developed (red arrow).

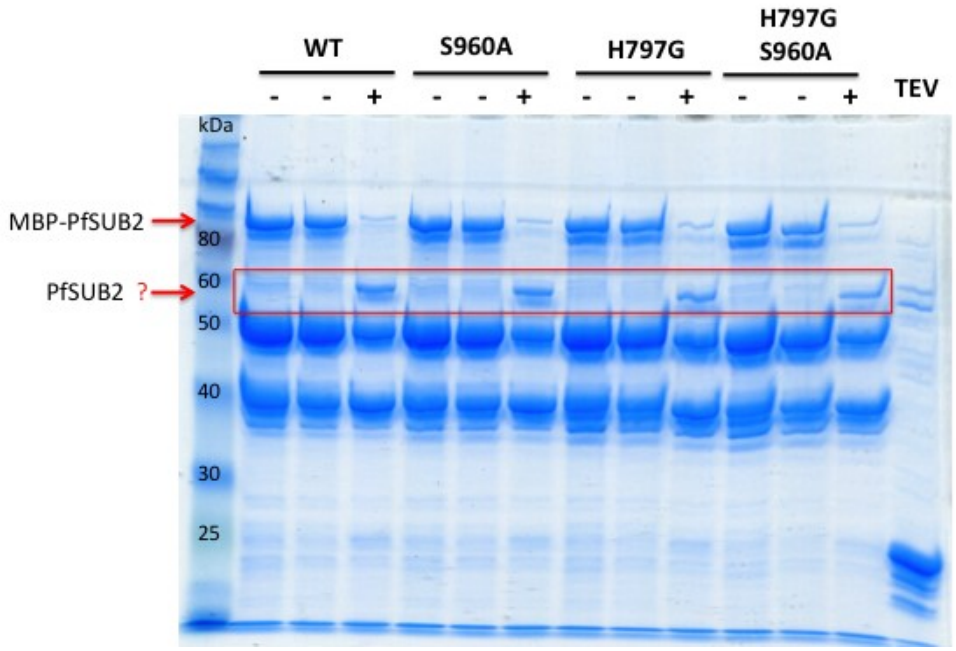


Figure 4.6 Recombinant protein stays soluble after removal of the MBP tag. Purified recombinant SUB2 was incubated with TEV protease to cleave off the MBP tag. Wild type and mutants of SUB2 were tested. Arrows show full length recombinant protein (top) and soluble *Pf*SUB2 released from MBP after cleavage (bottom). All SUB2 mutants behaved the same as wild type in this assay. No evidence of autoproteolytic activity or degradation is observed. 50 kDa and 40 kDa bands remained unidentified in this SDS-PAGE.

