BEZMIALEM VAKIF UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

CYTOLYSINS EXPRESSING LIVER STAGE PARASITES AS NOVEL LIVE ATTENUATED MALARIA VACCINES

PhD THESIS

Ümit Yaşar KINA

Biotechnology Department

Biotechnology PhD Programme

Thesis Advisor: Assist. Prof. Dr. Ab. Matteen RAFIQI

JUNE 2023

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JUNE 2023

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FOREWORD

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June 2023

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DECLARATION

I declare that; this thesis study is mine, I do not have any unethical behavior at all stages of the thesis, I have obtained all the information within academic and ethical rules, I have referred to all information and comments that have not been obtained through this thesis study and I have included them in the list of references, I have not violated any patent and copyright during the study and writing of this thesis.

Ümit Yaşar KINA

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CYTOLYSINS EXPRESSING LIVER STAGE PARASITES AS NOVEL LIVE ATTENUATED MALARIA VACCINES

SUMMARY

Malaria is amongst the deadliest of infectious diseases globally. It is a vector-borne disease transmitted by the bite of female *Anopheles* mosquitos. Causative agents are *Plasmodium* species protozoan parasites. There are 5 species that are known to cause disease in humans. Over 3 billion people are at risk of malaria transmission. Prevention methods such as vector control and seasonal mass drug administration are effective tools for malaria control but with increasing resistance to frontline drugs, efficient vaccine development becomes imperative for malaria elimination. Ideal malaria vaccine should protect against the most common *Plasmodium* species and should have more than 75% efficacy, and a long-lasting protection. Only available vaccine so far is RTS,S which underperforms the expectations. Complex biology and various immune evasion strategies of *Plasmodium* parasites makes vaccine development challenging. Pre-erythrocytic stages are the bottleneck of development in vertebrate hosts. Vaccine strategies against this stage are so far the most promising. Live-attenuated whole sporozoite vaccines have been proven to confer sterile protection. Various genetic modification strategies allowed precise attenuation profiles which led to development of genetically attenuated parasites (GAP). In this thesis study, we designed two alternative attenuation strategy to be evaluated as GAP vaccines that aimed for late liver stages arrest in rodent malaria model. Both candidates had similar designs: knockout of an essential liver stages gene, and expression of a bacterial cytolysin protein. Selected genes, LISP1 and LISP2, are only expressed during liver stages. Bacterial cytolysins sequences of Streptolysin O (SLO) from *Streptococcus pyogenes* and Listeriolysin O (LLO) from *Listeria monocytogenes* were replaced with coding sequences of target genes. Resulting strains, *ΔLISP1*::SLO and *ΔLISP2*::LLO, were phenotypically analyzed for all life cycle stages of the parasite. To understand the effect of cytolysins, we replaced the cytolysin sequences with fluorescent proteins that would also work as reporter genes for liver stages expression (*ΔLISP1*::mNeonGreen and *ΔLISP2*::mTurquoise2). Cytolysin expressions significantly improved attenuation profiles of both strains. BALB/c mice were immunized with both strains and immunized mice were challenged against wild type sporozoites. As a result, *ΔLISP*1::SLO strain were completely attenuated. *ΔLISP*1::SLO strain-immunization of mice could protect against lower numbers of sporozoites but could not protect against higher numbers of sporozoites. In contrast, attenuation profile of *ΔLISP*2::LLO strain were strong, but there were occasional breakthrough infections. *ΔLISP*2::LLO strain-immunization of mice conferred sterile protection against extremely high numbers of sporozoites. Attenuation profile of *ΔLISP*2::LLO can be improved with various modifications, and this strategy can easily be applied to human malaria as a promising vaccine candidate in future studies.

Keywords: genetic attenuation, *Plasmodium*, pre-erythrocytic stages, vaccine

SİTOLİZİN EKSPRESYONU YAPAN KARACİĞER AŞAMASI PARAZİTLERİNİN YENİLİKÇİ CANLI ZAYIFLATILMIŞ SITMA AŞISI OLARAK KULLANILMASI

ÖZET

Sıtma, küresel olarak, en ölümcül bulaşıcı hastalıklardan biridir. Dişi Anofel sivrisineğinin ısırması ile bulaşan vektör kaynaklı bir hastalıktır. Hastalık etkeni, *Plasmodium* türü protozoan parazitlerdir. İnsanlarda hastalığa neden olduğu bilinen 5 türü vardır. 3 milyardan fazla insan sıtma bulaşma riski altındadır. Vektör kontrolü ve mevsimsel toplu ilaç uygulaması gibi önleme yöntemleri sıtma kontrolü için etkili araçlardır; ancak majör ilaçlara karşı artan ilaç direnci, sıtmanın ortadan kaldırılması için etkili aşı geliştirilmesini zorunlu kılmaktadır. İdeal sıtma aşısı, en yaygın *Plasmodium* türlerine karşı koruma sağlamalı ve %75'ten fazla etkinliğe ve uzun süreli korumaya sahip olmalıdır. Şimdiye kadar mevcut olan tek aşı, beklentilerin altında performans gösteren RTS,S'dir. *Plasmodium* parazitlerinin karmaşık biyolojisi ve sahip olduğu çeşitli immün kaçış mekanizmaları, aşı geliştirmeyi zorlaştıran etkenlerdir. Pre-eritrositik aşamalar, omurgalı konakçılarda gelişimin darboğaz noktasıdır. Şimdiye kadar geliştirilen aşı adayları arasında en umut verici olanlar bu aşamayı hedefleyen stratejilerdir. Canlı zayıflatılmış tam sporozoit aşıların tam koruma sağladığı kanıtlanmıştır. Çeşitli genetik modifikasyon yöntemleri, genetik olarak zayıflatılmış parazitlerin (GAP) gelişmesine yol açan güçlü atenüasyon stratejileri geliştirilebilmesini sağlamaktadır. Bu tez çalışmasında, kemirgen sıtma modelinde, karaciğerin geç evrelerinde gelişimin durdurulmasını amaçlayan GAP aşıları olarak değerlendirilmek üzere iki alternatif atenüasyon stratejisi tasarlandı. Her iki aşı adayının da hayati bir karaciğer aşaması geninin delesyonu ile eş zamanlı olarak bir bakteriyel sitolizin proteininin ekspresyonunu sağlayacak şekilde benzer tasarımları bulunmaktadır. Seçilen hedef genler, LISP1 ve LISP2, ekspresyonları yalnızca karaciğer evrelerinde yapılan genlerdir. Çalışma kapsamında, *Streptococcus pyogenes* Streptolysin O (SLO) ve *Listeria monocytogenes* Listeriolysin O (LLO) bakteriyel sitolizin proteinlerinin kodlayan dizileri, hedef genlerin kodlayan dizileriyle değiştirildi. Ortaya çıkan suşların (*ΔLISP*1::SLO ve *ΔLISP*2::LLO) fenotipik analizleri parazitin tüm yaşam döngüsü aşamalarında yapıldı. Ayrıca, sitolizin dizileri floresan protein dizileriyle değiştirilerek hem sitolizinlerin etkisini analiz edebilmek hem de karaciğer evreleri ifadesi için raportör genler olarak kullanılmak amacıyla kontrol suşları elde edildi (*ΔLISP*1::mNeonGreen ve *ΔLISP*2::mTurquoise2). Sitolizin ekspresyonları, her iki suşun atenüasyon profillerini önemli ölçüde arttırmıştır. BALB/c fareleri, her iki suşla immünize edildi ve immünize fareler, yabanıl tip sporozoitlere karşı test edildi. Sonuç olarak, *ΔLISP*1::SLO suşu tamamen atenüe edilmiştir. Farelerin *ΔLISP*1::SLO suşu ile aşılanması, düşük sayıda sporozoitlere karşı koruma sağlayabilse de yüksek sayıda sporozoitlere karşı koruma sağlayamamıştır. Buna karşılık, *ΔLISP*2::LLO suşu ise kuvvetli atenüasyon profili göstermiştir, ancak nadiren kan aşaması enfeksiyonlarına yol açabilmektedir. *ΔLISP*2::LLO suşu ile immünize edilen farelerde son derece yüksek sayıda

sporozoitlere karşı tam koruma sağlanmıştır. *ΔLISP*2::LLO suşunun atenüasyon profilinin, çeşitli modifikasyonlarla iyileştirilebilmesi mümkündür ve bu strateji, gelecekteki çalışmalarda umut verici bir aşı adayı olarak insan sıtma parazitlerine kolaylıkla uygulanabilecektir.

Anahtar Kelimeler: genetik atenüasyon, *Plasmodium*, pre-eritrositik aşamalar, aşı

1 INTRODUCTION

Malaria is one of the oldest known diseases in human history. It is caused by protozoan parasites of *Plasmodium* genus and transmitted by the bite of female Anopheline mosquitoes. Distinct species of *Plasmodium* parasites infect a wide range of vertebrate hosts. The most common human-infective species are *P. falciparum* and *P. vivax*. Symptoms vary from fever, tiredness, and vomiting to jaundice and seizures in severe cases. The disease is widespread throughout the tropical and sub-tropical regions.

1.1 History

The history of malaria is almost as old as written human history. Chinese medical canon from 2700 BCE mentions periodic (every third or fourth day) fevers with the enlargement of the spleen. Other known written examples of what seems to be malaria can be found in ancient texts as cuneiform scripts from Mesopotamia, papyri from Egypt, or from Hindu scripts dating back from about 2000 to 600 BCE. It was known as the "fever" in Ancient Greeks, and "intense burning heat" in Roman Empire. Even Homer (about 750 BCE) mentioned it in Iliad: "*Sirius, harbinger of fevers, the evil star which dominates the night sky at harvest time*". This link between the appearance of Sirius in late summer and malarial fevers was also mentioned by Hippocrates in about 400 BCE [1-3].

Its devastating effect has been felt throughout history. Indian writings called it "king of diseases". Malaria antigens could be detected in mummies from Ancient Egypt from 3200 and 1304 BCE [4]. It is even speculated to contribute to the fall of Rome. For thousands of years, Malaria is associated with swamps or the miasmas rising from the swamps, thus the name malaria was originated from the Italian *mal'aria* meaning "bad air" or "spoiled air" [2].

With the 17th and 18th centuries, discovery of bacteria and the development of germ theory made it possible to start scientific studies on the causative agent of malaria. French army doctor Charles Louis Alphonse Laveran discovered the parasites in 1880

[5], and British army doctor Ronald Ross discovered that the anopheline mosquitoes are the vector of malaria parasites in 1897 [6]. Both received Nobel Prize in Medicine for their findings, in 1907 and 1902, respectively.

First news of an effective "cure" came from Peru in the late 17th century, the powdered version of the "Peruvian bark". The remedy was quickly introduced to Europe in the 18th century and the tree was named *cinchona* by Linnaeus in 1742. Quinine was isolated from cinchona bark in the early 19th century and became the most popular and effective treatment throughout the world. It became so popular that in 1854, the British Navy started to administer a daily dose of quinine as a formal prophylaxis during patrols on the Niger River in Africa. Private companies started producing sugar and fruit flavored bubble waters with quinine and/or lime as *tonics* against malaria. They even turned it into an alcoholic beverage to convince soldiers to consume the bitter drink as a prophylaxis. After having heavy casualties to malaria in World War I, German government started a search for a substitute of quinine, and it resulted with the synthesis of chloroquine (Resochin) as new modern class of antimalarials [1, 7].

1.2 Global Burden of Malaria

Over 3 billion people are at risk of malaria transmission. There were an estimated 247 million cases in 84 malaria endemic countries and 619 thousand deaths, globally in 2021. It is widespread in tropical and sub-tropical regions with the highest burden in sub-Saharan Africa. 29 countries accounted for 96% of all malaria related deaths globally in 2021, and about half of total death numbers were from Nigeria (31%), the Democratic Republic of the Congo (13%), the Niger (4%) and the United Republic of Tanzania (4%) (**Figure 1.1**) [8].

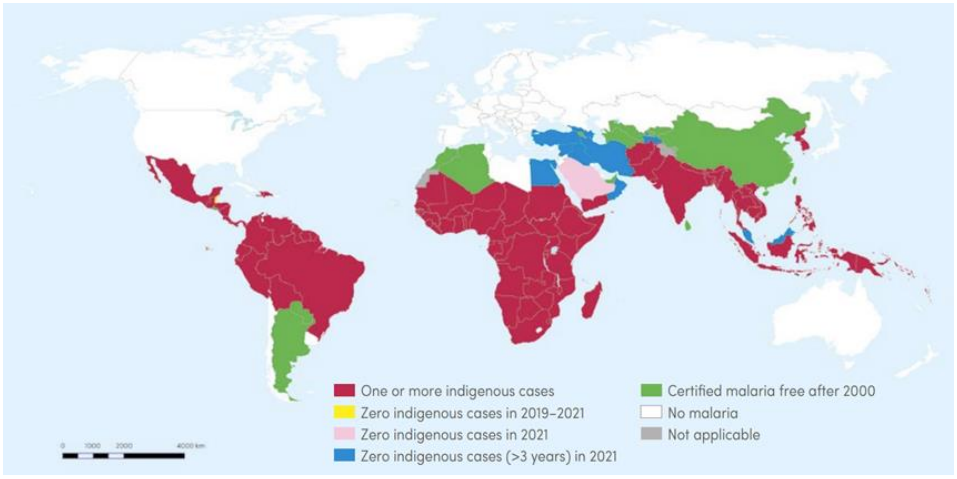


Figure 1.1 : Countries with indigenous cases in 2000 and their status by 2021 (WHO, 2022).

1.3 Global Fight Against Malaria

Fighting a global disease requires a global effort. Soon after its founding, the World Health Organization (WHO) started the Global Malaria Eradication Programme (GMEP; 1955-1969). Key terms defined by WHO in infectious diseases are control, elimination, and eradication. While control is the reduction in numbers of disease incidence, prevalence, morbidity, and mortality to acceptable levels; elimination is defined as interruption of local transmission or zero incidence of indigenous cases, and eradication is defined as permanent reduction to worldwide zero incidence [9]. The GEMP was successful in elimination of malaria in many countries, especially in southern Europe but failed to achieve global eradication target. Second eradication effort came in 2006 with a high international support and funding, causing significant declines in the case and death rates, and increase in the number of malaria-free countries between 2000-2015. Success of this initiative increased the possibility of global eradication once again started to be considered [10]. In 2015, WHO accepted the *Global Technical Strategy for Malaria 2016–2030*. The aim is to reduce 90 % of the death and case numbers by eliminating malaria in 35 more countries by 2030 [11, 12]. In 2016, WHO brought together a group of scientists, creating the Strategic Advisory Group on Malaria Eradication (SAGme), to evaluate the future aspects of malaria strategies such as feasibility of eradication. Their analyses showed that in best-case scenario, with current tools, number of malaria cases could be only reduced to under 11 million by 2050.

In the last two decades, there is a notable progress in drug and vaccines development. However, drug resistance is increasing [13], and there are still no vaccines that provides a lifelong protection. Closest available vaccine, RTS,S/AS01 was shown to reduce approximately 30% of clinical cases. It is promising, but it is still far from a sustained protection [14]. Therefore, new tools are urgently needed to achieve the dream of a malaria-free world [12].

1.4 Malaria in Türkiye

Malaria had always been a big burden since the founding of the new Republic. There were many efforts to control and even eliminate malaria, but they were not successful due to many social, cultural, geographical, and technical problems. *Plasmodium vivax* was the dominant species, and it is the only locally transmitted species since 1970s. Vector control efforts and mass drug administrations were successful in controlling the epidemics in Çukurova and south-eastern Türkiye in the 1970's and 1990's. In 2005, malaria elimination goal was considered as feasible, and the Ministry of Health signed "*The Move from Malaria Control to Elimination*" Programme the Tashkent Declaration target of elimination of malaria in WHO European region by 2015. The Programme was successful and there are no autochthonous cases by 2011 (**Figure 1.2**) [15]. Although around 200 recorded cases per year are all imported cases since 2011, one report from April 2023 shows 3 possible indigenous cases [16]. Human movement is not the only source of imported cases for vector-borne diseases anymore. There are reports of airport malaria in which the exotic *Anopheles* mosquitoes are carried internationally [17, 18]. Türkiye is already an *Anopheles*-endemic country. With the effects of climate change, and increasing human migration, the risk of new epidemics must be considered an important threat to malaria elimination.

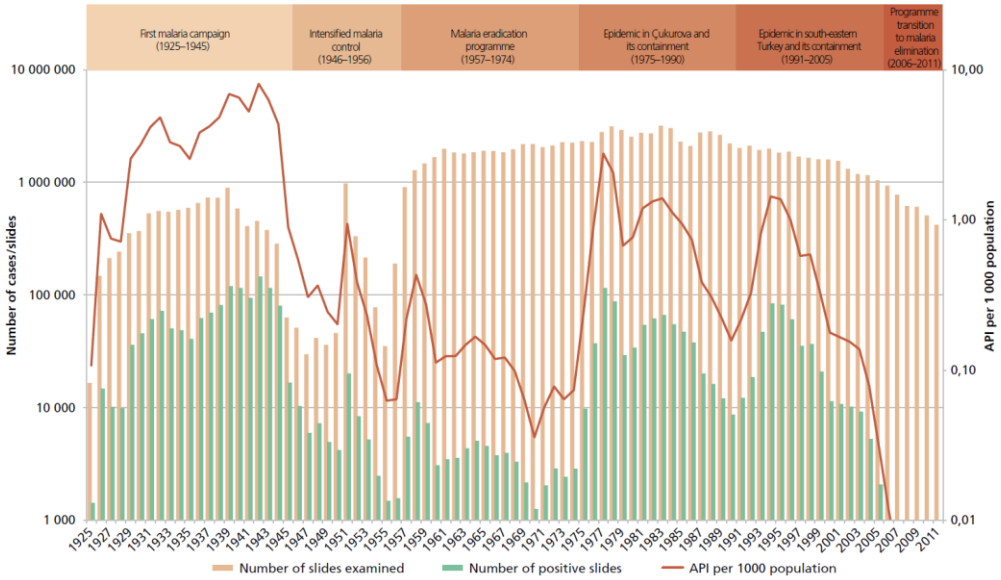


Figure 1.2 : History of malaria control and elimination in Türkiye. Adapted from WHO 2013 [15].

1.5 The Disease

Malaria is a serious disease which can also be fatal in some cases. Parasite is transmitted during the blood meal of the infected female *Anopheline* mosquitoes. They inject sporozoites which are infective and motile forms of the parasite to the skin of the host. Sporozoites reach the liver where they infect hepatocytes. Single sporozoite in a single hepatocyte can produce thousands of merozoites in 5-7 days. This first stage is called liver stages which is asymptomatic. Produced merozoites are released into the bloodstream where they infect erythrocytes to replicate and produce new merozoites (schizogony). Symptoms start during these blood stages where the merozoites rupture the erythrocyte and metabolites of the parasites released to the bloodstream which usually occur in 7-10 days after the initial bite. Severity of the symptoms vary depending on the species and immunity level of the host. Disease is classified as asymptomatic, uncomplicated, and complicated or severe malaria. Symptoms include fever, muscle ache, shaking, and in severe cases high periodic fevers, exhaustion, and extreme sweat [19]. Severe malaria presents with cerebral malaria, severe anemia, multi-organ damage, and is often fatal [20].

1.5.1 Diagnosis, treatment, and prevention

Diagnosis of malaria is done by fever and the existence of parasites. Most common methods of detection are microscopic examination of blood films or antigen detection based rapid tests [19].

