BEZMIALEM VAKIF UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

DEVELOPMENT OF WHOLE BLOOD STAGE VACCINE FOR MALARIA TREATMENT VIA CRISPR-GENE EDITING TECHNOLOGY

Ph.D. THESIS

Gözde DEVECİ

Biotechnology Department

Biotechnology PhD Program

Thesis Advisor: Prof. Dr. Binnur TEMEL
Thesis Co-Advisor: Assoc. Prof. Dr. Ahmed S. I. ALY

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After fulfilling all the necessary conditions determined by the related regulations, PhD student Gözde DEVECİ (student ID number: 185314001) successfully presented her/his thesis titled as "DEVELOPMENT OF WHOLE BLOOD STAGE VACCINE FOR MALARIA TREATMENT VIA CRISPR-GENE EDITING TECHNOLOGY" in front of the jury members.

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Date of Submission : 16 December 2022
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To my family,

FOREWORD

I would like to thank my advisor Prof. Dr. Binnur Temel and co-advisor Assoc. Prof. Dr. Ahmed S. I. Aly for their support and guidance during my Ph.D. thesis.
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November 2022

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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.

Gözde DEVECİ

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ABBREVIATIONS

ADP : Adenosine diphosphate
AMA1 : Apical Membrane Antigen
ATP : Adenosine triphosphate
CelTOS : Cell-traversal protein for ookinetes and sporozoites
ChAd63 : Chimpanzee adenovirus 63
CRISPR : Clustered Regularly Interspaced Short Palindromic Repeats
CRT : Chloroquine resistance transporter
CSP : Circumsporozoite protein
ddH_2O : Double distillated water
DHFR : dihydrofolate reductase
DMSO : Dimethyl sulfoxide
EDTA : Ethylene Diamine Tetra Acetic Acid
EEF2 : Translation elongation factor 2
FBS : Fetal Bovine Serum
FI : Fluorescence intensity
FSC : Forward scattering
GAP : Genetically attenuated parasite
gRNA : Guide Ribonucleic Acid
KAHRP : Knob Associated Histidine Rich Protein
IL2 : Interleukin 2
IRSs : Indoor residual sprays
ITNs : Insecticide-treated nets
IV : Intravenous
LB Broth : Luria-Bertani Broth
mETC : Mitochondrial electron transport chain
MDR1 : Multidrug resistance transporter 1
NHEJ : Non-homologous end joining
NLS : Nuclear localization signal
NT1 : Nucleoside transporter gene 1
NT2 : Nucleoside transporter gene 2
NT3 : Nucleoside transporter gene 3
NT4 : Nucleoside transporter gene 4
PAM : Protospacer Adjacent Motif
P. berghei : *Plasmodium berghei*
Pbnt1(-) : *Plasmodium berghei* nucleoside transporter gene 1 deficient parasite
PBS : Phosphate buffered saline
PCR : Polymerase chain reaction
P. falciparum : *Plasmodium falciparum*
PfEMP1 : *Plasmodium falciparum* erythrocyte membrane protein 1
PfGARP : Plasmodium falciparum glutamic-acid-rich protein
PfRH5 : *Plasmodium falciparum* reticulocyte-binding protein homolog 5
PfSEA1 : Schizont Egress Antigen-1

PVM **:** Parasitophorous vacuolar membrane
P. yoelii **:** *Plasmodium yoelii*
Pynt1(-) **:** *Plasmodium yoelii* nucleoside transporter gene 1 deficient parasite
RON2 **:** Rhoptry neck protein 2
RPMI **:** Roswell Park Memorial Institute Medium
RT **:** Room Temprature
SAP1 **:** Sporozoite asparagine-rich protein
SC **:** Subcutaneous
SD **:** Standard deviation
TALENs **:** Transcription activator-like effector nucleases
TNF **:** Tumor necrosis factor
UIS3 **:** Up-regulated in infective sporozoites gene 3
UIS4 **:** Up-regulated in infective sporozoites gene 4
WHO **:** World Health Organisation
WT **:** Wild Type
ZFNs **:** Zinc-finger nucleases