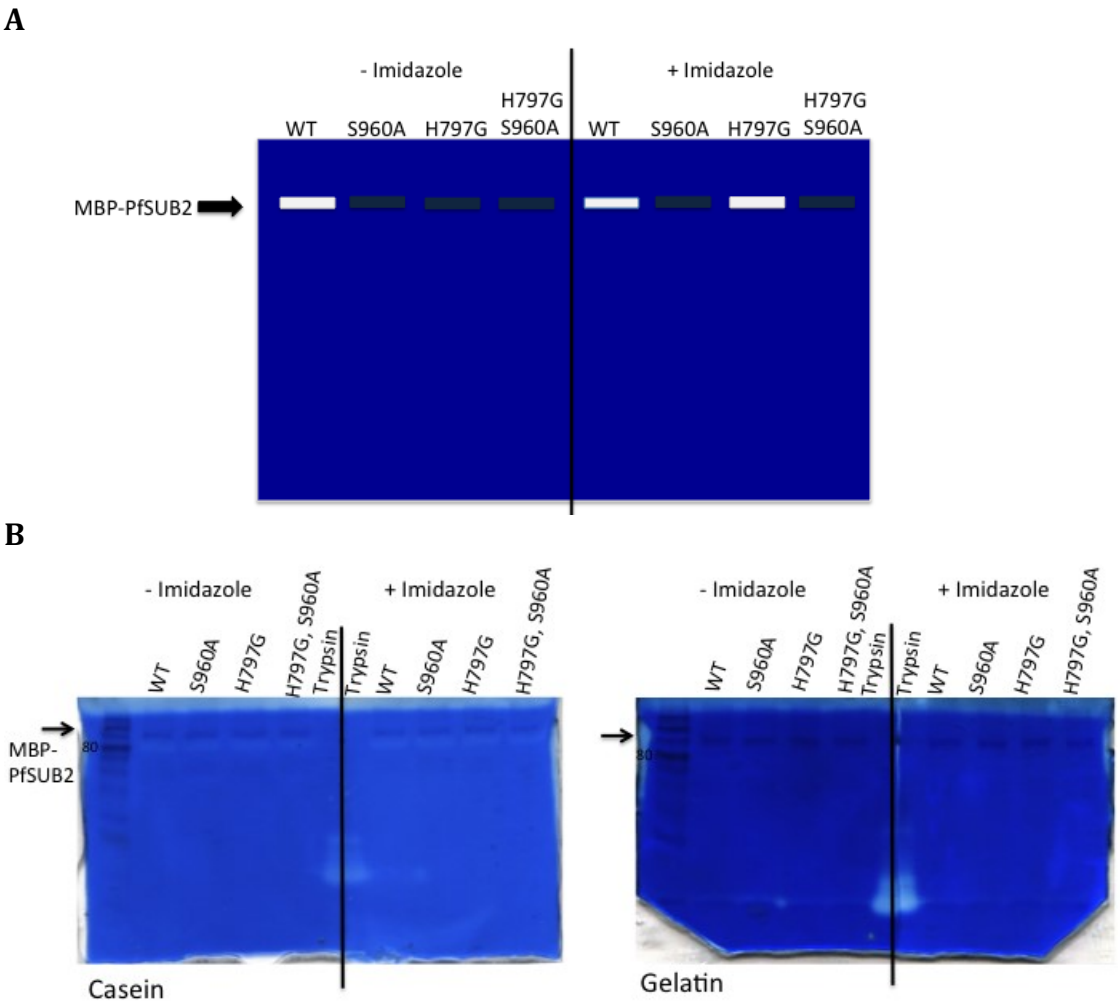


Figure 4.7 Protease activity of recombinant protein was tested by zymography. A) Experimental design and expected results in the presence or absence of imidazole, assuming that *Pf*SUB2 is active when expressed in *E. coli*. Bottom: Results of zymogram assessment of PfSUB2 show no activity from this recombinant *Plasmodium* protease. The only activity observed in this assay is that from trypsin positive control.