Recommended treatment of uncomplicated malaria is the administration of artemisinin combination therapies (ACTs) for 3 days. Combinations change according to the patients (risk groups, age, etc.) and region (common drug resistance of the parasites). In the case of severe malaria, patients are treated with parenteral administration of artesunate for at least 24 hours followed by oral ACTs for 3 days [21].

Prevention methods include vector control, prophylactic mass drug administration, and vaccines.

Vector control can be defined as a prevention method to stop mosquito breeding such as widespread use of insecticides and larvicides, destruction of mosquito breeding grounds, indoor residual insecticide spraying, and insecticide treated bed nets [19].

Seasonal mass drug administration methods have been used since the 1930's. It is usually effective at rapidly reducing the prevalence and incidence in the short term. Benefits and risks are still debated but it is shown to be effective when used with other prevention methods in small populations. During mass drug administration, it is expected from all members of the defined community is required to take the antimalarials at approximately the same time and with same intervals. This requires high levels of participation and coordination [21].

Ideal prevention method for any infectious disease is an efficient vaccine. And ideal malaria vaccine should protect against the most common *Plasmodium* species and should have more than 75% efficacy, and a long-lasting protection [19]. But as mentioned earlier, only approved vaccine so far, RTS,S, has a limited efficacy. The first successful human trial of RTS,S was in 1996 and several trials were conducted in the last two decades. It was shown in a phase III trial in 2015 that the RTS,S induced immunity in children, 6 to 12 weeks-old, began with 63% efficacy which decreased to 11% and 3% in after 1 and 5 years, respectively. Similarly, in 5 to 17 years-old children, protection efficacy started with 74% and reduced to 28% and 9% in 1 and 5 years, respectively. There is a little information on adults but in studies conducted in Gambia, RTS,S induced a short-lived immunity of adults with 34% protection in 2001, and trials in Kenya resulted with no significant protection in 2009 [22].

1.6 The Parasite

Causative agents of malaria are protozoan parasites of *Plasmodium* genus belonging to the phylum *Apicomplexa*. Common traits of *Apicomplexa* are the group of unique organelles termed the apical complex, in addition to the unique plastid called apicoplast (apicomplexan plastid). Almost all members are obligate intracellular parasites, including *Toxoplasma gondii* and *Cryptosporidium parvum*.

Plasmodium parasites have a complex life cycle with many distinct morphological features in two different hosts: *Anopheline* mosquitoes and vertebrates.

Studying the human species is difficult due to the impossibility of *in vivo* studies. But emerging new methods are enabling to mimic *in vivo* behaviors such as blood-humanized and/or liver-humanized mouse models [23, 24]. *In vitro* culturing of blood and liver stages is relatively easy, but studying mosquito stages requires biosafety level 3 (BSL-3) laboratories for containment of human infective species.

Rodent species are easier and cheaper to study, since they require only mice and BSL-2 laboratories. Most common species for rodent models are *P. berghei* and *P. yoelii*. Rodent models allow studying of all aspects of life cycle.

1.6.1 Life Cycle

The complex life cycle of the parasites consists of a sexual growth stage in mosquitoes (called mosquito stages) and asexual growth stages in vertebrate hosts (**Figure 1.3**).

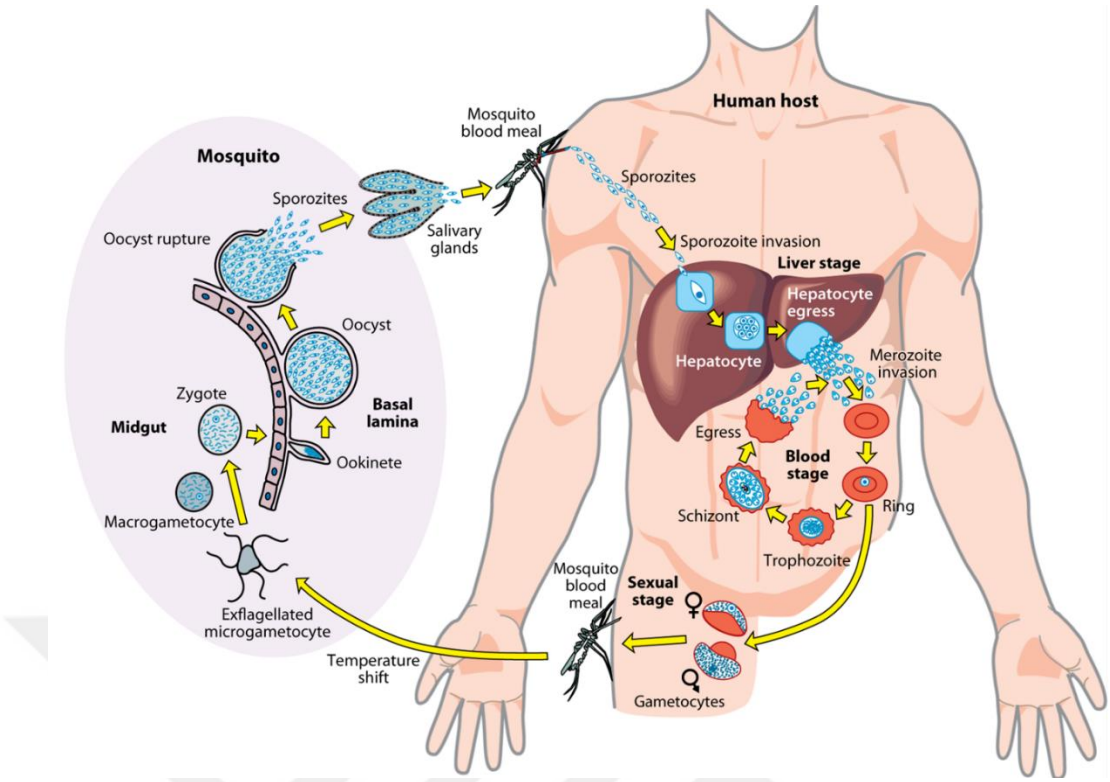


Figure 1.3 : Sexual and asexual life cycle of *Plasmodium* parasites in mosquito and human hosts. Adapted from Boddey and Cowman 2013 [25]

1.6.1.1 Pre-erythrocytic stages

Pre-erythrocytic or exo-erythrocytic stages are asymptomatic stages that begin with the injection of sporozoites into the vertebrate host's skin and end with the release of liver merozoites to blood circulation. Sporozoites are injected by a bite from an infected mosquito. A mosquito can inject up to a few hundred sporozoites from its salivary glands alongside with its saliva proteins. Sporozoites are liver-infective forms of the parasite. Once injected into the skin, sporozoites traverse host cells by gliding motility, until they enter a blood capillary where they can reach to the liver within minutes through blood circulation. They traverse the Kupffer cells to reach the hepatocytes, and further traverse through several hepatocytes until they invade and settle in a final hepatocyte [26, 27]. Cell traversal and invasion are two separate processes. Sporozoites can traverse various type of cells, but invasion only occurs in hepatocytes (**Figure 1.4**). Although some of the involved proteins are known, underlying mechanisms of both functions are poorly understood.

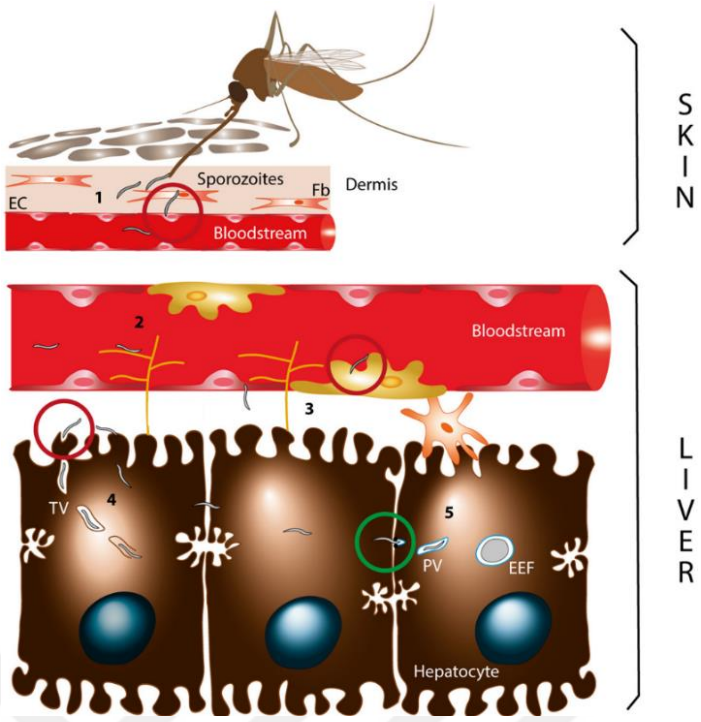


Figure 1.4 : Sporozoite cell traversal and invasion of hepatocytes. Adapted from Loubens et al. 2021 [28].

Liver stages start with the invasion of a hepatocyte. Sporozoite forms a parasitophorous vacuole (PV) with the invagination of the hepatocyte membrane by assembling a moving junction. Some of the membrane proteins are excluded selectively during this process to insure the safe development of the parasite. Following the hepatocyte invasion, parasitophorous vacuole membrane (PVM) remodeling continues with the integration of parasite specific proteins [28]. After the invasion stage, sporozoite develops into a liver trophozoite. Interestingly, sporozoites can develop into early liver trophozoite forms in vitro [29]. During this transition, sporozoite transforms into a rounded form, disassembles its machinery for motility and invasion, and adapts its host cell to support its expansion [30]. Beyond the trophozoite stage, parasites will grow into multinucleated schizont stage, which will eventually produce up to 10^5 merozoites within a few days. It is clear that parasite will need the resources of the host cell [26, 30]. Parasite also requires space to greatly increase its size but at the same time needs to keep the host cell alive for its own survival.

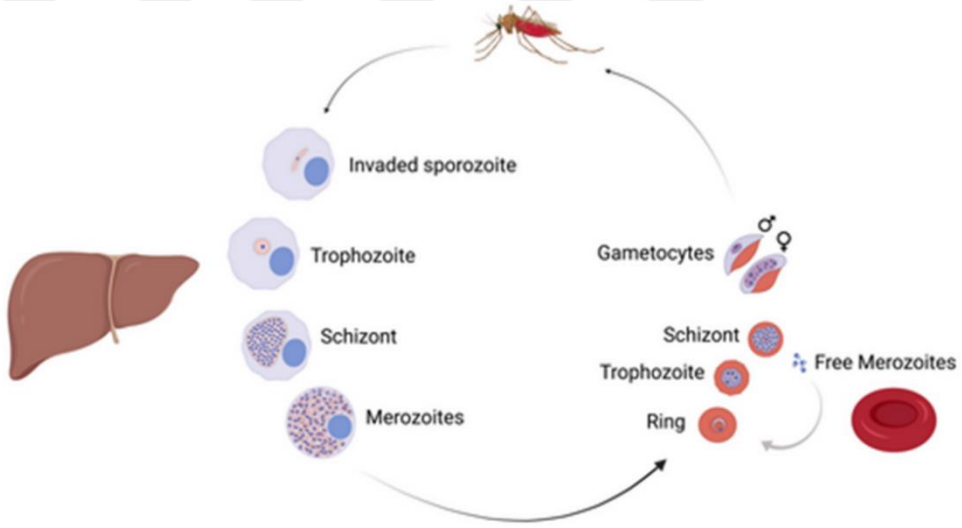
Establishment of a successful liver stage development is only possible by an extensive control on the critical processes and responses of the host cell. This control of parasite over the host cell is often called “hijacking”. Hepatocytes are major sources of

glycogen, lipids, purines, and cholesterol. They can support the massive amounts of required nutrients for liver stage development. Pores and export machinery on the PVM are key to accessing to host cell resources. But molecular mechanisms and interactions are not fully understood.

Some of the critical genes for the liver stage development for the early liver stages are UIS (up-regulated in infective sporozoites) group. This group of genes are highly transcribed in sporozoites while they are in mosquito's salivary glands. Some of these genes are translationally repressed such as PVM resident proteins UIS3 and UIS4. Translation of these "stored" mRNAs activates following hepatocyte invasion. This strategy allows the rapid transition of sporozoite cells to liver stage development. It has been thought that the role of UIS3 is to transport of lipids to support liver stage growth since it interacts with host liver fatty acid protein (L-FABP). Roles of other critical liver stage proteins UIS4 and EXP1 are yet to be fully revealed as well as late liver stage PVM-associated proteins liver-specific proteins, LISP1 and LISP2, and PPM-associated proteins B9 and an iron transporter ZIPCO [31]. LISP1 is involved in PVM disruption for egress of merozoites [32]. LISP2 has been shown to be localized in PV and PVM and also transported into host cell cytoplasm and host cell membrane, and in involved in formation of merozoites [33].

Studies show that other than the intake of nutrients, parasites also use some of the host cell organelles. PV is associated with host cell endoplasmic reticulum (ER) [34], Golgi apparatus [35], and mitochondria [36]. For instance, infection of the host cell induces endoplasmic reticulum stress which leads to apoptosis. A previous study showed that hepatoma cell line HepG2 cells infected with *P. berghei* sporozoites lacked apoptosis signaling. And induction of apoptosis in *P. berghei* infected mice by liver apoptosis inducer d-glucosamine and tumor necrosis factor (TNF)-a revealed that more than one third of non-infected cells were TUNNEL positive in comparison to less than 5% of TUNNEL positive infected cells [37]. Furthermore, it has been shown that *Plasmodium* infection modulates unfolded protein response (UPR) in host cell, and activation of UPR promotes parasite infection [38]. Multifunctional parasite protein, circumsporozoite protein (CSP), is exported to host cytoplasm, and contains a nuclear localization signal (NLS). Deletion of NLS significantly reduces parasite growth inside the hepatocyte. NLS is known to bind host importin-α3 and it is possible that it competes with NF-κB for binding importin-α3, therefore inhibiting the host cells inflammatory signals [39].

Liver-stage trophozoite undergoes a process called schizogony in which nuclear divisions take place without cell division. During this stage, essential major organelles like ER, mitochondria, and apicoplast start to elongate, creating a vast network of intertwining branches to reach each asynchronously dividing nucleus [40, 41]. At the end of schizogony, merozoites start to form by invaginations of parasite plasma membrane (PPM). A synchronous cytokinesis takes place in the end, accompanied by organelle segregations, resulting in tens of thousands individual merozoites with individual organelles [42]. PVM ruptures and merozoites are released into host cell cytosol. Finally, merosomes (vesicles filled with merozoites) are formed and released into the bloodstream [40]. One rodent malaria sporozoite can produce approximately 29.000 mature liver merozoites in less than three days, and P. *falciparum* sporozoites can produce approximately 90,000 in less than seven days (**Figure 1.5**). This accounts for one of the fastest eukaryotic replication events [31].



	Human *Plasmodium* (*P. falciparum*, *P. vivax*)		Rodent *Plasmodium* (*P. berghei*, *P. yoelii*)	
	Blood	***Liver***	***Blood***	***Liver***
Duration (hours post- infection)	48 hpi	5-7 days	24 hpi	2 days
Host cell size (volume)	90 µm³	10,000 µm³	90 µm³	10,000 µm³
Liver schizont size (diameter)	-	80-100 µm	-	30-50 µm
Number of merozoites per schizont	8-30	100,000	12-16	Up to 10,000-30,000

Figure 1.5 : Replication of human and rodent *Plasmodium* parasites during exoerythrocytic and erythrocytic development. Adapted from Roques et al. 2023 [40].

1.6.1.2 Erythrocytic stages

Erythrocytic stages or blood stages are asymptomatic stages of disease progression. Once the liver merozoites are released into the bloodstream, individual merozoites invade circulating erythrocytes. Invasion of erythrocytes are similar to the invasion of hepatocytes. First, merozoite initiates contact with an erythrocyte and reorients itself, then establishes a moving junction and enters the cell by forming a PVM (**Figure 1.6**) [43].

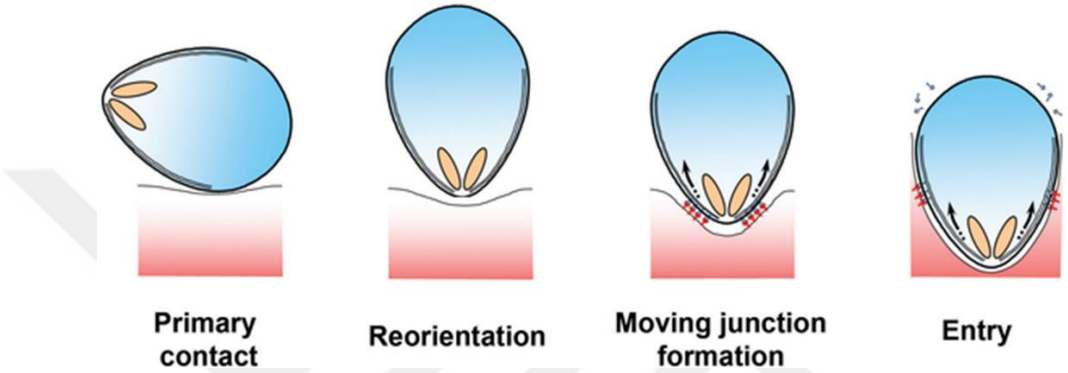


Figure 1.6 : Steps of erythrocyte invasion. Adapted from Wright and Rayner 2014 [43].

Development begins after the invasion step. Invading merozoite develops into ring, trophozoite and schizont stages. Erythrocytes are terminally differentiated cells which are devoid of major organelles and critical systems such as immune responses and major histocompatibility complex (MHC). Infected erythrocytes are substantially remodeled to sustain a successful blood stage infection. Erythrocyte deformability is significantly reduced, and cells gain the ability to adhere to other cell surfaces during intracellular development of *P. falciparum*. This gives the erythrocytes the ability to sequester in veins or major organs to avoid destruction by splenic macrophages [25]. Sequestration in the capillaries and major organs, e.g., brain, is responsible for severe malaria.

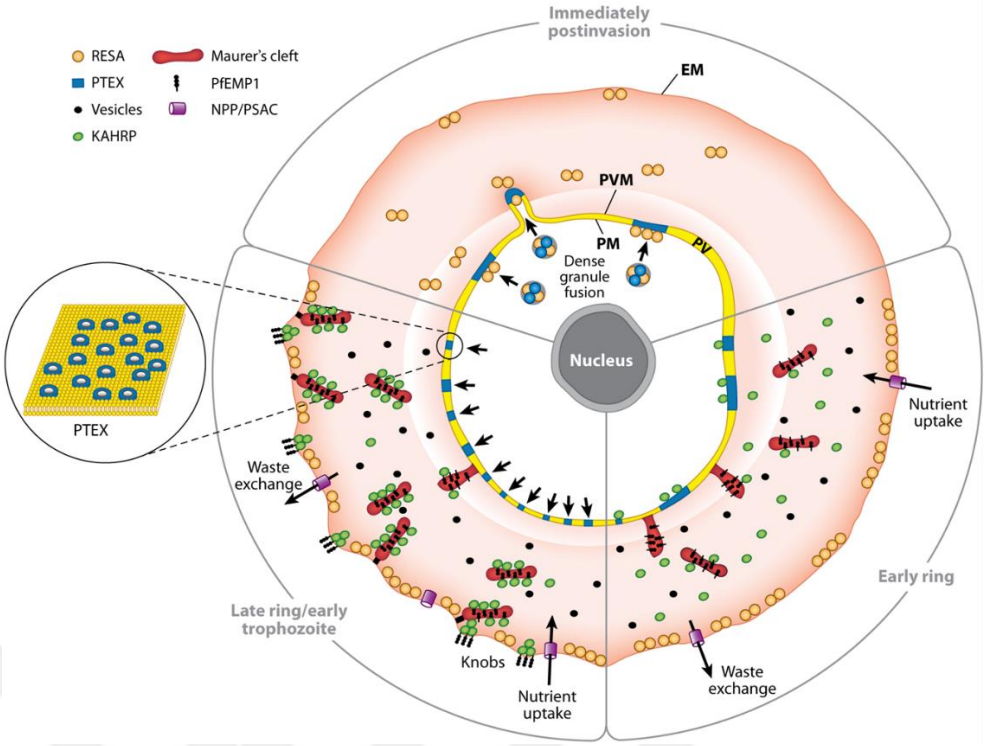


Figure 1.7 : Extensive remodeling of the infected erythrocyte. Adapted from Boddey and Cowman 2013 [25].