SYMBOLS

ºC	**:** Degree Celcius
*****	**:** p value < 0.05
******	**:** p value < 0.01
***	: p value < 0.001

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DEVELOPMENT OF WHOLE BLOOD STAGE VACCINE FOR MALARIA TREATMENT VIA CRISPR-GENE EDITING TECHNOLOGY

SUMMARY

Malaria remains a major global health problem among mainly in pregnant women and children, which resulted in death of hundreds of thousands of humans. One of the greatest challenges for malaria treatment is drug resistance against antimalarial drugs. Therefore, eradication of malaria can only be achieved with the application of very potent and safe vaccines. However, there is no malaria vaccine currently available, and the most advanced subunit vaccine candidate has recently reported efficacy between 0-30% against clinical malaria cases. On the other hand, live attenuated whole parasites used in experimental vaccination trails induced complete sterile protection. However, the inefficiency of gene editing technologies has limited their use in generating live attenuated vaccines.

Four members (NT1, NT2, NT3, and NT4) of the nucleoside transporter gene family play an important role in the purine transfer from the host to human malaria parasites. In this thesis, we developed nucleoside transporter 1 deficient *Plasmodium yoelii* and *Plasmodium berghei* via CRISPR-Cas9 gene editing methods. The CRISPR/Cas9 system is an emerging genome-editing technology that is used to edit the gene for various living organisms. CRISPR/Cas9 system sheds light on *Plasmodium* (*P. yoelii*, *P. falciparum*, etc.) to modify the targeted genes based on homologous recombination. We also generated a mixed live attenuated blood-stage malaria vaccine model using that NT1 deficient plasmodium strains. Equally mixed *Pbnt1(-)* and *Pynt1(-)* parasites in single subcutaneous fresh or frozen doses were injected in a group of mice and conferred sterile protection against intravenous infectious blood-stage challenge with wild-type parasites of *P. berghei* ANKA and *P. yoelii* 17X-NL strains. This data may indicate that a single subcutaneous sub-patent dose of two species of genetically-growth-attenuated parasites, can protect humans against two *Plasmodium* spp. infections. NT1 knockout parasites could be developed in cultures provided with supra-physiological concentrations of purine and shipped to endemic areas in frozen-stock doses stored in liquid nitrogen.

In this thesis, we also evaluated the role of the nucleoside transporter 4 gene (NT4) in the *Plasmodium* life cycle as a potential malaria vaccine target. Herein, NT4 deficient *P. berghei* parasites were generated, and in the erythrocytic stage, significant differences have not been observed. However, oocyst egress and sporozoite invasion of salivary glands are restricted in the mosquito stage. Moreover, the *Pbnt4(-)* salivary glands and hemolymph sporozoites did not develop infectivity. As a result of these results, the NT4 gene could be a promising target for the next malaria transmission-blocking studies.

Keywords: Malaria, *Plasmodium berghei*, *Plasmodium yoelii*, CRISPR-Cas9, Blood-stage vaccine, Nucleoside Transporter 1, Nucleoside Transporter 4

CRISPR GEN DÜZENLEME TEKNOLOJİSİ İLE SITMA TEDAVİSİNDE KULLANILACAK CANLI ZAYIFLATILMIŞ KAN EVRESİ AŞISI GELİŞTİRİLMESİ

ÖZET

Sıtma insanlık tarihinin ilk yıllarından beri özellikle gebe ve çocukları etkileyen ve binlerce insanın ölümüne yol açmış başlıca küresel sağlık problemlerinden biridir. Sıtma tedavisinde karşılaşılan en büyük zorluk sıtma ilaçlarına karşı gelişen ilaç direncidir. Sıtmanın yok edilebilmesi için aşılama en güvenilir ve önemli yöntemlerden biridir. Ancak sıtma tedavisinde kullanılacak mevcut bir aşının bulunmadığı ve geliştirilen alt birim aşı adayları etkinliğinin % 0-30 arasında olduğu rapor edilmiştir. Diğer yandan, deneysel aşılamalarda kullanılan canlı zayıflatılmış aşıların steril bağışıklığı indüklediği gösterilmiştir. Ancak gen düzenleme teknolojilerinin yetersiz olması canlı zayıflatılmış aşıların geliştirilmesini sınırlamaktadır.