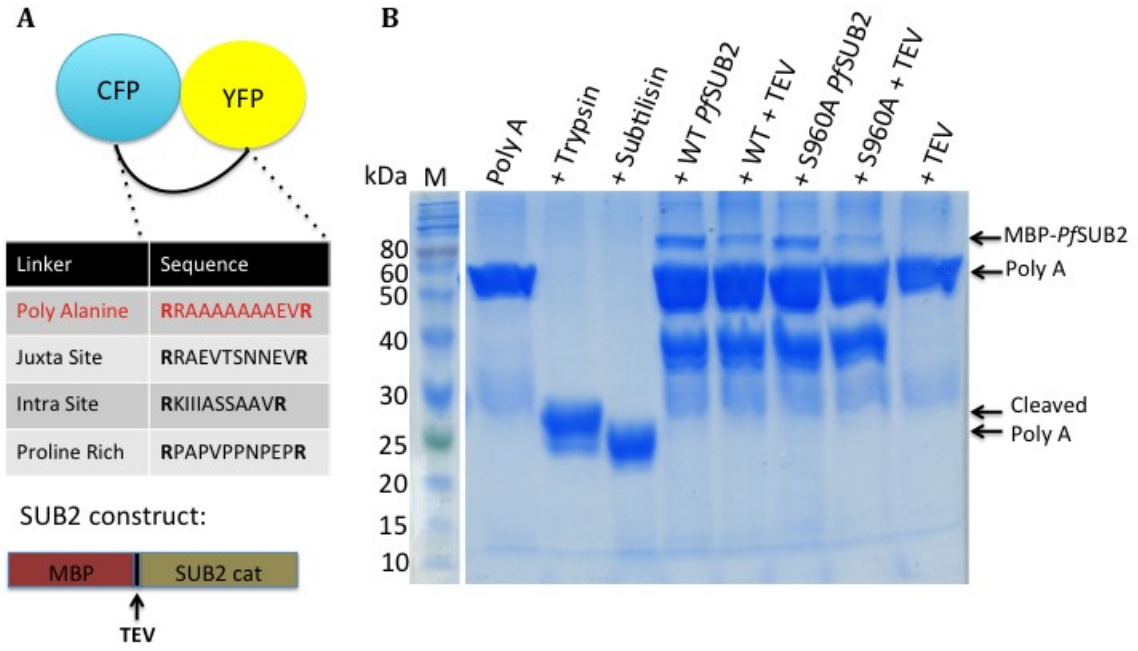


Figure 4.8 AMA1-based *Pf*SUB2-specific substrates. A) GKAR substrate with AMA1 sites of SUB2 cleavage (Juxta site and Intra site) sequences and other sequences as positive (Poly Alanine) and negative (Proline rich) controls. B) Test of SUB2 activity with the Poly Alanine substrate. Trypsin and a bacterial subtilisin were included as positive controls. This Poly Alanine substrate is cleavable by proteases as demonstrated by the two stable products (~ 25 kDa) left by both, trypsin and the subtilisin control. However, no reaction products were observed for wild type SUB2 in the presence or absence of TEV protease that was included to remove the MBP tag. TEV cleavage of the full length SUB2 can be observed. In conclusion, SUB2 expressed in *E. coli* is not active under the conditions tested but the substrate is viable for testing protease activity.

Chapter 5

Concluding remarks

Plasmodium subtilisin-like protease 2 is a viable target for antibody-mediated inhibition of erythrocyte invasion

Out results show that, as predicted, immunization against SUB2 leads to a reduction of malaria infection in a mouse model of malaria, compared to control (Chapter 2). This shows that the short window in which the parasite is exposed to the blood is sufficient for the antibodies to interact with surface-exposed SUB2 and interfere with parasite invasion. Previous to this study, there was evidence that SUB2 antibodies were present in the blood of children in malaria endemic regions. The presence of antibodies in the blood was strongly correlated to protection from symptomatic malaria [130]. This evidence proves that SUB2 works as a merozoite surface antigen in addition to working as a merozoite surface sheddase.

A modified SAPN immunization protocol (Chapter 3) should be effective for antibody generation leading to protection against malaria in mice, as seen in previous experiments with KLH-conjugated SUB2 peptides (Chapter 2). Moreover, further optimization efforts will provide information on the requirements of SUB2-mediated inhibition with antibodies. It will also be informative to test these antibodies in live parasite cultures and in biochemical assays. These can be used to explore if antibody-mediated inhibition also interferes with SUB2 protease activity, helping us to understand the mechanism on how these anti-SUB2 antibodies led to a decrease in parasite infection in mice. In addition, SAPN-SUB2 studies can also provide insights into how this approach will work with other molecular targets.

Current immunization protocol requires optimization for effective antibody-mediated inhibition of asexual development of *Plasmodium*

Here, we have provided evidence of the potential of a SUB2-based vaccine for malaria. According to our results, with the correct protocol, it is possible to induce production of SUB2-specific antibodies in mice (Chapter 2). Furthermore, the presence of antibodies provides measurable protection from blood stages of the malaria parasite and potentially results in clearance, as observed in Chapter 2 experiments. Since better results were achieved with the use of adjuvants and a KLH immunogenic carrier protein, it is possible that both or one of these components is required for induction of SUB2-targeted protection using SAPN as a delivery mechanism.

In our experiments, SAPN was solely used as an antigen-delivery tool (Chapter 3), with no additional elements for aiding to elicit an immune response. However, in some of the mice, a detectable amount of antibodies against the SAPN used for immunization was detected with immunoblotting. This shows that, even under non-optimal conditions, positive results can be achieved with this approach. Demonstrating the potential of a SAPN-SUB2 based malaria vaccine.

Now, how to express and purify catalytically active *Plasmodium* SUB2 for *in vitro* studies?