This extensive remodeling of the host cell is achieved by complex trafficking network of parasitic proteins outside the PVM to the host cytosol and membrane. This network includes various membranous structures such as Maurer's clefts, electron-dense vesicles, and J-dots (**Figure 1.7**). Maurer's clefts bud from the PVM, migrates to host cell membrane, and physically tethers for the trafficking of parasitic proteins. P. *falciparum* erythrocyte membrane protein 1 (PfEMP1) which is responsible from the sequestration of erythrocytes is assembled with knob-associated histidine-rich protein (KAHRP) and inserted under the erythrocyte membrane [25].

Parasite's cell cycle is different than that of model eukaryotes. Major differences are asynchronous replication of the multinucleated schizont, atypical structure of centrosome, specialized cytokinesis, and lack of major cell cycle checkpoints. It is not clear how the number of daughter cells are determined; it can be between 10-30 in *P. falciparum*. It is believed that replications are controlled either by a kind of time or counter dependent mechanism. Considering the random progeny numbers, it is convenient to think that lack of a cytokinesis checkpoint is helpful to keep the division process continuous even if non-viable merozoites are formed [44].

As in liver development, from trophozoite to schizont, major organelles ER, mitochondria, and apicoplast starts to form elongated and branched structures around

the newly formed nuclei. Apicoplast and mitochondria further elongates. Multiplication of centriolar plaque and Golgi occurs. Apicoplast divides, and associates with mitochondrial branches. Mitochondria divides after the last round of divisions and daughter merozoites are formed with one set of each major organelles. Eventually merozoites rupture the schizont and egress from the erythrocyte, leaving a residual body of food vacuole and other cellular remains behind (**Figure 1.8**) [45].

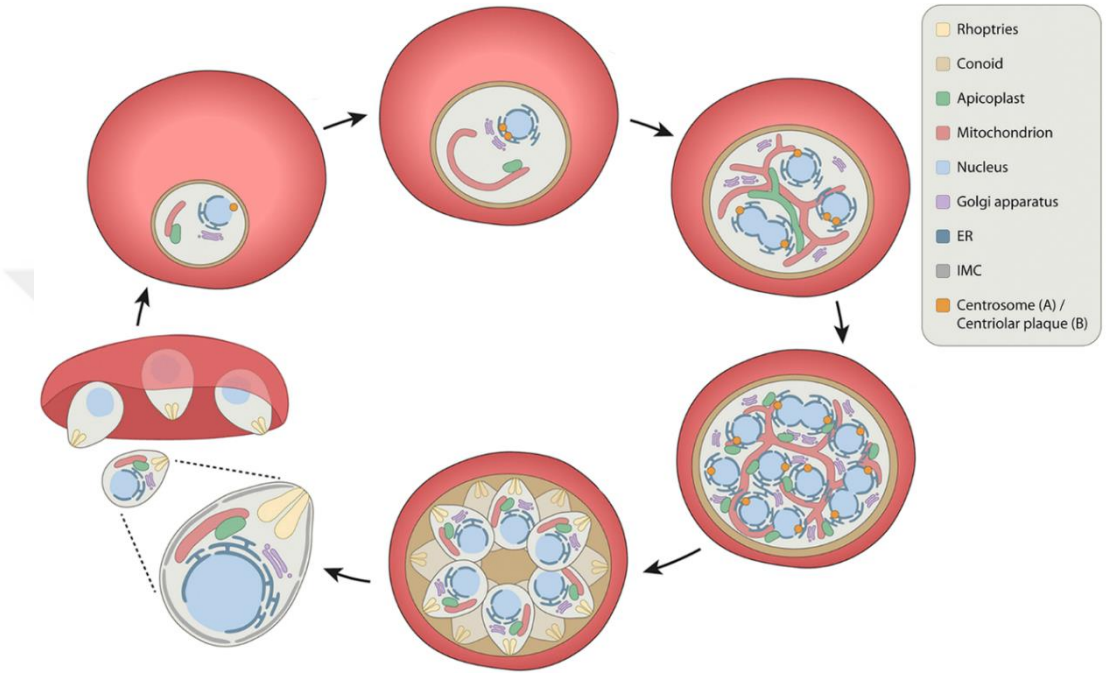


Figure 1.8 : Asexual replication cycle of *Plasmodium* parasites in erythrocytes. Modified from Verhoef et. al. 2021 [45].

Merozoites are released into blood following schizont rupture to infect a new erythrocyte. A small fraction of merozoites commit to sexual development and form gametocytes which are essential for transmission to mosquitoes (**Figure 1.3**). This process is known to be regulated by a transcription factor, AP2-G [44]. Conditional expression of PfAP2-G resulted with 90% conversion of the merozoites to sexual stages [46].

Macrogametocytes and microgametocytes are asexual non-replicative forms of blood stages. *P. falciparum* gametocytes maturate from stage I to stage V in about 7-10 days. Immature stages sequester away from the peripheral blood stream, while stage V gametocyte circulates the blood stream to be taken up by mosquito vector [47].

1.6.1.3 Mosquito stages

Mosquito stages begin with the blood meal of female *Anopheles* mosquito. Alongside the blood, mosquitoes ingest mature gametocytes. Upon ingestion, gametogenesis is activated to produce microgametes and macrogametes by temperature shift and a mosquito derived molecule, xanthurenic acid. Microgametocyte begins replicating three times to produce eight flagellar microgametes in ten minutes. This process is called exflagellation. Consequently, motile microgamete finds macrogamete, and zygote forms following the fusion of gametes [48].

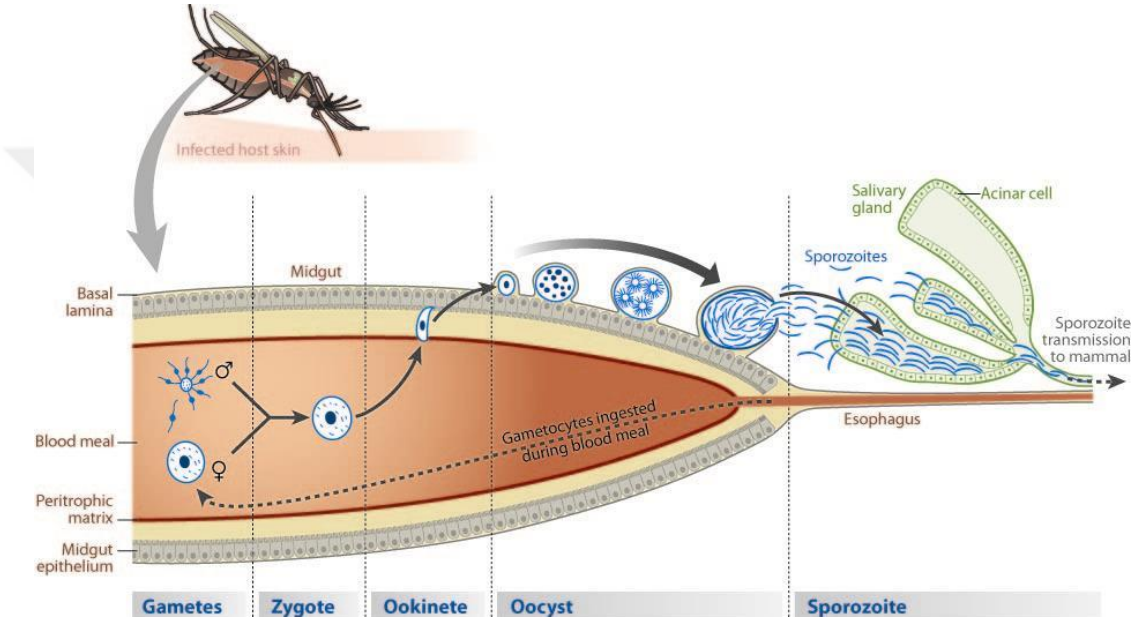


Figure 1.9 : Sexual reproduction of parasites in mosquito host. Modified from Aly et al. 2009 [48].

The sole diploid stage in the parasite life cycle, the zygote, undergoes meiosis within a few hours, and maturates into tetraploid motile ookinete in around 24 hours [49]. Ookinete then traverses through the peritrophic matrix. Once it passes the midgut epithelium and encounters the midgut basal lamina, ookinete arrests to transform into oocyst form to produce sporozoites [48]. Oocyst nuclei undergo several mitoses, forming sporoblast. Sporozoites are formed from sporoblasts starting from day 10 after the blood meal [27]. Next, sporozoites rupture the oocyst by proteolytic activity, and enter the hemolymph. They travel with the circulation of the hemolymph. When they reach the salivary glands, they adhere to the basal lamina of the salivary glands, traverse the acinar cells, and accumulate in the salivary duct. Here, mature sporozoites wait until the next blood meal of the mosquito to be transmitted to the skin of the vertebrate host (**Figure 1.9**) [27, 48, 50].

1.7 Hosts

There is a vast diversity of vertebrate hosts of almost three hundred *Plasmodium* species. Many of the *Plasmodium* species are highly host specific, while some of them have a variety of hosts which are closely related. Of almost three hundred species of *Plasmodium*, five species are known to cause disease in humans: *P. malariae, P. ovale, P. knowlesi, P. vivax,* and *P. falciparum*. Of note, *P. vivax* and *P. ovale* species have a dormant stage called "hypnozoite" in which they can stay dormant for years during liver stages. *P. knowlesi* is originally a simian malaria species but it is recently discovered that it is also infectious to humans [51]. Some of the species with different hosts are *P. reichenowi, P. gaboni,* and *P. lemuris* (in primates), *P. bubalis* (buffaloes), *P. caprae* (goats), *P. cyclopsi* (bats), *P. relictum, P. gallinaceum, and P. juxtanucleare* (birds), *P. weyoni* (snakes), *P. draconis* and *P. minasense* (lizards), *P. vinckei, P. chabaudi, P. berghei,* and *P. yoelii* (rodents).

The major mosquito host of *Plasmodium* is *Anopheles* species. Nevertheless, some avian malaria species are transmitted *Culex species* (*P. relictum*) [52], and *Aedes aegypti* (*P. gallicaneum*) [53]. Many species of *Anopheles* are widely spread around the globe. *Anopheles gambiae* is the dominant vector for *P. falciparum* in sub-Saharan Africa, which is responsible for the majority of morbidity and mortality, but *Anopheles stephensi* is the commonly used laboratory species.

1.8 Immune Responses Against *Plasmodium*

1.8.1 Immune responses of the mosquito host

Other than the physical barriers of midgut peritrophic membrane or lining of tracheal respiratory system, mosquitoes can form physical capsules around the pathogen by their melanin in a process called melanization. Midgut microbiota is another defensive line since bacteria in the midgut induces production of anti-microbial peptides (AMPs) which stimulates basal innate immune activity. Other than these innate responses, mosquito immune system also has humoral and cellular responses [54].

Humoral responses include thioester-containing protein (TEP) 1, leucine-rich repeat protein 1 (LRIM1), and *Anopheles Plasmodium*-responsive leucine-rich repeat protein (APL1). These proteins form complement-like structures that attack and terminate significant portion of ookinetes while traversing the midgut [55].

Main immune cells of the mosquito are hemocytes which have three distinct classes: granulocytes, oenocytoids, and prohemocytes. Granulocytes are responsible for phagocytosis and mediating immune responses by humoral signaling that resembles macrophages. oenocytoids are responsible for melanin production, wound healing, and pathogen killing. Lastly, prohemocytes are progenitor cells that differentiate into other hemocyte types [56].

Three major signaling pathways control the immune responses of the mosquito against *Plasmodium* parasites: Toll, immune deficiency (Imd), and Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathways. Little is known about the JAK-STAT pathway in mosquitoes but activation of Toll and Imd begins with recognition of pathogen-associated molecular patterns (PAMPs), which results in nuclear translocation of NF-κB transcription factors Rel1 and Rel2. These factors enter the nucleus and activate the immune effector mechanism such as expression of AMPs [55].

1.8.2 Immune responses of the human host

Pre-erythrocytic stages of the malaria infection is the bottleneck for the parasite. Sporozoites are exposed to complement system, innate immune cells and antibodies in the skin, bloodstream and in hepatic extracellular fluid. This is the only stage where parasites invade nucleated human cells which has MHC I complex to present parasite antigens to $CD8^+$ T-cells.

Skin acts as the first physical barrier, and it harbors diverse range of dendritic cells, macrophages, neutrophils, monocytes, and mast cells [57]. Almost half of the sporozoites cannot leave the inoculation site on the skin [54]. This results in being taken up by skin draining lymph node resident $CD8^+$ dendritic cells (DCs) which efficiently primes $CD8^+$ T-cells [58]. Also, $CD11c^+$ DCs in spleen, liver, and liver-draining lymph nodes have been shown to prime $CD8^+$ T-cells [57]. Infected hepatocytes can induce inflammatory responses by releasing type-I interferons (IFN-α and IFN-β) in response to cytosolic parasite derived RNAs, resulting in recruitment of natural killer (NK) and $CD3^+CD49b^+$ natural killer T (NKT) cells to the site of infection. These cells produce IFN-γ that activates nitric oxide (NO) pathway in macrophages [57]. Toll-like receptor 2 (TLR2) has been identified as another innate infection sensor which signals by MyD88 to induce strong pro-inflammatory transcription signal through NF-κB for the secretion of cytokines [58].

Antibody responses begin with stimulation of naïve B cell with parasite antigens. These cells might proliferate to produce short-lived antibody secreting plasmablasts or interact with $CD4^+$T-cells and transform into long lived memory cells. High antibody titers against major sporozoite proteins CSP, liver surface antigen (LSA1), thrombospondin-related anonymous protein (TRAP), and sporozoite threonine and asparagine-rich protein (STARP) have been shown in controlled human malaria infection studies to confer protection against reinfections. $CD4^+$T-cells are responsible for regulation of immune responses such as cytokine production, activation of $CD8^+$T-cells, B cells, DCs, while $CD8^+$T-cells are the primary effector cells which has been shown to confer sterile immunity against *P. berghei*, independent of other immune cells [57].

Erythrocytic stages are symptomatic stages, in contrast to the pre-erythrocytic stages, due to the parasite load in the blood. Symptoms are characterized by sepsis-like excessive inflammation. Variant surface antigens (VSAs) are the main virulence factors some of which are var, rif, and stevor gene families which encode around 60, 200, and 30 highly polymorphic genes, respectively [58]. These families encode PfEMP1, RIFIN, and STEVOR genes which function as surface proteins of the infected erythrocytes. PfEMP1 is responsible for sequestering by adhering to endothelial cell surfaces, while RIFINs are thought to be responsible for immune modulation by binding to LAIR1, LILRB1, and LILRB2 receptors and inhibiting the activation of B cells and NK cells [59]. These proteins are also responsible for binding to uninfected erythrocytes and forming rosette structures, as another form of immune evasion [60].

Innate immune responses are induced by the recognition of PAMPs. Three PAMPs are described in P. *falciparum*: hemozoin, immunostimulatory nucleic acid motifs, and glycosylphosphatidylinositol (GPI) anchors. Recognition of these patterns triggers intracellular signals leading to DC maturation. Hemozoins are crystal structures that are produced by parasites during detoxification of heme which is released from the hydrolysis of hemoglobin. Release of hemozoin and *Plasmodium* DNA into host phagolysosomes induces an innate immune response by activation of TLR9. GPI anchors on the parasite plasma membrane also triggers TLRs and induces cytokine synthesis [61].

Erythrocytes' lack of MHC-I means that they cannot present antigens to CD8+ T-cells, therefore cannot stimulate a cytotoxic response. However, reticulocytes which are

immature erythrocytes retain their translation machinery, and can induce $CD8^+$ T-cell responses of the reticulocyte infecting *P. vivax* parasites via MHC-I [62]. Some parasite proteins are constantly exported to the surface of the infected erythrocyte where they can induce antibody responses through recognition by B cells. Infected erythrocytes lose their shape by the remodeling of the growing parasite which causes clearance from the blood by the spleen. Parasites avoid this by sequestering from the blood stream [63]. Infected erythrocyte surface antigens also stimulate DCs which subsequently activate $CD4^+$T-cells via MHC-II resulting in elevated levels IFN-γ and TNFα secretion [63].

Secretion of cytokines activates more innate immune cells and promotes phagocytosis. Macrophages phagocytosis of parasites, and hemozoin containing remains of ruptured schizonts leads hemozoin accumulation in macrophages and leukocytes. These cells cannot digest hemozoin which inhibits their ability to repeat phagocytosis, to generate oxidative burst, or to produce NO. Furthermore, hemozoin accumulated monocytes were shown to be defective of MHC-II induction in response to IFN- γ, suggesting a role of hemozoin in immune evasion [64].

1.9 Vaccine Strategies Against Malaria

The quest for an efficient malaria vaccine is a priority for global efforts of malaria eradication. There are approximately 20 malaria vaccine candidates in clinical trials, and only one approved vaccine against malaria [65].

Vaccines can be designed against pre-erythrocytic stages, blood-stages, or sexual stages of the parasite. Pre-erythrocytic stages are the bottleneck of the parasitic life cycle in vertebrate host. It is also a clinically silent stage. Therefore, vaccines against pre-erythrocytic stages draws the attention as an efficient strategy [65]. Repeatedly infected individuals show a degree of naturally acquired immunity which results in asymptomatic or uncomplicated malaria [66]. Similarly, blood stages vaccines are design to induce immune responses to block or limit invasion of erythrocytes by merozoites. They are designed to target either infected erythrocyte surface proteins, or free merozoite surface proteins. Vaccine designs against the sexual stages aims to induce antibody responses against the gametocytes to block the transmission to mosquito host. These vaccines are called transmission blocking vaccines (TBV) [65].

1.9.1 Pre-erythrocytic stages vaccine strategies

Currently, RTS,S is the only vaccine that is approved and recommended by WHO [22]. RTS,S is a major sporozoite protein, circumsporozoite protein (CSP), based sub-unit vaccine that is designed to target pre-erythrocytic stages. Construct of central repeat region ("R") and carboxy-terminal T-cell epitope ("T") of CSP protein fused to the surface protein ("S") of hepatitis B virus stands for "RTS". This polypeptide is expressed in yeast that carries additional "free" hepatitis B surface antigen expression cassette. It is designed as a virus like particle (VLP), RTS and S particles form a mixed lipoprotein structure with CSP protein on its surface (**Figure 1.10**) [67, 68].