İnsan sıtma türünde nucleosit transport gen ailesinin 4 üyesi (NT1, NT2, NT3 ve NT4) tanımlanmıştır ve her bir aile üyesinin konakçıdan pürin alımında rol oynadığı düşünülmektedir. Bu tez çalışmasında nucleosit transport 1 geni *P. yoelii* ve *P. berghei* suşlarından CRISPR-Cas9 gen düzenleme yöntemiyle silinmiştir. CRISPR-Cas9 teknolojisi, çeşitli canlı organizma genlerinin düzenlenmesinde kullanılmaktadır. CRISPR-Cas9 teknolojisi homolog rekombinasyon temelli gen hedefleme yöntemini geliştirmede ışık tutmaktadır. Bu çalışmada ayrıca üretilen nükleosit transport 1 geni silinmiş parazit suşları ile karışık doz canlı zayıflatılmış kan evresi sıtma aşısı modeli geliştirilmiştir. Eşit miktarda *Pbnt1(-)* ve *Pynt1(-)* parazitleri karıştırılarak taze ya da dondurulmuş formda oluşturulan aşılar tek doz subkutan yolla farklı fare gruplarına injekte edilmiştir. Ardından aşının etkinliği yabanıl tür *P. berghei* ANKA ve *P. yoelii* 17-XNL ile intravenöz olarak injekte edilerek incelenmiş ve steril koruma gözlemlenmiştir. Elde edilen sonuçlar, genetik yöntemlerle zayıflatılmış iki farklı parazit türünün tek doz deri altı subpatent injeksiyonunun insanlarda da iki farklı türdeki sıtma infeksiyonuna karşı koruyucu olabileceğini düşündürmektedir. NT1 geni silinmiş parazitler pürin içeren kültürlerde üretilebilir ve endemik bölgelere dondurulmuş dozlar olarak sıvı nitrojen içinde gönderilebilir.

Bu tezde ayrıca nükleosit transport 4 geninin plazmodium yaşam döngüsündeki rolü potansiyel sıtma aşı hedefi olarak incelenmiştir. NT4 nakavt *P. berghei* parazitleri üretilmiş, bu parazitlerin kan evresindeki gelişiminde belirli bir farklılık görülmemiştir. Ancak, sporozoitlerin ookistlerden çıkışı ve tükrük bezini istilasında bir kısıtlanma gözlemlenmiştir. Ayrıca, tükrük bezi ve hemolenften toplanan *Pbnt4(-)* sporozoitlerinin infeksiyona sebep olmadığı belirlenmiştir. Sonuç olarak gelecekteki NT4 geni sıtma transmisyon blokaj çalışmaları için umut vaadedicidir.

Anahtar Kelimeler: Sıtma, *Plasmodium berghei*, *Plasmodium yoelii*, CRISPR-Cas9, kanevresi aşısı, Nucleosit Transport 1, Nucleosit Transport 4

1. INTRODUCTION

1.1 Purpose of Thesis

Malaria is one of the deadliest infectious diseases for centuries. In 2020, about $3.3 billion was spent on malaria prevention and elimination [1]. Over the next three years, more than three times that amount is expected to be spent on malaria. Development of drug resistance by plasmodium species is the major obstacle for malaria elimination [2]. By this time, several vaccination methods have been developed. Induction of IgG production, causing symptoms and introducing large amount of antigens to the immune system make attenuated whole-blood stage vaccines attractive in all other malaria vaccine approaches. Therefore, the development of a genetically attenuated blood stage vaccine model by CRISPR (Regularly Interspaced Short Palindromic Repeats)-Cas9 gene editing technlogy is the main goal of this thesis. Genetically attenuated vaccines have been produced by several genetic alteration related to metabolic pathways [3]. Previous studies have shown that immunization with nucleoside transporter 1 knockout parasites of *Plasmodium yoelii (P.yoelii)* confers sterile immunity against wild-type *P.yoelii* while providing partial protection against *Plasmodium berghei (P.berghei)* [4]. The limitations of cross-species immunization have also been reported in other studies. Therefore, the development of genetically attenuated blood stage vaccine against two different plasmodium species also another goal of the thesis. In addition, the evaluation of new targets for antimalarial drugs and vaccines is critical for the elimination of malaria. The promising results related with nucleoside transporter 1 for malaria treatment have drawn attention to other members of the family as potential vaccine or drug target. Therefore, determination role of nucleoside transporter 4 (NT4) in plasmodium life cycle is another objective of this thesis.