Only a few studies focused on SUB2 have been published during the past 10 years. This *Plasmodium* subtilisin is considered a major target for the design of antimalarials and, more recently, for vaccine development. It seems that, however, biochemical and biophysical studies have been impossible to perform *in vitro* because of the nonexistence of an appropriate expression system for active protease. It is unclear why SUB2 is not expressed in an active form in *E. coli* (Chapter 4). Though, it is not trivial to express active eukaryotic proteases in this expression system due to several factors including: post-translational modifications, incorrect formation of disulfide bonds, cytoplasmic protein concentration, protein solubility, among others.

It is also possible that SUB2 is expressed active in bacteria but critical components for SUB2 activity are missing from *in vitro* assay conditions that were tested. Future biochemical analyses should include activity assays with blood serum or parasite media to reach that conclusion, if true. Most likely, however, it will be necessary to express SUB2 using a eukaryotic heterologous expression system such as insect or human cells.

There are also other plausible options for obtaining catalytically active SUB2. For instance, a modified SUB2 with an extra protease cleavage site, could be introduced in the *Plasmodium* genome for expression in the parasite, to release SUB2 from the surface of the parasite upon secretion. Alternatively, a SUB2

construct could be developed for episomal expression to avoid detrimental effects of modifying all SUB2 in the parasite. Another option would be to express a SUB2 with a truncated C-terminal to exclude the transmembrane domain and secrete SUB2 unattached from the parasite membrane. These are only a few options for expression of active SUB2 for *in vitro* studies that will also require additional modifications for purification purposes. To this date, it is unclear how we can express and purify active SUB2 for biochemical and biophysical characterization.

SUB2 stands as a target for small-molecule and antibody-mediated inhibitor design against red blood cell stages of the malaria parasite

The role of SUB2 is essential for parasite survival and for RBC invasion, a critical step for *Plasmodium* asexual development [96, 131]. Blocking of parasite entry to the RBC results in parasite death, since it is an obligate intracellular parasite that requires a host cell for survival. In this case, a RBC confers protection from the host's antibodies that are circulating in the blood and provides hemoglobin as a food source during asexual development. Precluding RBC invasion, therefore, means a fatal end for parasite. Thus, these results could be achieved by targeting SUB2.

In this dissertation, it was demonstrated that immunization with SUB2 peptides leads to protection from blood stage parasites. This does not necessary mean that the antibodies are directly interfering with SUB2 activity, but targeting of

SUB2 could be effective for both, small-molecule and antibody-mediated inhibition of RBC invasion. It is known that serine-protease inhibitors result in inhibition of invasion by targeting a calcium-dependent merozoite surface sheddase (MeSh) activity. Prior to these studies, SUB2 was attributed to be responsible for the MeSh activity, which is essential during RBC invasion. We are just left with the task of figuring out how to gather biochemical data on SUB2 for the development of small molecule probes against its activity.

In vitro analyses will help to test if antibody and small molecule-mediated inhibition, both target SUB2 protease activity or if they work by different mechanisms of SUB2-targeted inhibition of RBC invasion. It is possible that these would have different mechanisms of action and could be used in complement to enhance the efficacy of inhibition using this target. In this case, different combinations could be utilized for different clinical scenarios of malaria or for different affected populations, for example. Putting together our results and those from previous studies, we can conclude that SUB2 is a suitable target for both, small molecule and antibody-mediated inhibition of RBC invasion.

Additional future directions and final remarks

This work provides insights into preliminary research on a novel malaria vaccine candidate that was previously described as an attractive target for novel antimalarials. The proof-of-principle experimental data in Chapter 2 shows that

SUB2 is a practical target for antibody-mediated inhibition of RBC invasion and that it results in clearance of malaria infection, in some cases. From these and previous results we can also deduct that targeting SUB2 for small-molecule inhibitor design will result in impaired RBC invasion, preventing successful parasite entry.

As mentioned above, future studies on antibody-mediated inhibition elicited by immunization with SUB2 peptides should take into consideration the addition of a suitable adjuvant, a carrier protein, additional boosts and verification of antibody generation prior to the challenge with *P. berghei*. The use of self-assembling protein nanoparticles (SAPN) should still be employed in future experiments since there are advantages in using a delivery system that can be developed in a laboratory by standard expression and purification protocols using *E. coli* expression system. This technology also permits us to combine different antigens and could be used to target several strains or species of the parasite for a more potent and effective vaccine.