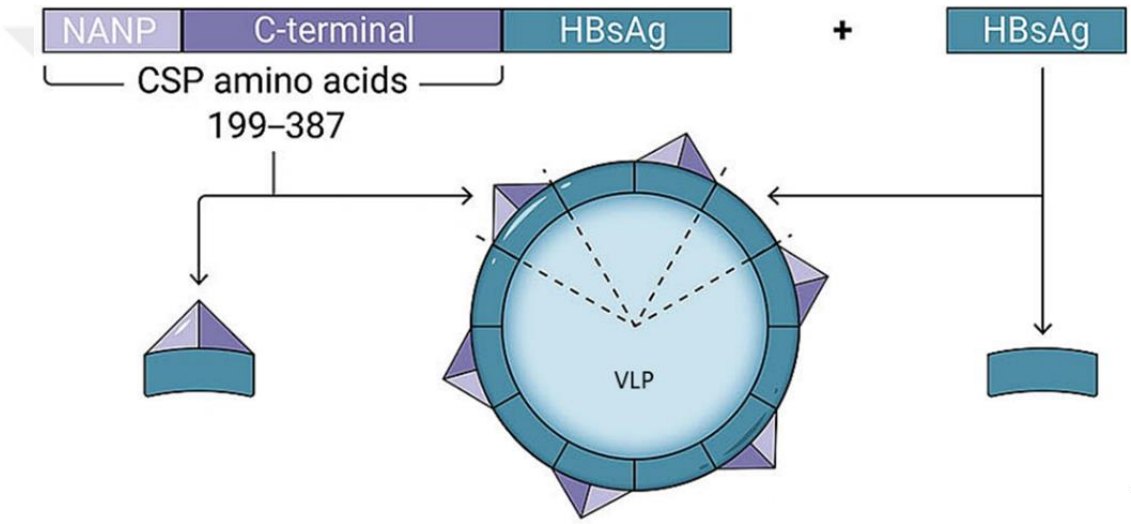


Figure 1.10 : Structure of RTS,S vaccine. Modified from Beeson et al. 2022 [67].

Approved version of RTS,S is administered with AS01 adjuvant, hence it is named as RTS,S/AS01 vaccine. which has a limited and short-lasting efficacy. R21, another pre-erythrocytic vaccine candidate that are in clinical trials, uses a similar approach with a full-length CSP [68].

ChAd63/MVA ME-TRAP, a viral vectored vaccine candidate, uses a mix of two viral vectors: a chimpanzee adenovirus followed by modified vaccinia Ankara (MVA) both encoding a sporozoite protein multi epitope thrombospondin-related adhesive protein (TRAP) has been reported to induce high immunogenicity and significant efficacy [69]. Recently, a promising new method, lipid nano particle mediated mRNA delivery, has been tested with known protein candidates, such as cell traversal protein for *Plasmodium* ookinete and sporozoites (CelTOS) [70] and CSP. Both showed

promising results as a powerful alternative [71, 72]. Other than these approaches, Radiation attenuated live sporozoite (RAS) vaccines are the only vaccines that confers sterilizing immunity [73, 74]. This method is applied to induce random DNA damage by X-irradiation. Sporozoites are able to invade hepatocytes but are attenuated to be arrested during early liver development. This approach is now in clinical trials as PfSPZ. Chemically attenuated PfSPZ-CVac, and genetically attenuated PfSPZ-GA01 vaccine candidates are also in the pipeline [65, 68].

1.9.2 Erythrocytic stages vaccine strategies

The idea of blood stage development goes back to the early 1900s. The effect of heat was though to cure mental diseases and had been used in various methods. In 1917 Austrian neuro-psychiatrist Julius Wagner Jauregg used malaria inoculation to cure dementia paralytica (neurosyphilis) and received a Nobel prize in medicine in 1927 for his work. He injected patients with blood from the malaria patient and expected fever from malaria to cure neurosyphilis. After several malarial paroxysms, he applied quinin treatment. “Malariatherapy” was the widely accepted method until 1950s when penicillin started to be administered for syphilis [75]. Retrospective studies in 2000s described the acquired immunities in these studies. It was described as highly species specific [76].

Blood stage vaccines are mostly based on surface proteins of merozoites and infected erythrocytes such as AMA1, MSP1, PfRh5, PfEMP1. But developing a vaccine based on surface antigen is challenging due to the high parasitic load and short infection time of the free merozoites. Also, the highly variable nature of surface antigens, and redundant invasion pathways are contributing to these challenges [68].

Novel blood stage antigens have been described recently as vaccine targets: PfSEA1 which targets schizont egress and arrests rupture of *P. falciparum* schizonts, and PfGARP which is an infected erythrocyte surface antigen that induces apoptosis of the *P. falciparum* infected erythrocytes with the binding of antibodies. Both of them conferred partial protection or reduced/delayed parasitemia similar to other sub-unit vaccines [68]. There are also promising PAMVAC and PRIMVAC against placental and pregnancy-associated malaria that are still under clinical trials. Both are designed against the chondroitin sulfate A (CSA)-binding parasites that sequester in placenta. VAR2CSA recombinant sub-unit was designed to induce antibodies that block parasite binding to CSA [77, 78]. Another approach to blood stages vaccine development is

similar to PfSPZ-CVac which is chemically attenuated parasites (CAP). CAP is prepared *in vitro* before administration. It was shown to induce T-cell responses but not antibody responses in humans [68]. There is also a debate about re-evaluating the whole-killed blood-stage vaccine due to its previously shown efficacy but it cannot induce $CD8^+$ T-cell responses [79].

A genetically attenuated blood stage whole-cell vaccine candidate was also clinically evaluated as a novel approach. *Pfkahrp(-)* strain was found to be immunogenic but not safe. It was shown that can lead to high parasitemia when administered at high doses [80].

1.9.3 Transmission blocking vaccine strategies

Transmission blocking vaccines are mostly targeted against the gametocyte surface antigens. Among the candidates Pfs25, Pfs230, Pfs48/45, and Pvs230, only Pfs25 and Pfs230 have undergone clinical trials. Due to the low antibody responses, there are considerations of conjugating these candidates [65].

1.10 Genetic Attenuation of Parasites

Genetic attenuation can be achieved by modification of critical genes that affects parasites survival or ability to infect. Attenuated parasites are arrested at a point of their life cycle depending on the lost/gained function of genetic modification. That can be achieved either directly by deletion or insertion of (one or more) gene(s), or indirectly by deletion of a gene that affects other critical genes.

Identification of biological role of novel genes is important for the selection of candidate genes to design late arrets. Some critical genes knockouts have already been evaluated as precise genetic attenuation targets which are listed in **Figure 1.11** [81].

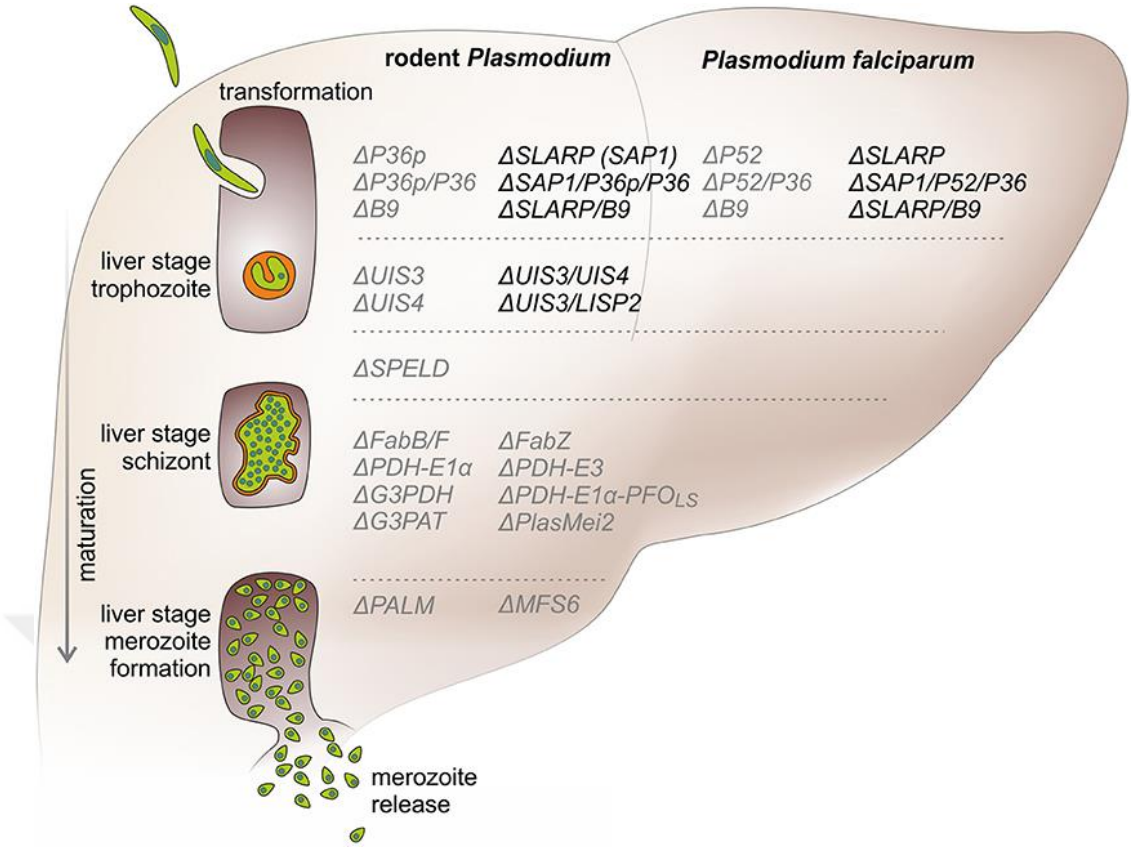


Figure 1.11 : Previously tested gene knockouts for liver stages attenuation. Names written in gray shows strains with breakthrough infections while names written in black symbolizes shows complete attenuations. Listed strains from top to bottom shows the timing of the parasite arrest. Adapted from Kreutzfeld et al. 2017 [81].

First genetic attenuation studies started with the members of the early transcribed membrane proteins (ETRAMP) family. Selection criteria were stage specific liver stages expression to allow the knockout strains to be selected and produced without a defect in other stages, and high levels of expression that indicate essential roles during liver stages [81]. Up-regulated in infective sporozoites (UIS) group genes are the best example to fulfill these criteria. They are highly expressed in infective sporozoites, indicating a level of importance for the infection phase [82]. Many of the UIS genes remain uncharacterized. High-level sporozoite specific expression suggests a role in establishment phase in liver stages infection. For instance, knockout of two ETRAMP family genes, UIS3 and UIS4, resulted in early liver stage attenuation, however, *ΔUIS4* resulted in breakthrough infections [81].

A double knock-out of infectivity associated P52 and P36 genes created early arrest in rodent model in but there were breakthrough infections in human trials of *P. falciparum* [83]. Another early arrest knock-out, SAP1, has been reported to induce

post-transcriptional regulation of infectivity-associated genes UIS3, UIS4, and P52 showed complete attenuation in mice [84]. A further study combined all three deletions in *P. falciparum* which has been reported to show complete attenuation and warranted clinical testing [85].

Following studies on genetically attenuated parasites (GAP) focused on late phases of liver growth. Targeting the fatty acid synthesis II (FASII) pathway by deletion of trans-2-enoyl-ACP reductase (FabI) enzyme resulted in a defect during late liver stages but not complete arrest [86]. Additional deletions of the other enzymes of the same pathway resulted in similar arrests [81].

Targeting of another apicoplast protein, *Plasmodium*-specific apicoplast protein important for liver merozoite formation (PALM), resulted in very late-stage attenuation but with dose-independent breakthrough infections. Nevertheless, immunization study with only two doses resulted in robust long-term protection [81].

Studies have shown that vaccines against pre-erythrocytic stage have higher potential of eliciting potent immune responses, and whole sporozoite vaccines can elicit stronger immune responses due to the ability of infected host cells to prime $CD8^+$ T-cells over MHC-I, and diversity of antigenic make up. Accordingly, rodent models have proven that late arresting sporozoite vaccines can induce superior immunity [87].

Although they can induce stronger immune responses, late arresting strategies have shown that due to the nature of development, causing late arrest will frequently result in occasional breakthrough infections.

Late liver specific genes LISP1, LISP2, and Plasmei2 are also possible candidates of GAP studies. But in parallel with other late arrests, knockouts have resulted in breakthrough infections. *ΔLISP*1 and *ΔLISP*2 alone has not been evaluated as vaccine candidates yet. ΔPlasmei2 resulted in complete attenuation with regular doses and conferred protection but resulted in breakthrough infections when injected with higher doses. Mixed knockouts of ΔUIS3/*ΔLISP*2 and ΔPlasmei2/*ΔLISP*2 resulted in complete, safe attenuations with extremely higher doses. ΔUIS3/*ΔLISP*2 parasites showed reduced protection, while ΔPlasmei2/*ΔLISP*2 resulted in complete protection.

1.11 Aims and Rationale

Resistance against frontline drugs, increased the speed of drug development in the last two decades [13]. Artemisinin combination therapies have proven to be useful but there are increasing number of reports of resistance to some of the combination therapies [88-90]. Next generation drugs are in development with novel mechanisms of action and single dose efficacies. In addition, many vaccine candidates are in development with different aims and strategies in pre-clinical and clinical phases of global vaccine development pipeline [19].

As with many infectious diseases, vaccines are thought to be the ultimate solution for eradication. But developing a vaccine against malaria has proven difficult. Unlike simpler pathogens, protozoan parasites have evolved complex immune evasion strategies [19]. But available biotechnological tools are improving and there are various vaccine strategies in development such as sub-unit vaccines, viral-vectored vaccines, or whole sporozoite vaccines. Some of which are targeting pre-erythrocytic stages (liver stages) or blood stages, and some are targeting gametocytes for transmission blocking [65, 68]. Considering the complex nature of *Plasmodium* parasites, simple antigen targeting strategies are usually less effective, and previous studies shown that the most effective vaccines with sterilizing protection so far are late arresting live-attenuated sporozoite vaccines [87, 91]. There are only three live-attenuated sporozoite vaccines in pre-clinical and clinical development: radiation-attenuated sporozoites (PfSPZ), sporozoites attenuated *in vivo* by concomitant administration of antimalarial drug (PfSPZ-CVac), and genetically attenuated early arresting sporozoites (PfSPZ-GA1) [92]. Although, feasibility of producing large number of live sporozoites is low, there are new studies that show promising results on *in vitro* sporozoite production [93].

This work aimed to create safe, genetically attenuated late arresting safe and efficient whole sporozoite vaccine models in rodent malaria model. Late arresting sporozoites induce superior protection [87] but occasional breakthrough infections especially at high sporozoite doses are major risk for a vaccine development. To increase safety, we aimed to produce a late arresting knockout parasite with enhanced safety by addition of a transgene as a gain of function modification during liver stages. Essential late liver stage genes LISP1 (PlasmoDB accession number: PY17X_1027000) and LISP2 (PlasmoDB accession number: PY17X_1004400) were knocked out individually to

arrest the parasites during liver stages progression. To enhance the attenuation, bacterial pore-forming cholesterol-dependent cytolysins (CDCs) Streptolysin O (AB050250.1) from *Streptococcus pyogenes* [94], and Listeriolysin O (M24199.1) from *Listeria monocytogenes* [95] were replaced with the open reading frames of LISP1 and LISP2 genes, respectively. These constructs were designed to express the highly immunogenic bacterial pore forming CDCs during late liver stages as suicidal toxins. CDCs can form large pores (~30 nm diameter) on the cellular membranes which will allow to disrupt the parasite and the parasitophorous vacuole membranes. In addition to the enhanced attenuation, the toxins might function as strong adjuvants due to their high immunogenicity, hence, enhancing the adaptive immune responses.

2 MATERIALS AND METHODS

2.1 Ethics Statement

All animal care and handling procedures used in this study were approved by the Experimental Animals Ethical Committee of Bezmialem Vakif University, Istanbul, Turkey.

2.2 Animals, Cells, Parasites, and Bacteria

6-8 weeks old female BALB/c and CD1 mice were used for all mice experiments. All mice experiments were conducted in the rodent facilities of Beykoz Institute of Life Sciences and Biotechnology, Bezmialem Vakif University.

Wild-type *Plasmodium yoelii* 17X-NL non-lethal parasite strain was used in transfection experiments to acquire transgenic *ΔLISP*1::SLO and *ΔLISP*2::LLO constructs; and *ΔLISP*1::mNeonGreen and *ΔLISP*2::mTurquoise2 strains. The previously described eGFP expressing WT-Like PyP230p(-) strain was used as WT in phenotypic experiments.

Anopheles stephensi mosquitoes were used for mosquito stages analysis and sporozoite production. Mosquito colonies were grown in 28°C and 75% humidity with 12 hours day/night cycle. Infected mosquitoes were kept at 21°C and 75% humidity.

Human tetraspanin CD81 receptor expressing hepatoma cell line HepG2 cells were used in in vitro liver stages experiments [96].

Escherichia coli XL10-Gold (Agilent, USA) commercial competent cells were used for all bacterial plasmid construction and molecular cloning experiments.

2.3 Buffers and Media

Parasite Transfection Culture Media:

- RPMI
- 20% heat-inactivated fetal calf serum
- Gentamycin (20 µg/mL)

Accudenz solution:

- 27.3 g Accudenz
- Dissolved in 100 mL of 5mM Tris-HCL
- pH=7.4

Saponin solution:

- 0.5 g saponin
- Dissolved in 100 mL of PBS

Freezing solution for frozen stocks:

- Alsever's solution
- 10% glycerol

Sucrose solution for mosquitoes:

- 80 gr Sucrose
- 50 mg PABA
- Dissolved in 1 L of double distilled water

Pyrimethamine solution:

- 70 mg pyrimethamine dissolved in 10 ml DMSO
- Dissolved in 1 L of double distilled water
- pH:4.5

Complete DMEM media:

- DMEM
- 1x Penicillin/Streptomycin
- 12% fetal bovine serum

Complete Ookinete media:

- 500ml RPMI
- 1 g of sodium bicarbonate
- 30 mg hypoxanthine
- 5 mL 100x Penicillin/Streptomycin solution
- 0.5 mL of a 1000x 0.1M Xanthurenic acid stock
- 20% fetal bovine serum
- pH:7.8

RBC Lysing Solution (10x):

- 16.04 g of ammonium chloride
- 1.68 g of Sodium bicarbonate
- 0.74 g of EDTA
- Dissolved in 100 mL of double distilled water

2.4 Blood Stage Analyses

2.4.1 Parasitemia calculation

Parasitemia of the infected mice were followed daily by Giemsa-stained tail (blood) thin smears. The tip of the mouse's tail was punctured to take a drop of blood on a glass slide. Blood on the slide was smeared by using the short edge of a clean glass slide. Smears were left to dry for a few minutes, then incubated for one minute in

methanol for methanol fixation. Following methanol fixation, slides are incubated in 10% Giemsa stain for 15 minutes. Slides were then left to dry.

Microscopic analysis was done under a bright field microscope with 63x oil-immersion objective. Average percent parasitemia was calculated by counting all parasitized and total erythrocytes in 20 fields. Parasitemia of the phenotypic analysis step was analyzed by flow cytometry. The drop of blood was diluted 1:300 in heparinized PBS solution, and prepared sample was analyzed by flow cytometry.

2.4.2 Parasite counting for infection of naïve mice.

Erythrocytes per µL were counted on a hemocytometer. Parasites per µL were calculated dependent on number of erythrocytes and percentage of parasitemia.