In future studies, it should be considered to collect a variety of observations and data throughout the course of the experiment, as it was implemented in the SAPN studies (Chapter 3). For example, by recording the *P. berghei* challenge by mosquito feeding, we were able to determine that mosquitoes did not bite one of the mice. By knowing this, we did not include its data in the parasitemia analysis. Inclusion of this information would have affected the data in how the mice were protected and parasite clearance. Live imaging, if optimized, could provide information about when during the blood stages antibody-mediated inhibition is effective. It is possible that in some mice the parasites go thru liver stages but they never establish in the blood because the antibodies interfere with the initial

merozoites coming out of the liver, prior invading erythrocytes in the first place.

Also, multiple invasion events should be measured in follow-up studies as it could hint to details of the mechanism of antibody-mediated inhibition of parasite invasion of RBCs by the malaria parasite. To confirm the presence low levels of parasitemia, DNA sequencing of infected RBCs using *P. berghei*- specific primers may aid in detecting low levels of parasite infection that would not be measurable on the Image Stream due to signal-to-noise ratio. This will permit to detect early blood stage infection prior to elimination by antibody-mediated inhibition. However, additional blood samples would need to be collected.

In conclusion, our results provide proof that SUB2 is a suitable target for vaccine development and should still be considered a target for the development of new antimalarials. It is important to continue studies that promise to achieve better understanding of this molecular target. We yet have to establish a method for biochemical and biophysical characterization of SUB2. Here, we have provided the basis for vaccine development based on this target. Studies performed with this *Plasmodium* subtilisin could also provide the basics for developing treatment based on similar targets for other diseases.

Finally, Perhaps, the key for successful control of malaria relies in a specific complex combination of factors and not solely on a single “magic” drug or vaccine. The better understanding of the cellular mechanisms of malaria pathogenesis and the characterization of molecular targets, the more prepared we are to fight against this and other infectious diseases.

This dissertation illustrates some of the multiple challenges that are faced in the attempts to develop treatment and protection against malaria infection. The more information we gather, the better prepared we are to continue fighting against this infectious disease caused by *Plasmodium* parasites. This antibody-mediated inhibitor design is a rational approach permitting to shape the advancement in the characterization of this targets and optimization of the use of the inhibitors. Only by acquiring all the necessary tools for malaria control, we will be moving towards the right direction to fight against this disease and to envisage the possibility of malaria eradication.

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EDUCATION

2016 Ph.D. in Biochemistry and Molecular Biology, **Johns Hopkins Bloomberg School of Public Health**, Baltimore, MD

2009 B.S., Biology with Honors, **University of Puerto Rico**, Río Piedras Campus, San Juan, PR

DOCTORAL RESEARCH

Dissertation title: Targeting the role of subtilisin-like protease 2 for inhibition of erythrocyte invasion by the malaria parasite, *Plasmodium*
Advisor: Dr. Jürgen Bosch

ADDITIONAL RESEARCH EXPERIENCE

2009-2010 Laboratory Technician, **University of Puerto Rico-Río Piedras**, Department of Chemistry, San Juan, PR
Structural biology laboratory
Principal Investigator: Dr. Eric R. Schreiter

2005-2009 Undergraduate Research, **University of Puerto Rico-Río Piedras**, Department of Biology, San Juan, PR
"Post-transcriptional role of human interleukin-3 3'-untranslated region"
Advisor: Dr. Carlos I. González

Summer 2008 Undergraduate Research Intern, **Johns Hopkins Bloomberg School of Public Health**, Department of Biochemistry and Molecular Biology, Baltimore, MD
"Characterization of a mutant DNA replication initiator: expression, purification and functional analysis"
Advisor: Dr. Roger McMacken

PUBLICATIONS

1. Van Voorhis, Wesley et al. "Open Source Malaria Box: over one-hundred-thousand data points useful for many indications." (Submitted)
2. Çelik H., Hong S., **Colón-Lopez D.D.**, Han J., et al. "Identification of novel ezrin inhibitors targeting metastatic osteosarcoma by screening open access malaria box." Molecular Cancer Therapeutics. (2015)
3. Smith R.C., **Colón-López D.D.**, Bosch J. "Immunization against a merozoite subtilisin protease attenuates *Plasmodium* infection in mice." Malaria Journal. 13(1):313 (2014)
4. González-Feliciano J.A., Hernández-Pérez M., Estrella L.A., **Colón-López D.D.**, López A., et al. "The role of HuR in the post-transcriptional regulation of interleukin-3 in T Cells." PLoS One. 9(3) (2014):e92457

PATENTS

Bosch J., **Colón-López D.D.**, Smith R.C. 2014. Malarial antigens derived from subtilisin-like protease 2 and vaccines and methods of use. U.S. Patent 12845-01, filed June 2014. Provisional Patent.