2.4.3 Genomic DNA extraction from parasites

Whole blood (~1 mL) of anesthetized mouse (with >1% parasitemia) was collected by cardiac puncture. 300-500 µL of infected blood sample was ran through a cellulose column by gravity flow to separate nucleated blood cells from the blood. Blood was diluted with PBS for to ease the flow. Flow through was collected in 15 mL centrifuge tube, centrifuged at 200x*g* for 10 minutes at RT. Supernatant was discarded. The pellet was resuspended with ice-cold saponin (0.05% w/v) and centrifuged at 300x*g* for 8 minutes at RT. Supernatant was discarded. Pellet was washed with 1 mL PBS, and purified parasites were resuspended with 200 µL PBS. Then DNA isolation was performed by DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions.

2.4.4 Preparation of parasite frozen stocks

Whole blood (~1 mL) of anesthetized mouse (with >1% parasitemia) was collected by cardiac puncture. 150 µL blood was diluted (1:3) with 300 µL freezing solution, transferred in to a cryovial and stored in -80 °C freezer.

2.5 Transgenic Parasites

2.5.1 Construction of transfection cassettes

Cytolysin coding sequences Streptolysin O (AB050250.1) from *Streptococcus pyogenes* and Listeriolysin O (M24199.1) from *Listeria monocytogenes* were codon optimized to exclude matching restriction enzyme recognition sites that with the

backbone plasmid. Sequences were synthesized commercially (Twist Bioscience, USA) with the addition of NotI and SpeI enzyme recognition sites on 5' and 3' ends, respectively. Construction of cassettes were performed on a backbone plasmid. Plasmids were linearized with restriction enzymes prior to transfection.

A total of four constructs were designed. *ΔLISP*1::SLO and *ΔLISP*1::mNeonGreen are LISP1 knockouts. *ΔLISP*1::SLO is primary vaccine candidate strain, and *ΔLISP*1::mNeonGreen is its control strain which expresses mNeonGreen instead of SLO. Similarly, *ΔLISP*2::LLO and *ΔLISP*2::mTurquoise2 are LISP2 knockouts. *ΔLISP*2::LLO is secondary vaccine candidate strain, and *ΔLISP*2::mTurquoise2 is its control strain which expresses mTurquoise2 instead of LLO.

All constructs had similar designs to ease the plasmid construction. pAA32, pAA33, and pAA35 plasmids were used as backbone plasmids. All plasmids contain a human dihydrofolate reductase (hDHFR) gene which confers resistance to pyrimethamine drug. All plasmids share the same design with the only differences are the fluorescent protein markers. *ΔLISP*1::SLO and *ΔLISP*1::mNeonGreen transfection cassettes were constructed on pAA33, *ΔLISP*2::LLO was constructed on pAA32, and *ΔLISP*2::mTurquoise2 was constructed on pAA35 backbone plasmids.

Homologous regions were amplified from *P. yoelii* 17X-NL genomic DNA by using primers listed in **Table 2.1**. All plasmid cloning steps were performed as described below:

PCR amplifications were performed by Q5 polymerase enzyme (NEB, USA). PCR was conducted at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 45 s, and 60°C for 2 min with the final extension step of 5 min at 60°C. Vectors and amplified PCR products were double digested by restriction enzymes (All restriction enzymes were from NEB, USA). PCR products were purified by using QIAquick Gel Extraction Kit (Qiagen, Germany), and vector backbone was purified from the agarose gel by using the same kit. Purified vector and left HR were ligated by using T4 ligase (NEB, USA). Chemically competent *E. coli* cells were transformed with the ligation mix. Frozen aliquots of competent bacteria were thawed on ice. Ligation mix was gently added on to the cells and incubated on ice for 30 minutes, followed by 30 seconds heat shock in a water bath at 42°C, then put back on ice for 2 minutes. 1 mL of room temperature LB media was added on cells and incubated at 37°C for 30 minutes. After incubation, transformed cells were inoculated into LB agar plates containing 100 µg/mL carbenicillin antibiotic, and incubated over-night at 37°C. Next

day 4-8 colonies were selected for inoculation into 5 mL LB media containing 100 µg/mL carbenicillin antibiotic. Cultures were incubated over-night in shaking incubator at 37°C, 220 rpm. Cultures were collected following day and used for plasmid isolation by using Qiagen QIAprep Spin Miniprep Kit (Qiagen, Germany). Isolated plasmids were double digested by matching restriction enzymes to verify the integration of right size by the help of a reference plasmid. Integration positive plasmids samples were sent to Sanger sequencing service (Eurofins Genomics, Germany) for verification. All enzymatic reactions, plasmid isolation, and purification were performed according to the manufacturer's instructions.

5' (left) homologous regions (HR) started with around 1 kb upstream of the start codon and included the first 111 base of the coding sequence (first 37 amino acids) to cover the amino acid signal. sequence for the protein export. Amplifications were performed with primers containing SacII and NotI restriction enzyme recognition sites. Amplified left HRs were cloned into backbone plasmids in between SacII and NotI restriction enzyme recognition sites. Following left HRs cloning reactions, fluorescent proteins that were used as liver stages markers were PCR amplified from corresponding base plasmids with primers containing NotI and SpeI restriction enzyme recognition sites. Commercially ordered SLO and LLO cytolysin sequences, and PCR amplified fluorescent protein sequences were double digested and cloned into respective backbone plasmids between NotI and SpeI restriction enzyme recognition sites. Subsequently, PyHsp90 3' UTR sequence was PCR amplified and cloned into all backbone plasmids between SpeI and BamHI restriction enzyme recognition sites. Finally, 3' (right) HR sequences were PCR amplified and cloned into respective backbone plasmids between HindIII and KpnI restriction enzyme recognition sites.

Table 2.1 : Primer sequences used in this study.

Name	Sequence (5'-3')
Cloning Primers	
yLISP1_ko1_sac2F	GGTCCGCGGAGGGTAAGTAGCTTAAATGCTAGTTAAAAATGT
yLISP1_+111_NotI_R	TGGGCGGCCGCTACTTCCTAGTAAGTTGTATTCTATAGTTTTCTT
yLISP1_ko3_hnd3F	GGTAAGCTTTACTAAAGCAATTAAATAGTAAACATATATAACT
yLISP1_ko4_kpn1R	GGTGGTACCTAAGTTATATTCTCATAAGTATATCTCAATCTA
yLISP2_ko1_sac2F	GGTCCGCGGGAGGTTCTCTTACACATGTGAATGTATATGTAT
yLISP2_+111_NotI_R	TGGGCGGCCGCATCCACCATTTTCTTTTTCTAAATAATCATATTT
yLISP2_ko3_hnd3F	GGTAAGCTTGAAAAACCTAAAAGAGGTAATACCCAAAAATATG
yLISP2_ko4_kpn1R	GGTGGTACCCTTCAAATTAAAACTACAAAATATCGTTAGATGA
mTurq2_NotI_F	TGGGCGGCCGCATGGTGAGCAAGGGCGAAGAACTATTTACTGGA
mTurq2_SpeI_R	TGGACTAGTTTACTTGTACAGCTCGTCCATGCCGAGAGTGAT
mNeonGreen_NotI_F	TGGGCGGCCGCATGGTAAGTAAGGGTGAAGAAGATAATATGGCT
mNeonGreen_SpeI_R	TGGACTAGTTTACTTATATAGTTCATCCATACCCATTACATC
yH901_3UTR_SpeI_F	TGGACTAGTGCAAATTAAATTTAGAGAGAATATATATATCAT
yH901_3UTR_BamHI_R	TGGGGATCCACAACATATTATTTTTACACAGACCTTACTCTG
Diagnostic Test Primers	
yLisp1_5testF	GAGAAAAGAAAATTGAGATCATTTTTCGTT
yLisp1 3testR	CTTTAAATTTGTATGGGAAACAAAATGTGC
yLisp1 5orfF	TGTTGTATTTATTGGTGAATTAACAGGACT
yLisp1 3orfR	CCCGCATTAAATTCAAAACGTAATACTGAC
SLO_orf3R	TATTGTGCTCTGCAGCATCTCCTCCT
yLisp2 5testF	TCACATCTTTTAAGTGTTCATTAGCATATA
bLISP2_3testR	TACTAAGTGGGACATCCACGGATGCACTAA
yLisp2 5orfF	TTGGATGAAGAAAGGAAAAGCATGTTCGAA
yLisp2 3orfR	TTCTTAAATGCACAATGTAAGAACATATCC
LLO_orf3R	GCCGTCGATGATTTGAACTTCATC
nw_mTurq2_5testR	AACTAGAATAGGTACTACTCCAGTAAATAGTTC
mNeonGreen test R	GAACCAAAAATATGTAATTCATGTGTTGCTGGA
DHFRtestF-17	GTGTTCTTTCTGATGTTCAAGAAGAAAAAGGTA

*Restriction enzyme recognition sequences are underlined.

2.5.2 Transfection of parasites

Frozen stocks of sporozoite-induced (G0) *P. yoelii* 17X-NL parasites were thawed at room temperature (RT), then intraperitoneally injected into two donor mice. Parasitemia levels of donor mice were followed by Giemsa-stained thin blood smears from tail puncture. Blood from donor mice was collected by cardiac puncture when parasitemia levels were over 5%. Collected blood was gently added over 10 mL parasite culture media and centrifuged at 200x*g* for 10 minutes at RT. Supernatant was aspirated, and 30 mL parasite culture media was gently added on to pelleted blood then collected back and added gently over 100 mL media in 500 mL Erlenmeyer's flask. The last step was repeated with 20 mL culture media to collect the remaining blood.

Prepared parasite culture was incubated for 12-16 hours at 37°C, 5% CO_2, 10% O_2 with shaking at 75 RPM.

DNA constructs were linearized with SacII and KpnI enzyme and purified by isopropanol purification. Briefly, DNA volume was completed to 500 µL, 100 µL of 3 M NaOAc and 400 µL isopropanol was added. Mix was vortexed and centrifuged at maximum speed for 20 minutes at RT. Supernatant was discarded and pellet was washed with 500 µL 70% ethanol, centrifuged for 5 minutes and supernatant was discarded. Pellet was left to dry for a few minutes, then resuspended with 100 µL molecular grade water. Linearized high concentrations of construct DNAs were checked on agarose gel electrophoresis for undigested plasmid DNA.

Following incubation, the quality of the culture was analyzed by microscopic examination of Giemsa-stained smear of the culture. Parasite culture was loaded into 50 mL centrifuge tubes (35 mL each), then 10 mL of 60% Accudenz solution was carefully added with the help of a serological pipette and automatic pipettor, from the bottom of the tube with slowest speed, creating a separated phase at the bottom of the tube. Tubes were balanced on a scale with addition from remaining parasite culture, and centrifuged at 200x*g* for 25 minutes at RT. After centrifuging, a thin gray-brown layer forms as a circle between the two phases in each tube. This layer holds schizonts, therefore collected by the help of a glass Pasteur pipette into two clean 50 mL tubes. The volume of the collection on the tubes was completed to 30 mL with the media on the upper phases of the tubes. Collected schizont tubes were centrifuged at 300x*g* for 10 minutes at RT. Supernatant was discarded and pelleted schizonts were resuspended in 1 mL fresh culture media and aliquoted into required number of tubes for transfection. Result of the schizont isolation was analyzed by microscopic examination of Giemsa-stained smear of a small drop of the resuspended pellet.

10 µg linearized DNA constructs (up to 10 µL) were mixed with 100 µL transfection solution of Lonza Human T-cell Nucleofactor Kit (Lonza, Switzerland) in a microcentrifuge tube at RT. Aliquoted schizonts were spun down briefly and supernatant was discarded. Pellet was resuspended with DNA containing transfection solution. This schizont mix was collected with a small Pasteur pipette and transferred into a transfection cuvette. Cuvette was loaded into Nucleofactor (Lonza, Switzerland) electroporation device and electroporation was performed by using built-in U-33 setting. Right after the completion of the electroporation, 50 µL culture media was

added to the electroporation mix. The mix was collected by with a small Pasteur pipette and was transferred into a microcentrifuge tube to easily load into an insulin syringe. Naïve recipient CD1 mice were intravenously (IV) injected with the transfection mixes swiftly. Next day, Giemsa-stained tail blood smear was checked for the viability of the parasites. And oral pyrimethamine treatment started 24 hours after the transfection. At least 2 independent transfections were performed for each construct.

2.5.3 Selection and verification of positive transfectants

Positive transfectants were imaged by fluorescence microscopy from a drop of tail blood for visual verification of fluorescence markers. Fluorescent positive samples (parental cells) were FACS-sorted to acquire isogenic clones. Mice were bled by cardiac puncture for taking frozen stocks and (genomic DNA) gDNA extraction from parental cells.

2.5.4 Acquisition of isogenic clones

Isogenic clone acquisition from positive transfectant (parental cells) samples was performed by fluorescent-activated cell sorter (FACS) device (S3e - Biorad, Germany). 5 µL tail blood was diluted (1:200) with Alsever's Solution (Sigma Aldrich, USA) and used in sorting. Sorting was performed according to the fluorescent protein marker of each sample. 100 parasite cells were sorted in 200 µL complete RPMI medium. 50 parasite cells were injected into naïve mice. Mice were orally treated with pyrimethamine. Parasitemia of the mice were followed by daily Giemsa-stained blood smears. Positive mice were bled by cardiac puncture for taking frozen stocks and gDNA extraction from isogenic clones.

2.5.5 Genomic DNA verification of positive clones.

Parental cells and isogenic clones were analyzed by diagnostic PCR. gDNAs were used as template and PCR amplifications were performed by Bioline MyTaqRed 2x Master mix (Meridian Bioscience, USA). PCR was conducted at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 45 s, and 60°C for 6 min with the final extension step of 10 min at 60°C, by using WT-, 5' integration-, and 3' integration-specific primers (**Table 2.1**).

2.6 Phenotypic Analyses

Phenotypic analyses were performed for isogenic clones of each strain by using WT-like *PyP230p(-)* strain as control.

2.6.1 Blood stage experiments

2.6.1.1 Blood stage growth analysis

20,000 parasitized erythrocytes of each strain were IV injected into naïve CD1 mice (4 mice for each strain). Parasitemia was recorded every two days by flow cytometry.

2.6.1.2 Male gamete exflagellation

$1x10^6$ parasitized erythrocytes of each strain were IV injected into naïve CD1 mice (3 mice for each strain). Male gamete exflagellation centers were counted under hemocytometer. 5 µL blood was diluted (1:20) with RPMI containing 0.1 mM xanthurenic acid. 10 µL of the mix was loaded on a hemocytometer and exflagellation centers per µL of blood were counted after incubation at RT for 10 minutes.

2.6.2 Mosquito stage experiments

2.6.2.1 Parasite transmission to mosquitoes

Two mosquito cages for each strain were infected by blood meal (BM) from infected mice. Mice that were used for exflagellation counting was used for mosquito feeding for transmission of the parasites to mosquitoes.

2.6.2.2 Ookinete counting

Mice that were used for exflagellation counting were also used for ookinete counting. All mice bled by cardiac puncture and 200 µL blood from each (three mice of the same group) were pooled in 5.4 mL complete ookinete medium, and quickly ran through cellulose column, similar to gDNA extraction. Flow through was collected in a sterile flask, gently mixed, and incubated at 21°C for 20 hours. Culture was transferred into 50 mL centrifuge tube and put on ice. After cooling down, the culture was centrifuged at 300x*g*, for 5 minutes (with 6 acceleration, 3 deceleration) at 4°C, and supernatant was discarded. The Pellet was gently resuspended with 50 mL 1x erythrocyte lysing buffer and incubated on ice for 20 minutes. Following lysis, cells were pelleted at 300x*g*, for 5 minutes (with 6 acceleration, 3 deceleration) at 4°C, and supernatant was discarded. Pellet was washed with 50 mL ice-cold PBS, and centrifuged 300x*g*, for 10

minutes (with 6 acceleration, 3 deceleration) at 4°C. Wash step was repeated, and cells were resuspended with 1 mL PBS. Isolated ookinete cells were counted on a hemocytometer.

2.6.3 Oocyst sporozoites, and salivary glands sporozoites counting

Mosquitoes were collected (>30 from each cage), and midguts were dissected out at day 10 pmi. This time, midguts were grounded and sporozoites from midgut oocysts were extracted and counted on a hemocytometer.

Mosquitoes from the same cages were collected (>30 from each cage), and salivary glands were dissected out at day 14 pmi. Salivary glands were dissected out to extract sporozoites. Sporozoites were counted on a hemocytometer.

2.7 *In Vitro* Liver Stages Infection

2.7.1 Sporozoite infection

HepG2 cells were grown at 37°C with 5%CO_2 in complete DMEM media. $8x10^4$ cells were seeded on 24 well plates on glass coverslips. Next day, cells were infected with 30 $x10^4$ sporozoites of each strain that were extracted from salivary glands of mosquitoes in complete DMEM media containing 25 µL amphotericin B. Plates were centrifuged at 300x*g* for 5 minutes at RT, then incubated at 37°C, 5% CO_2. 2 hours later, cells were washed with PBS and incubated in complete DMEM media containing 2.5 µL amphotericin B at 37°C, 5% CO_2.

2.7.2 Imaging

Cells were fixed with 4% paraformaldehyde at 30 and 42 hours after sporozoite infection, respectively. After fixation, cells were washed with PBS 2 times. Glass coverslips were taken out and put on coverslips with mounting media containing DAPI. Slides were imaged with Leica SP8 confocal laser scanning microscope (Leica, Germany).

2.8 Immunization and Challenge Experiments

All four transgenic were fed to mosquitoes as described above. 14 days later, sporozoites were extracted from mosquitoes. 3 doses for each mice group ($5x10^3$, $20x10^3$, and $80x10^3$ sporozoites) were selected. 3 groups BALB/c mice for each

vaccine candidate were prepared to contain 8 mice each and control groups were prepared to contain 4 mice each. Sporozoite doses were prepared in RPMI media.

Table 2.2 : Immunization groups, doses, and intervals

Strains	Doses (No. of sporozoites)	No. of Mice	No. of Injections	Interval (days)
*ΔLISP*1::SLO	5,000	8	3	14
*ΔLISP*1::SLO	20,000	8	3	14
*ΔLISP*1::SLO	80,000	8	3	14
*ΔLISP*2::LLO	5,000	8	3	14
*ΔLISP*2::LLO	20,000	8	3	14
*ΔLISP*2::LLO	80,000	8	3	14
*ΔLISP*1::mNeonGreen	5,000	4	1	N/A
*ΔLISP*1::mNeonGreen	20,000	4	1	N/A
*ΔLISP*1::mNeonGreen	80,000	4	1	N/A
*ΔLISP*2::mTurquoise2	5,000	4	1	N/A
*ΔLISP*2::mTurquoise2	20,000	4	1	N/A
*ΔLISP*2::mTurquoise2	80,000	4	1	N/A

Mice were placed under heat lamp to promote peripheral vasodilation, and pre-prepared sporozoite doses were injected into the tail veins. Parasitemia of all mice were followed daily by Giemsa-stained tail blood smears for 15 days. 3 doses of immunization were performed 3 times at 15 days intervals.

One month after the last immunization, immunized mice were challenged with WT sporozoites. WT *P. yoelii* 17N-XL parasites were fed to the mosquitoes. 14 days later sporozoites were extracted and challenge doses were prepared in RPMI media. WT sporozoites were injected as above, and parasitemia of all mice were followed daily by Giemsa-stained tail blood smears for 15 days.

2.9 Statistical Analysis

Statistically significant differences between the median values of three genotypes were evaluated by analysis of variance (ANOVA) method in all experiments. GraphPad Prism 6 software was used for all analysis. *P*-values of <0.05 was considered statistically significant.