AWARDS, FELLOWSHIPS AND RESEARCH PROGRAMS

1. **The National Science Foundation Graduate Research Fellowship Program** (NSF-GRFP), 2012-2015
2. **The Amgen Biotechnology Mentorship Initiative to Develop Scientists** (Bio-MINDS) Program, The Amgen Foundation, 2008-2009
3. **Johns Hopkins University Diversity Summer Internship Program** (**DSIP**), Johns Hopkins Bloomberg School of Public Health, Summer 2008
4. **The Leadership Alliance Summer Research Early Identification Program** (SR-EIP), Summer 2008
5. **Minority Biomedical Research Support - Research Initiative for Scientific Enhancement** (RISE) Program, **The National Institutes of Health** (NIH), 2007-2009
6. **Puerto Rico Louis Stokes Alliance for Minority Participation** (PRLS-AMP) Program, **The National Science Foundation** (NSF), 2002-2007
7. National Science and Mathematics Access to Retain Talent Grant, 2006

TECHNICAL SKILLS

Molecular Biology: Molecular cloning, DNA and RNA isolation, Reverse Transcription-PCR, Real-Time-PCR, site-directed mutagenesis
Protein Biochemistry: Protein expression and purification, immunoblotting, proteolysis assays, zymography, DNA replication assays, electrophoretic mobility shift assays, nuclease assays
Biophysical Techniques: X-ray crystallography, isothermal titration calorimetry, circular dichroism spectroscopy, dynamic light scattering, differential scanning fluorimetry, surface plasmon resonance
Computational: General bioinformatics tools, molecular docking, general computational genomics tools
Cell Culture Techniques: General aseptic techniques, HeLa, Jurkat T-lymphocyte and HEK 293T human cell lines, *Plasmodium falciparum* parasites cultured in red blood cells
Animal Model Organisms: *Caenorhabditis elegans, Mus musculus*
Specialized training: ImageStream high-resolution microscopy, IVIS Series Pre-clinical *In Vivo* Imaging

COLLABORATIONS

1. Colón-Lorenzo EE, **Colón-López DD**, Bosch J, Ortíz JG, Serrano AE, "Identification and validation of *P. berghei* glutathione s-transferase small molecule inhibitors by virtual library screening." American Society for Biochemistry and Molecular Biology, Experimental
   Biology Meeting. San Diego, CA, April 2014
2. Çelik H, Kallarakal A, **Colón-López DD**, Toretsky J, Bosch J, Uren A, "Evaluation of anti malarial compounds as inhibitors of erzin in osteorsarcoma cells." Proceedings of the 105th Annual Meeting of the American Association for Cancer Research. San Diego, CA, April 2014
3. Boucher LE, Hain AUP, Miller AS, **Colón-López DD**, Bosch J, "Identification of protein-

protein (PPI) inhibitors and stabilizers for antimalarial drug development using SPR." Biophysical Society 58th Annual Meeting, San Francisco, CA, February 2014

4. Colón-Lorenzo EE, Herrans-Maya G, **Colón-López DD**, Bosch J, Serrano AE, "*Plasmodium berghei* glutathione S-transferase is a validated target for novel antimalarial development." Puerto Rico Society of Microbiologist Local ASM Branch, Second Student Research Symposium: Passing the Torch of Research and Ethics to the Next Generation. Arecibo, PR, December 2013

MENTORING EXPERIENCE

Graduate Students: Serge M. Stamm (Master student, Technical University of Munich, Germany) (6 months)

Graduate Rotation Students: Eric Simko, Kaitlin Johnson and Nick Sapp (8 weeks), Jeliazko Jeliazkov (9 weeks)