3 RESULTS

3.1 Constructed Plasmids

All constructed plasmids were verified by restriction digestions and sequencing results were aligned with original sequences for verification by using Benchling platform (**Figure 3.1**).

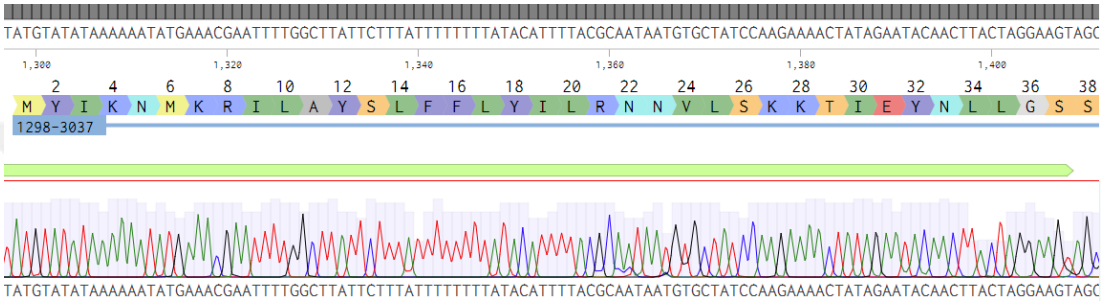


Figure 3.1 : Sanger sequencing result of LISP1 signal peptide coding sequence.

3.2 Genetic Modification of Parasites

Schizonts were purified from transfection culture by collecting the schizont ring (**Figure 3.2**). The results of the purifications were analyzed after transfection.

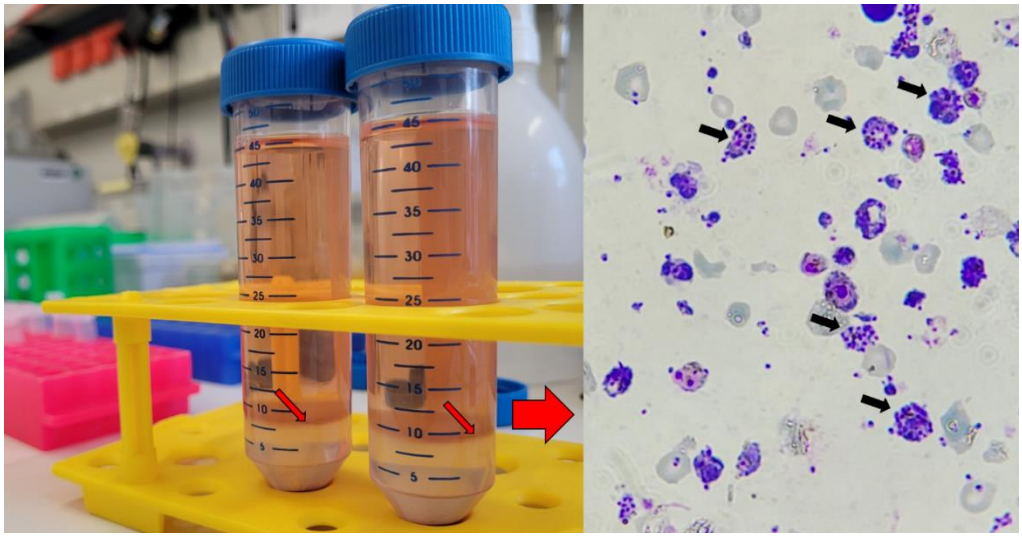


Figure 3.2: Purification of schizonts after transfection culture. Red arrows on the left shows seperated schizont ring. Giemsa staining of collected schizonts (black arrows).

Although functions are not fully understood, LISP1 and LISP2 are two of the most critical genes for liver stages (LS). They are both specifically expressed during mid-late liver stages. Both genes contain a signal peptide sequence to be exported to PVM. LISP1 is shown to be involved in breakdown of the PVM [32]. *ΔLISP*1 parasites were not defective in LS or blood stages growth but maturated merozoites were trapped inside PVM. Therefore, it is not fully attenuated and can lead to breakthrough infections [32]. Bacterial Streptolysin O (SLO) pore forming toxin is important to escape phagocytosis. It is shown to be active at neutral pH and purified form was used in selective membrane permeabilization in *Plasmodium* studies [97]. Our first strategy was to combine LISP1 deficiency with SLO's pore forming ability that would lead to complete attenuation (*ΔLISP*1::SLO). Thus, we kept the signal peptide sequence, and replaced the remaining LISP1 coding sequence with SLO coding sequence.

To understand the effect of SLO, we also created a control strain where mNeonGreen fluorescent protein was used instead of SLO (*ΔLISP*1::mNeonGreen). This allowed us to evaluate the phenotype of LISP1 knockout without SLO. This strain would also be useful to verify the activity of the promoter and the export signal.

LISP2 was shown to be critical for merozoite formation in *P. berghei* [33]. *ΔLISP*2 parasites produce defective merozoites. The majority of the knockout parasites had amorphous nuclei, suggesting impaired nuclear divisions. This protein was shown to be exported to vacuolar space, PVM, also host cytosol and nucleus. It is exported in the same vesicles together with LISP1. Similar to LISP1, attenuation is not complete and there were breakthrough infections [33]. As an alternative to LISP1 and SLO design, we replaced Listeriolysin O with the coding sequence of LISP2, keeping the export signal intact (*ΔLISP*2::LLO). LLO is another bacterial pore forming protein which is shown to be less active in neutral pH [98]. Nevertheless, its activity was shown to be comparable to SLO in neutral pH [99].

Using the same strategy, we created a control strain where mTurquoise2 was used instead of LLO, to understand the effect of the toxin and to use as reporter gene for both promoter and export signal (*ΔLISP*2::mTurquoise2). Details of all constructs were shown in **Figure 3.3**.

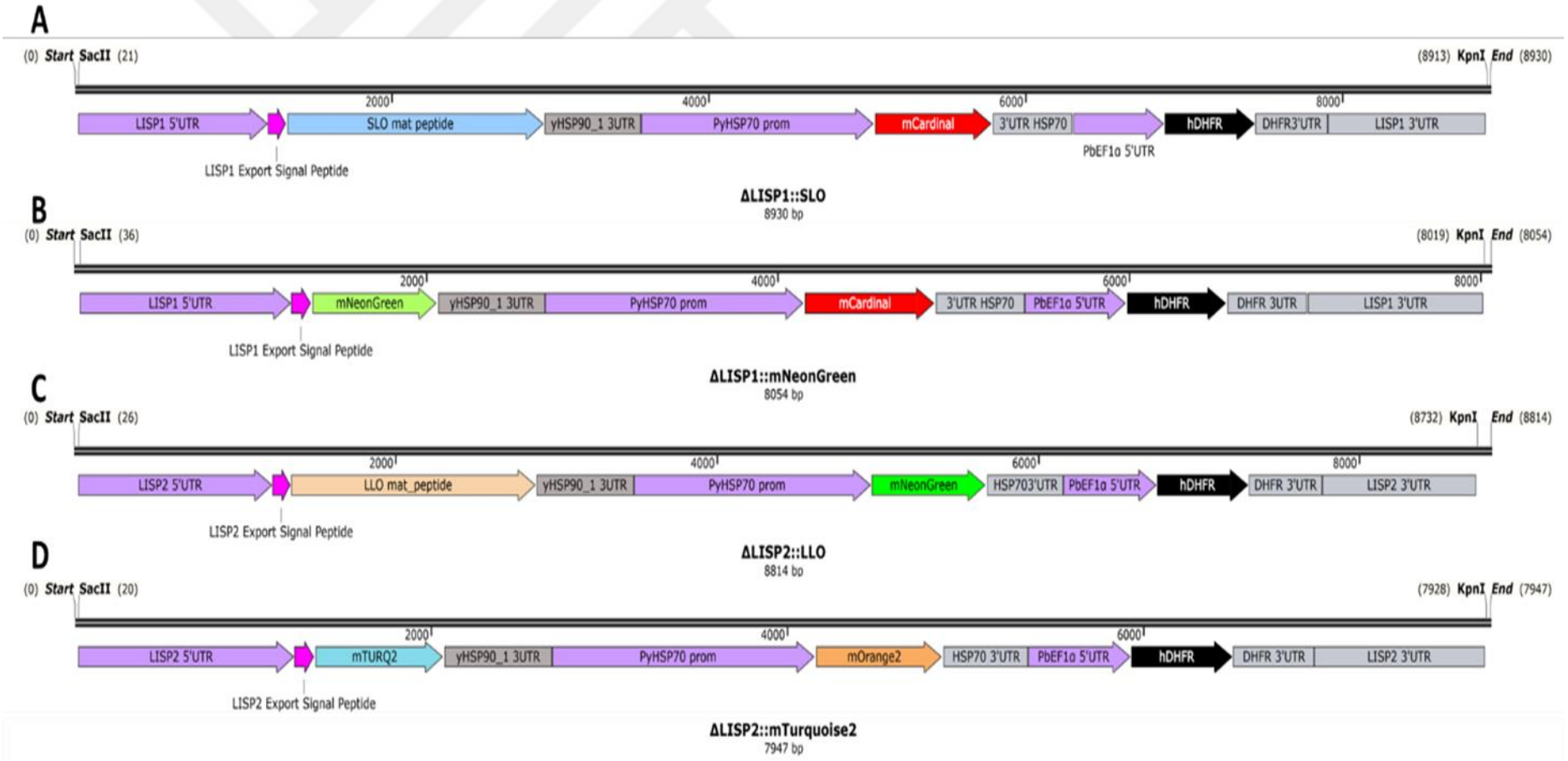


Figure 3.3 : Designs of transfection constructs.

3.3 Fluorescence Verifications

All samples were verified with positive fluorescence under confocal microscope. Fluorescent markers under the strong constitutive PyHSP70-1 promoter were added to visualize all stages and enabled live microscopy. Liver stages fluorescence of control strains would not be seen as expected due to the stage specific expression of LISP1 and LISP2 promoters.

3.4 Isogenic Clone Acquisition

Isogenic clones were acquired by FACS sorting and verified by diagnostic PCR reactions. As seen in **Figure 3.4**, 5' and 3' integrations were successful and there was no wild-type contamination in any of the samples.

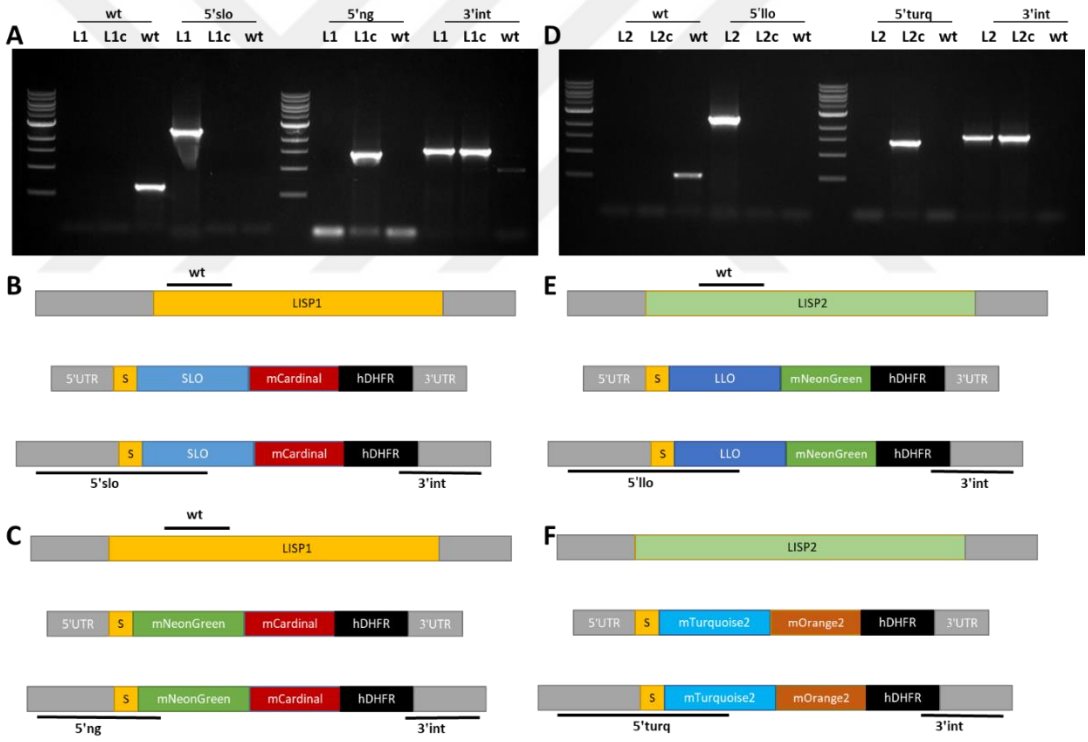


Figure 3.4 : Diagnostic PCR results and and PCR strategy of all constructs. (A,B,C) *ΔLISP*1::SLO and *ΔLISP*1::mNeongreen. (D,E,F) *ΔLISP*2::LLO and *ΔLISP*2::mTurquoise2.

3.5 Phenotypic Analyses

3.5.1 Blood stages growth

Blood stages growth were followed by flow cytometry. PyP230p(-) strain was used as control. This is a WT-like eGFP expressing strain which has identical growth characteristics as WT. There were no significant differences in any of the transgenic strains compared WT-like strain. Loss of liver stages specific genes, and bacterial toxin expressions under liver specific promoters had no effect on blood stages growth (**Figure 3.5**).

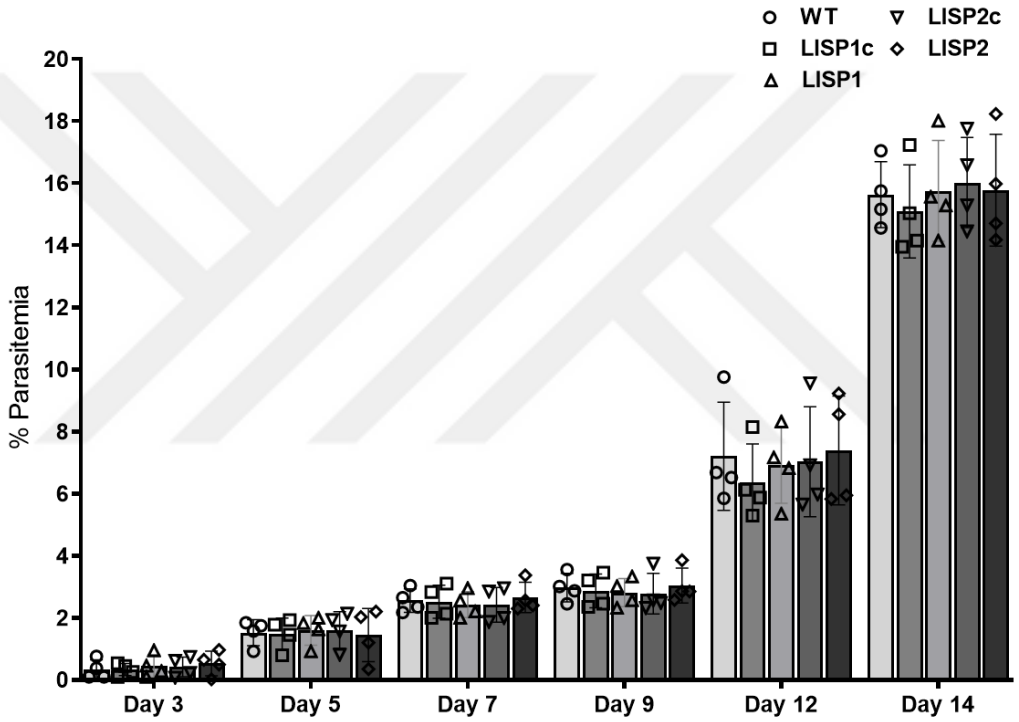


Figure 3.5 : Blood stages growth analysis of all strains. No significant growth differences were obsevered between any of the recombinant strains. WT:*PyP230p(-),* LISP1: *ΔLISP1*::SLO, LISP1c: *ΔLISP1*::mNeongreen, LISP2: *ΔLISP2*::LLO and LISP2c: *ΔLISP2*::mTurquoise2. The mean values for all parasite strains were analyzed with the two-way ANOVA and statistical significance was set at a $P<0.05$.

3.5.2 Mosquito stages growth

In parallel with the blood stages, modified parasites produced comparable numbers of exflagellation (**Figure 3.6**), ookinete (**Figure 3.7**), oocyst sporozoites (**Figure 3.8**), and salivary gland sporozoites (**Figure 3.9**). Knockout of liver stages specific genes and liver stages specific bacterial toxin expressions had no visible effect on sporogony.

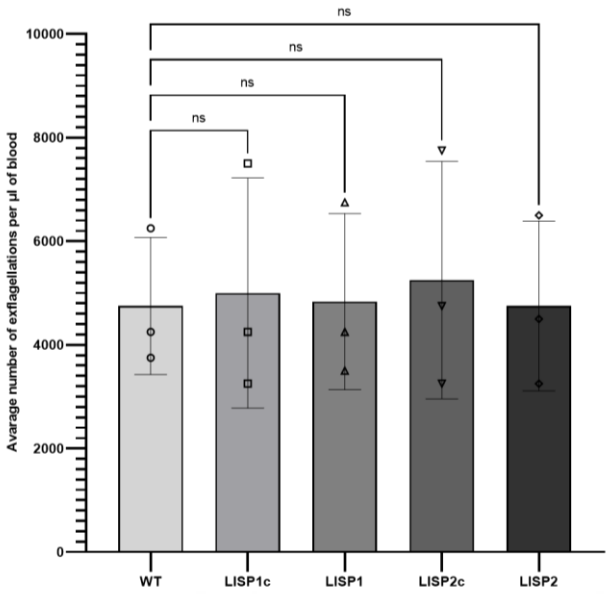


Figure 3.6 : Avarage number of exflagellations per µL of blood. No significant differences in exflagellation events were obsevered between any of the recombinant strains. WT:*PyP230p(-),* LISP1: *ΔLISP1*::SLO, LISP1c: *ΔLISP1*::mNeongreen, LISP2: *ΔLISP2*::LLO and LISP2c: *ΔLISP2*::mTurquoise2. The mean values for all parasite strains were analyzed with the one-way ANOVA and statistical significance was set at a $P<0.05$.

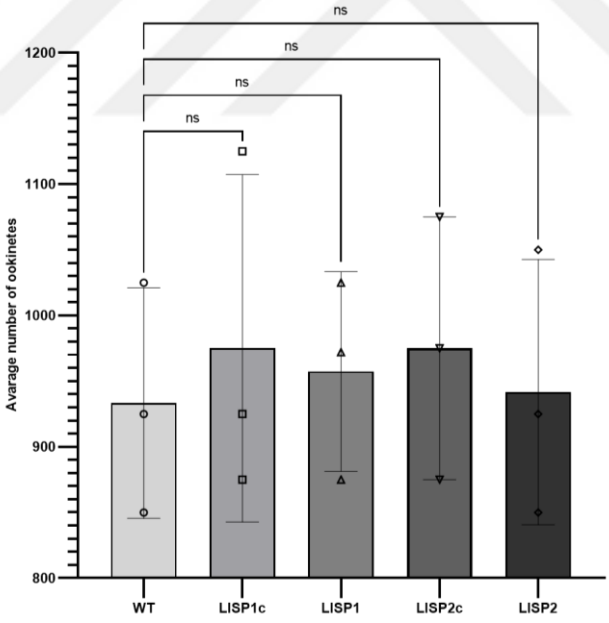


Figure 3.7 : Avarage number of ookinete per mL of blood. No significant differences in exflagellation events were obsevered between any of the recombinant strains. WT:*PyP230p(-),* LISP1: *ΔLISP1*::SLO, LISP1c: *ΔLISP1*::mNeongreen, LISP2: *ΔLISP2*::LLO and LISP2c: *ΔLISP2*::mTurquoise2. The mean values for all parasite strains were analyzed with the one-way ANOVA and statistical significance was set at a $P<0.05$.

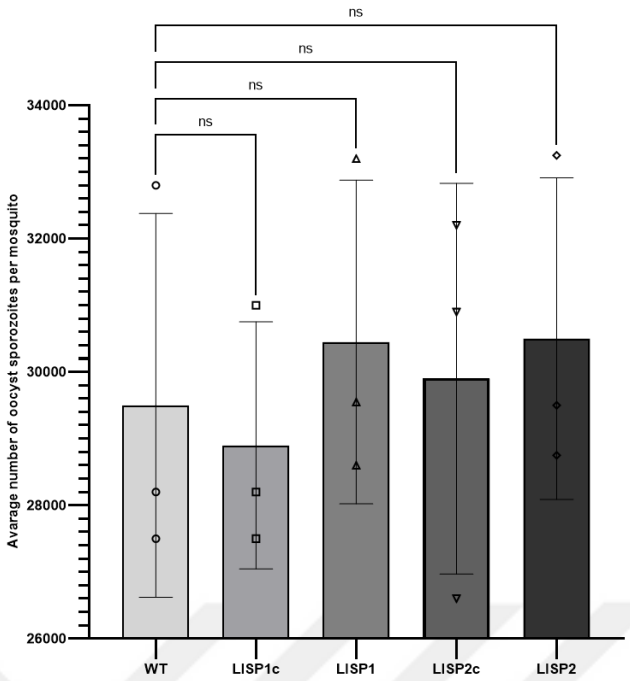


Figure 3.8 : Avarage number of oocyst sporozoites per mosquito. No significant differences in exflagellation events were obsevered between any of the recombinant strains. WT:*PyP230p(-)*, LISP1: *ΔLISP1*::SLO, LISP1c: *ΔLISP1*::mNeongreen, LISP2: *ΔLISP2*::LLO and LISP2c: *ΔLISP2*::mTurquoise2. The mean values for all parasite strains were analyzed with the one-way ANOVA and statistical significance was set at a $P<0.05$.

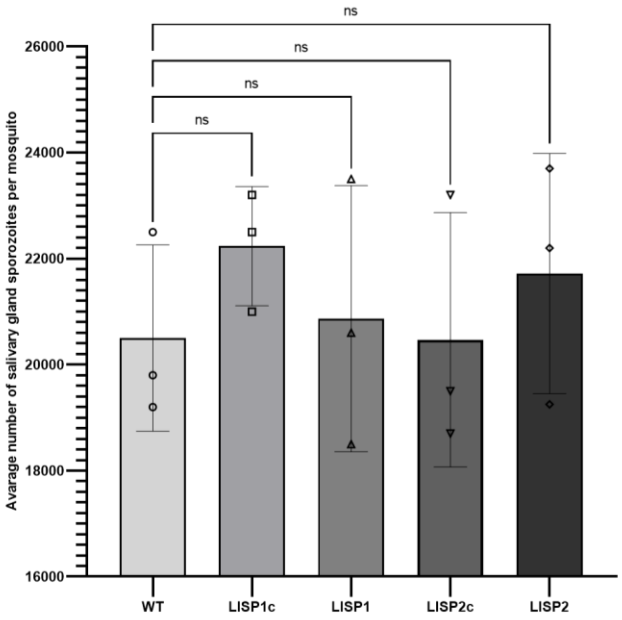


Figure 3.9 : Avarage number of salivary gland sporozoites per mosquito. No significant differences in exflagellation events were obsevered between any of the recombinant strains. WT:*PyP230p(-)*, LISP1: *ΔLISP1*::SLO, LISP1c: *ΔLISP1*::mNeongreen, LISP2: *ΔLISP2*::LLO and LISP2c: *ΔLISP2*::mTurquoise2. The mean values for all parasite strains were analyzed with the one-way ANOVA and statistical significance was set at a $P<0.05$.

3.5.3 *In vitro* liver stages infections

We further analyzed liver stages growth in vitro by infecting CD81 expressing HepG2 cells. There were no visible differences in infectivity between wt-like *PyP230p (-)* and transgenic strains. *ΔLISP*2::mTurquoise2 infections could not be observed. One possible explanation for that is the paraformaldehyde fixation bleached the fluorescent protein, due to the high acid sensitivity of mOrange2 fluorescent protein. Therefore, quantitative analysis could not be performed. Confocal imaging of remaining strains was performed between at 30th and 42nd hours. Liver stage cells of *ΔLISP*1::SLO strain could not be detected after 36th hours post infection. *ΔLISP*1::mNeonGreen showed high levels of PVM localized mNeonGreen expression at around 36th hour which proves the activity of LISP1 signal peptide sequence (**Figure 3.10**). However, we were able to observe *ΔLISP*2::LLO strain between at 24th and 42nd. PVM lysis were easily detected after 36th hour. But there were still intact cells remaining at 42nd hour (**Figure 3.11**).

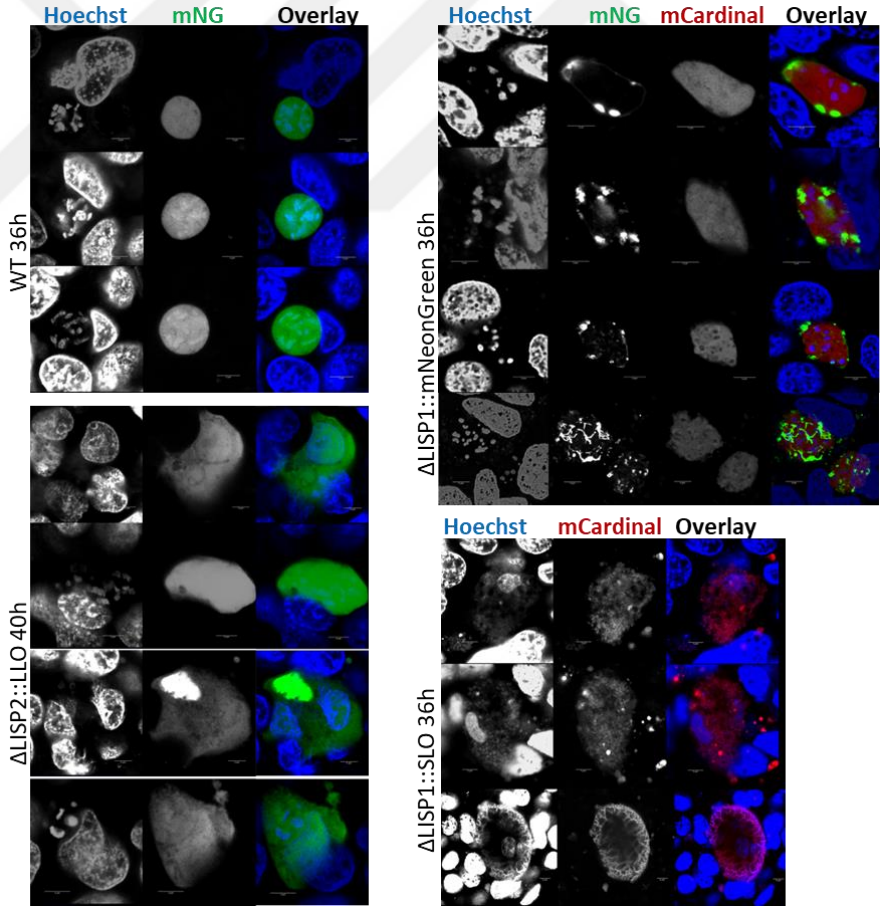


Figure 3.10 : Confocal microscopy imaging of *in vitro* liver stages infections of wt-like *PyP230p (-)* and recombinant strains

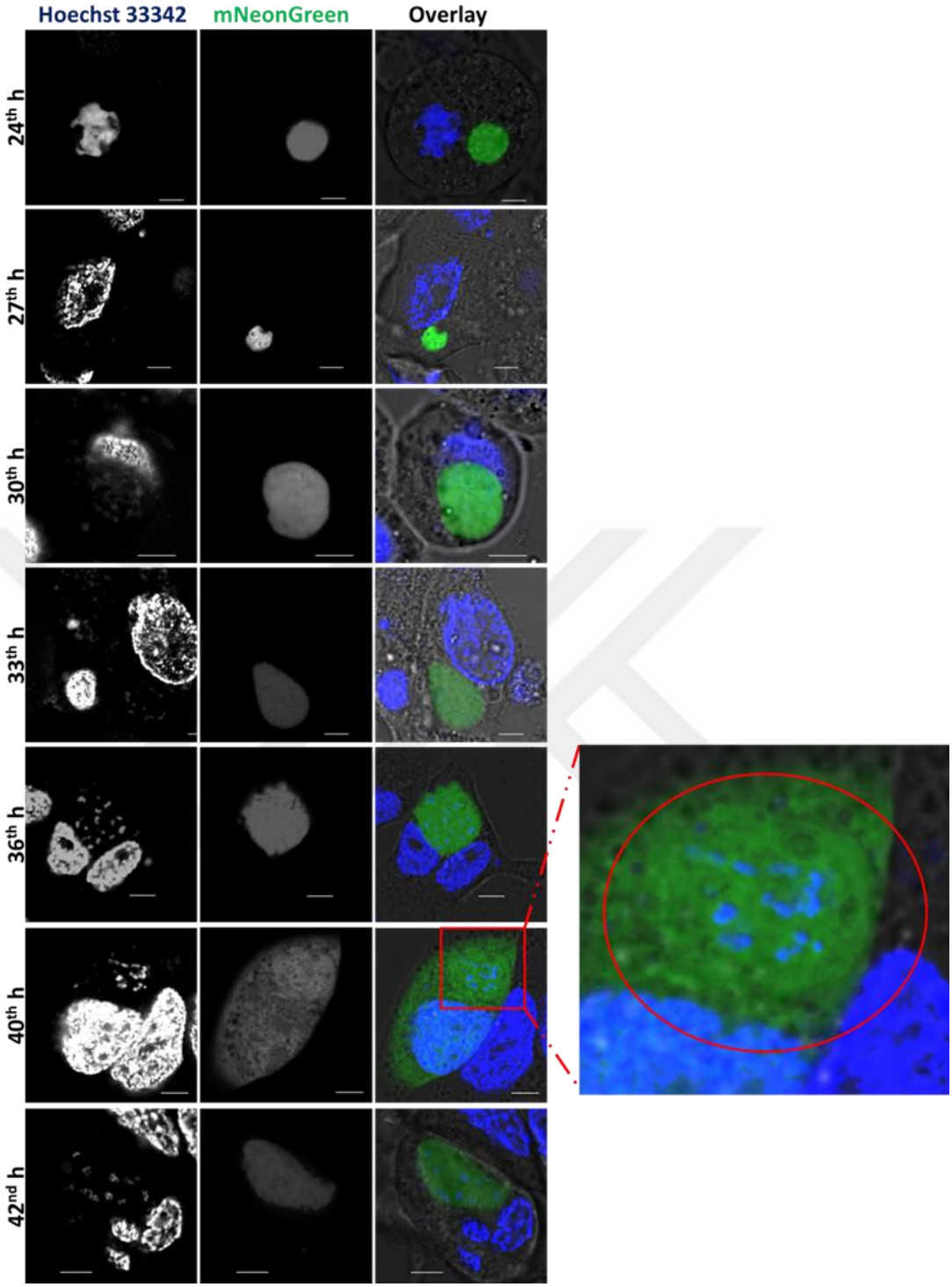


Figure 3.11 : Confocal microscopy imaging of *ΔLISP2*::LLO strain in vitro liver stages infections between 24 and 42 hours post infection. The lysed PVM of the parasite can be followed by the fluorescent protein leak to the host cell cytosol at 40th hour post infection.

3.6 Attenuation Profiles

All mice groups were injected with immunization doses that were shown in **Table 3.1**. *ΔLISP*1::SLO strain showed complete attenuation even at highest doses, while control strain *ΔLISP*1::mNeonGreen showed limited attenuation. This result proves the efficiency of SLO. On the other hand, LISP2 knockout, *ΔLISP*2::mTurquoise2, showed slightly stronger attenuation compared to *ΔLISP*1::mNeonGreen. *ΔLISP*2::LLO strain was completely attenuated against the lowest dose, but LLO was not efficient as SLO at stopping the breakthrough infections when injected with higher doses.

Table 3.1 : Attenuation profile of all strains.

Strains	Doses (No. of sporozoites)	No. of Mice	No. of Injections	Interval (days)	B.S growth/total injected
*ΔLISP*1::SLO	5,000	8	3	14	0/8
*ΔLISP*1::SLO	20,000	8	3	14	0/8
*ΔLISP*1::SLO	80,000	8	3	14	0/8
*ΔLISP*2::LLO	5,000	8	3	14	0/8
*ΔLISP*2::LLO	20,000	8	3	14	1/8
*ΔLISP*2::LLO	80,000	8	3	14	5/8
*ΔLISP*1::mNeonGreen	5,000	4	1	N/A	2/4
*ΔLISP*1::mNeonGreen	20,000	4	1	N/A	3/4
*ΔLISP*1::mNeonGreen	80,000	4	1	N/A	4/4
*ΔLISP*2::mTurquoise2	5,000	4	1	N/A	1/4
*ΔLISP*2::mTurquoise2	20,000	4	1	N/A	2/4
*ΔLISP*2::mTurquoise2	80,000	4	1	N/A	3/4

To further verify the strong attenuation profile of *ΔLISP*1::SLO, we injected 250,000 and 500,000 thousands sporozoite doses in 2 mice. No blood stage parasitemia was observed until day 14 post injection.

3.7 Immunization

*ΔLISP*1::SLO- and *ΔLISP*2::LLO-immunized mice we challenged with wild-type *P. yoelii* 17X-NL strain sporozoites 30 days after last immunization dose. *ΔLISP*1::SLO-immunized mice were divided into 2 groups for high (10,000) and low (500) dose sporozoite challenges.

6 mice with the breakthrough infections on *ΔLISP*2::LLO-immunized mice groups were excluded from challenge experiments. Also, 1 mice died after the third immunization dose injection. Therefore, we did not divide *ΔLISP*2::LLO-immunized

mice group and challenged this group with only high number (10,000) of wild-type sporozoites.

Table 3.2 : Doses and results of WT challenge experiment.

Strain, Dose, (3 doses, 14days intervals)	Challenge Dose / Days after last boost	No. of Protected/Challenged	Blood Stage Patency
*ΔLISP*1::SLO			
5,000	500/30	0/4	5 days
20,000	500/30	4/4	None
80,000	500/30	4/4	None
5,000	10,000/30	0/4	5 days
20,000	10,000/30	2/4	5 days
80,000	10,000/30	3/4	5 days
*ΔLISP*2::LLO			
5,000	10,000/30	8/8	None
20,000	10,000/30	6/6	None
80,000	10,000/30	3/3	None

Table 3.2 shows the protection levels of main vaccine strains, *ΔLISP*1::SLO and *ΔLISP*2::LLO. Interestingly, highly attenuated *ΔLISP*1::SLO strain did not perform well for protection. 5,000 sporozoite-immunized mice conferred no protection against even low doses of wild-type sporozoites. 20,000 and 80,000 sporozoite-immunized mice conferred %100 protection against low doses, but %50 and %75 protection against high doses of wild-type sporozoites, respectively.

In marked contrast, *ΔLISP*2::LLO-immunized mice groups showed %100 protection against high doses of wild-type sporozoites.

4 DISCUSSION

Co-evolution of *Plasmodium* species with wide variety of vertebrate hosts for millions of years resulted in various immune evasion strategies developed by the parasite [100]. Therefore, development of an efficient vaccine has many challenges. Variant surface antigens (VSAs) are the main virulence factors some of which are var, rif, and stevor gene families which encode around 60, 200, and 30 highly polymorphic genes, respectively [58]. Sub-unit vaccines against major surface antigens have proven less efficient than whole cell vaccine counterparts. High-efficacy protection against malaria seems only possible by strong induction of all immune mechanisms together.

Protective immunity against *Plasmodium* is associated with priming of $CD8^+$ T-cells directly by hepatocytes or DCs [101] through MHC-I. Erythrocytes' lack of MHC-I means that they cannot present antigens to CD8+ T-cells, which means immune responses against blood stages lacks and important component. New tools such as viral vectoring or mRNA vaccines might be a solution for efficient blood stage vaccines. Even then, the amount of parasitic load, speed of merozoite infection and evasion mechanisms of the parasite, makes vaccine development against this stage challenging.

Designing vaccines against pre-erythrocytic stages are also challenging but more promising than blood stages. The inoculum size by the mosquito bite is exceptionally low (10-100 sporozoites), therefore it is considered as the bottleneck of asexual stages. But prevention of sporozoites reaching the liver is challenging, and even one sporozoite is enough for transition to blood stages infection. So far, most efficient vaccine candidates have proven to be whole sporozoite vaccines that can induce sterile protection. First example of whole sporozoite vaccines is radiation attenuated sporozoites (RAS) which are produced by inducing random DNA damage by X-irradiation [73, 74]. Success of this method lead to chemically attenuated sporozoites (CAS) studies, and, with the advancement in molecular biology, genetically attenuated sporozoites (GAS).

Parasites can inhibit apoptosis of the host cell to survive, they can also trigger its own apoptosis when they are "unfit" to survive [102]. This is one way that $CD11c^+$ DCs in spleen, liver, and liver-draining lymph nodes have been shown to prime $CD8^+$ T-cells [57]. Despite the fact that DCs can prime $CD8^+$ T-cells, immune responses are not enough due to the minimal parasitic load in pre-erythrocytic stages. This might be a

reason that RAS vaccines were only successful when administered at multiple high doses [73]. The timing of the liver stages arrest is critical to induce efficient immune responses. The reason for this is the antigenic repertoire of parasite at the time of developmental arrest. Therefore, genetic attenuation can be superior to radioactive and chemical attenuation methods in inducing strong immune responses. But designing the precise timing is important. While early arrested parasites cannot induce enough immune responses, late arrested parasites lead to occasional breakthrough infections [81]. Finding the balance is the key to safe and efficient vaccine.

Availability of murine malaria model allows the quick development of genetically modified parasites as GAP candidates. Many genes of the commonly used species, of *P. yoelii* and *P. berghei*, have high homology with human parasite, *P. falciparum*, which allows the studies in these species applicable to human malaria. Although these species have been shown to infect human hepatocytes, they have high species specificity for erythrocytes, therefore it is also safer to work with murine model before transition into human malaria.

Two alternative designs in this thesis were aimed at mid to late liver stages arrest. Some of the considered candidates were LISP1, LISP2, Plasmei2, and some of the up-regulated in infective sporozoites (UIS) group genes.