TEACHING EXPERIENCE

1. Teaching Assistant, Introduction to the Biomedical Sciences, **Johns Hopkins Bloomberg School of Public Health**, Department of Molecular Microbiology and Immunology, Baltimore, MD, August 2014
2. Science Workshop for 12th Grade Students, **Pablo Colón Berdecia High School**, Barranquitas, PR, Nov. 2013
3. Teaching Assistant, Introduction to the Biomedical Sciences, **Johns Hopkins Bloomberg School of Public Health**, Department of Molecular Microbiology and Immunology, Baltimore, MD, August 2013

ORGANIZATIONAL SKILLS

1. Member of the Social Events Committee at BMB department, JHSPH, 2014-2015
2. Organizational Committee Member of the Biochemistry and Molecular Biology Annual Departmental Retreat, JHSPH, March 2015

PUBLICATIONS IN PREPARATION

1. Colón-Lorenzo E.E., **Colón-López D.D.**, Bosch J., Ortiz J.G., Serrano A.E., "*Plasmodium berghei* Glutathione S-transferase: insights in the biological role and validated drug target."
2. Stamm S.M., **Colón-López D.D.**, Bosch J., "Identification of a *Pf*GAP50-Factor H protein-protein interaction inhibitor from the MMV Malaria Box."

ORAL PRESENTATIONS

1. **Colón-López DD**, Smith RC, Burkhard P, Bosch J, "Antibody-mediated inhibitor design against *Plasmodium* invasion", Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, December 2015
2. **Colón-López DD**, Smith RC, Burkhard P, Bosch J, "Targeting a merozoite surface sheddase for inhibition of erythrocyte invasion", Department of Biochemistry and Molecular Biology, Penn State Hershey Medical Center, Hershey, PA, October 2015
3. **Colón-López DD**, Bosch J, "What to do when crystallography cannot answer your questions", Monthly Crystallography Meeting. Johns Hopkins School of Medicine, Baltimore, MD, March 2015
4. **Colón-López DD**, Smith RC, Burkhard P, Bosch J, "Biochemical characterization of

Plasmodium subtilisin-like protease 2 as a malaria drug target and vaccine candidate". Biochemistry and Molecular Biology Departmental Retreat. Sheppard Pratt Conference Center, Towson, MD, March 2015

5. **Colón-López DD**, Bosch J, "Structure based development of probes against key malaria parasite targets." Dept. of Microbiology and Medical Zoology, UPR Medical Sciences Campus, San Juan, PR. November 2013
6. **Colón-López DD**, Bosch J, **"**Characterization of *Plasmodium* subtilisin-like protease 2**."** Johns Hopkins Malaria Research Institute Friday Seminar Series. JHSPH, Baltimore, MD, May 2013
7. **Colón-López DD**, Bosch J, "Characterization of *Plasmodium* subtilisin-like protease 2." Biochemistry and Molecular Biology Departmental Retreat. Sheppard Pratt Conference Center, Towson, MD, May 2013
8. **Colón-López DD,** González-Feliciano JA, González C, "Post-transcriptional role of human interleukin-3 3'-untranslated region." 28th Puerto Rico Interdisciplinary Scientific Meeting 43rd ACS Junior Technical Meeting. UPR, Arecibo Campus, Arecibo, PR, March 2008
9. **Colón-López DD**, González-Feliciano JA, González C, "Post-transcriptional role of human interleukin-3 3'-untranslated region." 27th Puerto Rico Interdisciplinary Scientific Meeting 42rd ACS Junior Technical Meeting. Interamerican University, Bayamon, PR, March 2007

POSTER PRESENTATIONS

1. **Colón-López DD**, Stamm SS, Bosch J, "Identification of inhibitors against *P. falciparum* GAP50 and human complement factor H interaction in the mosquito". Biophysical Society 59th Annual Meeting. Baltimore Convention Center, Baltimore, MD, February 2015
2. **Colón-López DD**, Smith RC, Bosch J, "Targeting *Plasmodium* subtilisin-like protease 2 for the development of novel inhibitory probes of red blood cell invasion." The Johns Hopkins Vaccine Initiative Vaccine Day. Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, October 2014
3. **Colón-López DD**, Smith RC, Bosch J, "Targeting *Plasmodium* subtilisin-like protease 2 for the development of novel inhibitory probes of red blood cell invasion." Annual Biochemistry and Molecular Biology Departmental Retreat. Sheppard Pratt Conference Center, Towson, MD, March 2014
4. Stamm SM, **Colón-López DD**, Johnson K, Bosch J, "Suppressing human and *Plasmodium* protein-protein interaction: a novel way to provoke immune response?" Annual Biochemistry and Molecular Biology Departmental Retreat. Sheppard Pratt Conference Center, Towson, MD, March 2014
5. **Colón-López DD**, Smith RC, Bosch J, "Characterization of *Plasmodium* subtilisin-like protease 2." 24th Annual Molecular Parasitology Meeting. Marine Biological Laboratory, Woods Hole, MA, September 2013
6. **Colón-López DD**, Smith RC, Simko E, Bosch J, "Targeting *Plasmodium* subtilisin-like protease 2 for antimalarial drug design." Johns Hopkins Malaria Research Institute World Malaria Day Meeting. JHSPH, Baltimore, MD, April 2013
7. **Colón-López DD**, Bosch J, "A novel inhibitor-based and nanobody-mediated strategy for blocking *Plasmodium falciparum* transmission." Institute for Biophysical Research Retreat. Towson, MD, September 2012
8. **Colón-López DD**, González-Feliciano JA, González C, "Translational control of Human Interleukin-3 mediated by its 3'-untranslated region." Bio-MINDS Annual Poster Day.

Bioprocess Development and Training Complex, Mayaguez, PR, March 2009

9. **Colón-López DD**, Learn B, McMacken R, "Characterization of a mutant DNA replication initiator: expression, purification and functional Analysis." Annual Biomedical Research Conference for Minority Students. Orlando, FL, November 2008
10. **Colón-López DD**, Learn B, McMacken R, "Characterization of a mutant DNA replication initiator: Expression, Purification and Functional Analysis." Combined Poster Session. JHU Baltimore, MD, August 2008
11. **Colón-López DD**, Learn B, McMacken R, "Characterization of a mutant DNA replication initiator: expression, purification and functional Analysis." JHSPH Poster Session. Baltimore, MD, August 2008.
12. **Colón-López DD**, Learn B, McMacken R, "Characterization of a mutant DNA replication initiator: expression, purification and functional Analysis." The Leadership Alliance National Symposium. Hartford Marriott and Convention Center, Hartford, CT, July 2008
13. **Colón-López DD**, González-Feliciano JA, González C, "Translational control of human interleukin-3 mRNA by the adenosine/uridine rich elements." 4th RISE Area Conference. UPR-RP, San Juan, PR, March 2008

ADDITIONAL GRADUATE RESEARCH EXPERIENCE

Apr. 2011	Graduate Research, Johns Hopkins Bloomberg School of Public Health, Department of Molecular Microbiology and Immunology, Baltimore, MD Rotation project: "Substrate determination and localization of octanoate transfer enzyme in *Plasmodium falciparum.*" Advisor: **Dr. Sean Prigge**
Feb. 2011	Graduate Research, Johns Hopkins Bloomberg School of Public Health, Department of Biochemistry and Molecular Biology, Baltimore, MD Rotation project: "Selective inhibition of NF-κβ signaling by manipulating ribosomal protein S3-dependent signaling." Advisor: **Dr. Fengyi Wan**
Jan. 2011	Graduate Research, Johns Hopkins Bloomberg School of Public Health, Department of Biochemistry and Molecular Biology, Baltimore, MD Rotation project: "Biochemical characterization and X-ray data collection and analysis of the CRISPR/Cas system protein Cas1." Advisor: **Dr. Scott Bailey**
Nov. 2010	Graduate Research, Johns Hopkins Bloomberg School of Public Health, Department of Biochemistry and Molecular Biology, Baltimore, MD Rotation project: "Development of a *C. elegans* transgenic line for studying a mechanism of neurodegeneration and protein quality control." Advisor: **Dr. Jiou Wang**
Sept. 2010	Graduate Research, Johns Hopkins Bloomberg School of Public Health, Department of Biochemistry and Molecular Biology, Baltimore, MD Rotation project: "Expression, purification and crystallization of *Anopheles gambiae* densovirus capsid protein." Advisor: **Dr. Jürgen Bosch**

PROFESIONAL ASSOCIATIONS

2015-Present	The American Society of Cell Biology
2014-Present	Biophysical Society
2011-Present	American Association for the Advancement of Science