UIS group of genes were first identified by the high expression profiles during mature sporozoite forms. Which suggests an involvement in hepatocyte infection. There are 30 genes in UIS group [82]. Knockout of eukaryotic initiation factor 2α (eIF2α) kinase (IK2/UIS1) and eIF2α-P protein phosphatase 1 (PP1/UIS2) led to partial early attenuations [81]. UIS3 and UIS4 were two of the early arrest UIS genes. UIS9 is a Puf-family RNA-binding protein that controls sporozoite conversion to liver stages [103]. UIS10 is phospholipase that is involved in PVM breakdown and knockout leads to partial attenuation [104]. UIS12 is an RNA binding protein that is thought to have multiple roles, but deletion led to termination of sporogony during mosquito stages [105]. UIS14 was designated as lysine decarboxylase by bioinformatical analysis but there was no characterization study [82]. UIS16 has been previously annotated as merozoite capping protein (MCP1) has a role in moving junction formation during erythrocyte invasion [106]. UIS17 has a role in motility of sporozoites [107]. UIS19 is a dispensable ookinete surface protein [108]. UIS22 is a homolog of human origin recognition complex (ORC) and specifically expressed in gametocytes indicating a role in gametogenesis [109]. UIS23 is a transcription factor that is expressed

throughout blood stages [110]. UIS24 is HSP70-3 homolog that is thought to have mitochondrial localization [111]. Remaining UIS genes had no information indicating their roles.

UIS14 was one of the candidate genes that was selected to analyze its role during liver stages. Targeted deletion resulted in abolishment of male gamete exflagellation, resulting in transmission block to mosquitoes [112]. Therefore, UIS14 is excluded from this thesis study.

ΔPlasmei2 was shown to result in strong attenuation and protection but with breakthrough infections at high doses [113]. *ΔLISP*1, and *ΔLISP*2 were also shown to be arrested during late liver stages but resulting in breakthrough infections with regular doses [32, 33]. Double knockouts, UIS3/*ΔLISP*2 and ΔPlasmei2/*ΔLISP*2, double knockouts were shown to result in complete attenuation but UIS3/*ΔLISP*2 was induced a reduced immune response [114, 115]. Reduced immune response might have been caused by earlier arresting profile or absence of both antigens.

Our design is based on enhanced safety with late liver arrest. To enhance safety, we decided to express a transgene in addition to the knockout of a vital and stage specific gene. Modification in question must have been a type of "gain of function" but a disadvantageous function for the parasite. The parasite's extensive control on hepatocyte is only possible by a tightly controlled PVM transport machinery. Other than an effective way to use host resources to its own advantage, it is also a way to hide from the immune system. PVM also shields the parasite from host cytosol. Expressing and directing bacterial pore forming proteins to the PVM during late liver stages can result in a disruption of that control while exposing parasite to the host cell cytosol. Similar strategy was previously tested in an intracellular bacteria that is also dependent on vacuoles, *Mycobacterium tuberculosis* vaccine strain, *M. bovis* Mycobacterium *bovis* bacille Calmette-Guérin (BCG), to enhance safety with Listeriolysin O [116]. Later on, a study in murine malaria model *P. berghei* used a similar approach to create complete attenuation with knockout of E1 subunit of pyruvate dehydrogenase (PDH-E1) enzyme complex and Perfringolysin O (PFO) expression under LISP2 promoter [117]. Both knockout phenotype and toxin expression alone were tested, and both resulted in moderate late arrest. When both genotypes were combined in one strain, it still led to breakthrough doses even with 25,000 sporozoites. Protection data was not reliable due to the low challenge dose [117].

We selected another bacterial pore forming toxin as primary design, Streptolysin O (SLO) from *Streptococcus pyogenes* [94]; and as an alternative, Listeriolysin O (LLO) from *Listeria monocytogenes* [95]. Accompanying gene knockouts were LISP1 and LISP2. Coding sequences of LISP1 and LISP2 were replaced with coding sequences of mature peptides of SLO (*ΔLISP*1::SLO) and LLO (*ΔLISP*2::LLO), respectively. Control strains which have fluorescent protein sequences in place of toxins were also designed to evaluate enhancing effects of the toxins (*ΔLISP*1::mNeonGreen and *ΔLISP*2::mTurquoise2). Both liver specific genes contain a short transport signal peptide at the amino terminal. They were not removed while replacing the rest of the coding sequences, allowing those signals to transport toxins to PVM.

Resulting phenotypes had similar growth characteristics during blood and mosquito stages as wild-type parasites. Considering that LISP1 and LISP2 genes were specifically expressed during liver stages, absence of them should not have any effect on the remaining life cycle stages. Nevertheless, regular growth of main vaccine candidates, *ΔLISP*1::SLO and *ΔLISP*2::LLO, suggests a tight control of LISP1 and LISP2 promoters during blood and mosquito stages.

LISP1 was shown to be involved in breakdown of the PVM in *P. berghei* [32]. *ΔLISP*1 parasites were not defective in liver or blood stages growth but maturated merozoites were trapped inside PVM. Therefore, it is not fully attenuated and (possibly) spontaneous ruptures on PVM lead to breakthrough infections. RNA sequencing data from *P. berghei*-infected HepG2 or Huh7 hepatoma cell cultures taken from PlasmoDB database shows that the first detectable low-level LISP1 expressions start around 24^{th} hour, gradually increases around 36^{th} hour, and peaks around 48^{th} hour of liver stages [118, 119]. Streptolysin O (SLO) was previously shown to be active at neutral pH and purified form was used in selective membrane permeabilization in *Plasmodium* studies [97]. Our strategy was to combine LISP1 knockout with SLO's pore forming ability that would lead to forming of pores around mid-liver stages (~30-36 hours) which would eventually completely lyse PVM (~40-48 hours) before completion of merozoite formation, resulting in a complete attenuation. Thus, we kept the LISP1 export signal peptide sequence, and replaced the remaining LISP1 coding sequence with SLO coding sequence to direct the toxin to PVM.

As a similar alternative strategy, we replaced the coding sequence of LISP2 with Listeriolysin O (LLO), keeping the LISP2 export signal peptide intact. LISP2 was shown to be critical for merozoite formation in *P. berghei* [33]. *ΔLISP*2 parasites

produce defective merozoites. Similar to *ΔLISP*1, *ΔLISP*2 attenuation is not complete and there were breakthrough infections [33]. RNA sequencing data shows a similar timing on expression profile but up to five times more transcript numbers starting around 36 hours post liver infection [118, 119]. LLO is known to be less active in neutral pH [98]. Therefore, expressing a less active cytolysin under a promoter which is much stronger during late liver stages should compensate for the lower activity of LLO.

Figure 4.1 shows the comparison of the RNA sequencing data from *P. berghei* infected HeLa cells taken from PlasmoDB database [118, 120].

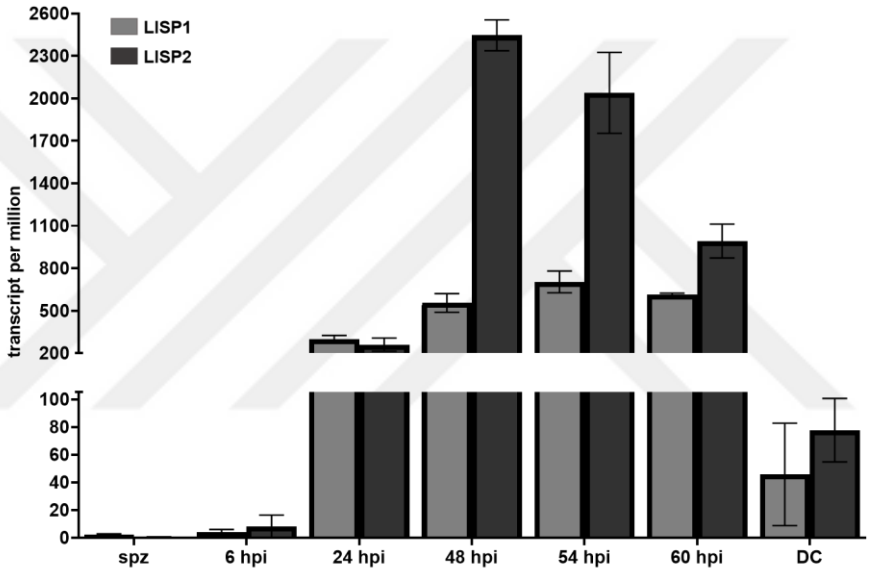


Figure 4.1 : Comparison of RNA transcript levels of *LISP1* and *LISP2* during liver stages. Graph created with data taken from PlasmoDB and Caldelari et al. 2019 [118, 120].

Considering the expression profiles, strong attenuation profile of *ΔLISP*1::SLO strain is not surprising. Compared to *ΔLISP*1::mNeonGreen, expression of SLO dramatically improved the attenuation profile. Almost 1.6 million sporozoites were injected into 26 mice and not a single sporozoite was able to reach blood stages.

On the other hand, LISP2 knockout, *ΔLISP*2::mTurquoise2, showed slightly stronger attenuation than *ΔLISP*1::mNeonGreen. Although the expression of LLO improved the attenuation profile against lower doses, *ΔLISP*2::LLO strain was not completely attenuated against higher doses as *ΔLISP*1::SLO. High level expression of LISP2

promoter could not compensate for weaker pore forming activity of LLO at neutral pH.

Comparison of the activities of SLO, LLO, and PFO at both neutral (7.0) and acidic (5.5) pH was shown in **Figure4.2.** The hemolytic activity of SLO was lower at acidic pH levels even at 5 times higher concentration [99]. But SLO is stable at neutral pH and showed a higher hemolytic activity [99].

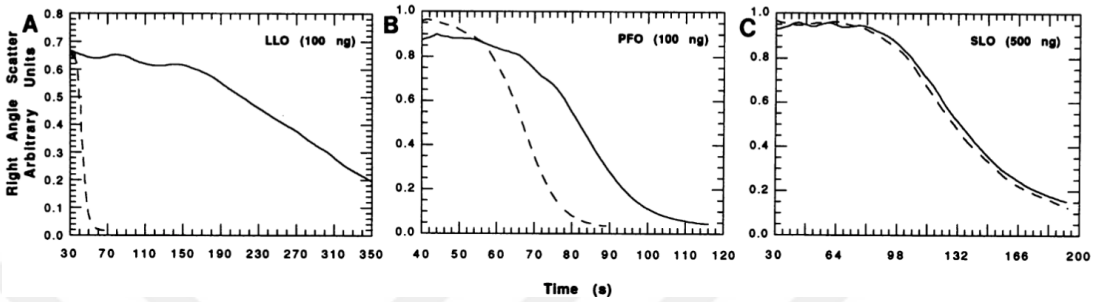


Figure 4.2 : Comparison of hemolytic activities of LLO, PFO, and SLO at pH=5.5 (dashed line) and pH=7.0 (solid line). SLO concentration is 5 times higher to achieve a comparable level of activity. Adapted from Portnoy et al. 1992 [99].

LLO is inactivated at neutral pH above 30°C due to the unfolding of unstable acidic regions at transmembrane domain [121] but a fraction of it can stay active at neutral pH and can form pores at very low concentration [122]. Also formed pores sizes were reported to be independent of pH [123]. Pore forming activity of LLO is considered as a phagocytosis escape tool, therefore it makes sense that it is active at acidic pH and inactivates once it mixes to the host cell cytosol at neutral pH to prevent host cell death [123]. But LLO is also a tool to invade host cells with the same pore forming activity. It was shown that in presence of cellular membranes, LLO can stay active and bind the membranes of host cells to form functional pores instead of unfolding at physiological temperature and pH [121]. Therefore, high level expression of LLO with PVM export signaling in *ΔLISP*2::LLO strain should have kept enough amounts of LLO active for a stronger attenuation which seemed to be true at lower numbers of sporozoite injections. Presence of breakthrough infections at higher sporozoite doses suggests a that active LLO concentrations at physiological temperature and pH are not always enough for complete attenuation. We observed that some of the PVMs were not lysed as late as 42 hours post infection. Those remaining intact cells would also be attenuated due to LISP2 deficiency. Thus, combination of LLO expression and *ΔLISP*2 lowered the breakthrough infections significantly. Yet, even at low probability, remaining intact parasites led to the occasional breakthrough infections.

Finally, we challenged the immunized mice with wild-type sporozoites. We divided fully attenuated 5,000, 20,000, and 80,000 *ΔLISP*1::SLO strain immunized groups into two sub-group. The first groups were challenged with 500 sporozoites. The 20,000 and 80,000 groups conferred 100% protection. But unexpectedly, the 5,000 group conferred no protection. The second groups were challenged with 10,000 sporozoites as extreme dose. As expected, the 5,000 group conferred no protection. But the 20,000 and 80,000 groups conferred 50% and 75% protection, respectively. The reason for low protection profile of *ΔLISP*1::SLO might be due to the early attenuation profile that was observed on in vitro cultures in which we could not observe any liver stages after 36 hours post infection. The expression levels of LISP1 promoter are very low until around the 24th hour but accumulation of SLO might have caused forming of the pores around as early as 24 hours post infection, leading to early attenuation profile. Increased immunization doses showed correlated increase in protection which, in fact, is consistent with early arresting liver stages profiles. Another possible contribution to low protection might be due to the loss of LISP1 protein as an important PVM antigen. Of note, immunogenicity of SLO was possibly not enough to enhance the overall protection.

*ΔLISP*2::LLO strain immunized groups (all 8 mice from 5,000 group, and 6 mice from 20,000 and 3 mice from 80,000 groups which survived the initial injection of immunization doses) were also challenged with wild-type sporozoites. Surprisingly, *ΔLISP*2::LLO strain conferred sterile protection against 10,000 sporozoites. This dramatic difference might be due to the late attenuation profile that was observed on in vitro cultures in which earliest PVM lysis was detected after 36 hours (**FigureXX**). Similar to LISP1, the expression levels of LISP2 promoter are very low until 24th hour, but significantly increases through to the late liver stages. Low activity of LLO toxin requires very high levels of expression to form functional pores. This weakness of LLO leads to late attenuation which causes breakthrough infections. It is possible that late attenuation possibly enhances the overall protection even with lower doses of immunization. Although adjuvant-like properties of LLO were previously reported [124], it was not possible to evaluate the contribution of LLO's immunogenicity in this experimental setup.

Expression of a bacterial cytolysin, Perfringolysin O (PFO), during liver stages was previously evaluated as an attenuation strategy in *P. berghei* [117]. They knocked out a fatty acid gene, PDH-E1α, which was previously reported to cause a complete

attenuation in *P. yoelii* liver stages [117, 125]. *pdh-e1α* knockout, PFO expression under LISP2 promoter, or two deficiencies combined, did not create a complete attenuation [117]. Considering this, to improve attenuation efficiency of *ΔLISP*2::LLO strain, replacing the toxin with SLO or PFO might improve the attenuation, but at the same time impair the protection efficiency.

Insertion of a bacterial toxin in an attenuated vaccine strains may enhance the immune stimulation, but it is unsuitable to use in humans [126]. Listeriolysin O is a pore forming toxin of *Listeria monocytogenes* [126]. It has been previously used to enhance the protective efficacy of *Mycobacterium tuberculosis* vaccine, *Mycobacterium bovis bacille* Calmette-Guérin (BCG) [116]. In vivo safety of LLO expressing recombinant strain (rBCG) was evaluated by infecting immunodeficient SKID mice [116]. A version of the rBCG vaccine has recently been evaluated for Phase III clinical trials [127]. Expressing L461T mutant which is active at neutral pH of LLO might create additional safety concerns, but it was reported to be almost 100-fold less virulent due to the killing of host cell, when expressed by *Listeria monocytogenes* intracellularly [128].

5 CONCLUSION

Expression of cytolysins under late liver stages promoters has proven to be a promising vaccine strategy. *ΔLISP*2::LLO strain can be an efficient vaccine if attenuation profile can be improved without losing the protection efficiency. An amino acid substitution in LLO gene, L461T, has been reported to increase the hemolytic activity of LLO almost 10-fold under physiological conditions [128]. This mutant can be replaced with wild-type LLO in *ΔLISP*2::LLO construct to improve the attenuation profile, but protection efficiencies must be re-evaluated. If found successful, this strategy can easily be translated into the human species, *Plasmodium falciparum*.

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APPENDICES

APPENDIX A: Ethics Committee Approval

CURRICULUM VITAE

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Place and Date of Birth :

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EDUCATION:

- **B.Sc :**2012, Istanbul University, Science Faculty, Department of Biology
- **M.Sc. :**2016, Istanbul University, Institute of Science, Department of Molecular Biology and Genetics

PROFESSIONAL EXPERIENCE AND REWARDS:

- 2018- Research Assistant at Institute of Life Sciences and Biotechnology in Bezmialem Vakif University

PUBLICATIONS, PRESENTATIONS AND PATENTS PRODUCED FROM THE THESIS:

- **Kina, U. Y., Kamil, M., Deveci, G., Rafiqi, A. M., Matuschewski, K. & Aly, A. S. I.** (2023). A Candidate Bacterial-Type Amino Acid Decarboxylase Is Essential for Male Gamete Exflagellation and Mosquito Transmission of the Malaria Parasite. *Infection and Immunity*, *91*(7), e0016723.(Article)
- **Kına Ü. Y.,** Rafıqı A. M. Cytolysins Expressing Liver Stage Parasites As Novel Live Attenuated Malaria Vaccines. 6. International Health Sciences and Life, Burdur, Türkiye, 2nd – 05th March 2023. (Poster presentation)

OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS:

- **Kina, U. Y., Kamil, M., Deveci, G., Rafiqi, A. M., Matuschewski, K. & Aly, A. S. I.** (2023). A Candidate Bacterial-Type Amino Acid Decarboxylase Is Essential for Male Gamete Exflagellation and Mosquito Transmission of the Malaria Parasite. *Infection and Immunity*, *91*(7), e0016723.
- **Gezen-Ak, D., Alaylıoğlu, M., Yurttaş, Z., Çamoğlu, T., Şengül, B., İşler, C., *et al.*** (2023). Vitamin D receptor regulates transcription of mitochondrial DNA and directly interacts with mitochondrial DNA and TFAM. *The Journal of Nutritional Biochemistry*, *116*, 109322.
- **Akyüz, S. N., Kina, U. Y., Aly, A. S. I. & Palabiyik, B.** (2023). Iron affects localization of Ght5 in fission yeast. *FEMS Microbiology Letters*, *370*.
- **Kamil, M., Kina, U. Y., Deveci, G., Akyuz, S. N., Yilmaz, I. & Aly, A. S. I.** (2022). Mitochondrial Spermidine Synthase is Essential for Blood-stage growth of the Malaria Parasite. *Microbiological Research*, *265*, 127181.

- **Kamil, M., Deveci, G., Kina, U. Y., Kappe, S. H. I. & Aly, A. S. I.** (2022). Subcutaneous Immunization with Unaltered Axenic Malaria Parasite Liver Stages Induces Sterile Protection against Infectious Sporozoite Challenge. *Vaccines*, *10*(11), 1884.
- **Kamil, M., Atmaca, H. N., Unal, S., Kina, U. Y., Burak, P., Deveci, G., *et al.*** (2022). An Alternative Autophagy-Related Mechanism of Chloroquine Drug Resistance in the Malaria Parasite. *Antimicrobial Agents and Chemotherapy*, *66*(12), e00269-00222.
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- **Ceylan, M., Kına, Ü. Y., Çakır, Ö. & Kara, N. T.** (2021). Assessing the influence of cycloastragenol on telomere/telomerase system of Arabidopsis thaliana. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *146*(1), 83-95.
- **Kina, U. Y. & Palabiyik, B.** (2019). Hydrogen peroxide-induced oxidative stress upregulates ght5 gene belonging to hexose transporters in Schizosaccharomyces pombe. *Cellular and Molecular Biology*, *65*(1), 41-45.