1.2 Literature Review

Malaria is a serious worldwide health problem. It mainly affects young children and pregnant women and is caused by plasmodium species. According to the World Health

Organization, more than 240 million cases of malaria were reported worldwide in 2020 [1]. Although intervention strategies are leading to a decrease in malaria-related deaths, the development of resistance to almost all available chemotherapeutic agents is a serious concern [5]. Another issue is limited protection for cross-species malaria infection. Both human malaria parasites *P. falciparum* and *P. vivax* are ubiquitous in malaria endemic such as Oceania [6], a mixed immunization strategy may provide protection against both malaria species. In India, mixed infections are also common. There are findings that indicate *P. falciparum* infection is immediately followed by *P. vivax* infection in Southeast Asian areas where malaria transmission is low, seasonal, and unstable [7]. Previous studies showed that cross-species immunization did not provide sufficient protection [8]. Therefore, a mixed immunization approach could become a cornerstone for protection against different malaria species and we aimed to develop vaccine to protect against two different type of *Plasmodium* species.

A whole parasite vaccine delivers a broad range of antigens to the immune system and, unlike a subunit vaccine, provides stronger protective immunity [9]. Moreover, the development of the whole blood stage parasite is a good alternative because of the well-explained mechanism and ease of production. Whole parasite malaria vaccines can be developed as killed or live attenuated blood stages by genetic, irradiated or chemical attenuation [10]. Genetically attenuated parasites are developed based on deletion or inactivation of essential metabolic pathways. Four members (NT1, NT2, NT3, and NT4) of the nucleoside transporter gene family have been identified in the genome of human plasmodium species known to play an important role intake of purine from the host. Nucleoside transporte 1 gene is mainly responsible for the import of purines to complete life cycle [11]. According to previous studies, other members of the family are also involved in purine transport, but their mechanisms are not well studied. One of the approaches to genetic attenuation is targeted genome editing, which has been demonstrated in several organisms using CRISPR gene editing technology [12]. Genetic modification is based on recombination of single- or double-stranded chromosomal loci, which requires homologous sequences. However, this is a time-consuming process, and tedious selection is required for parasites that are transformed at low levels. To overcome these difficulties, target-specific genome editing methods using CRISPR-Cas9 have been demonstrated in several organisms. In addition, another member of nucleoside transporter gene family, NT4, whose role of

Plasmodium life cycle has not been explained in detail. Therefore, a detailed explanation of the NT4 is essential to find new targets for the treatment of malaria. Meanwhile, combining novel techniques with the targets may open a new door to eliminate malaria.

1.3 Hypothesis

Genetically attenuated blood-stage vaccines have great potential for the treatment of malaria. However, the development of these vaccines is difficult due to low transfection efficiency. CRISPR-Cas9 technology has been studied in many cell types for gene deletion without off-target and with high transfection efficiency. Therefore, in this thesis, we aimed to use CRISPR gene editing technology to develop nucleoside transporter 1 deficient parasites. Second, in malaria endemic areas, infection with two different *Plasmodium* species is very common. However, studies have shown that malaria vaccines provide only partial protection for cross-species immunization. Therefore, a strategy that mixes two different knockout *Plasmodium* species may overcome these limitations. Finally, previous studies have shown that parasites that have the nucleoside transporter 1 knocked out have protection against disease and are a potential target for use in humans. Nucleoside transporter 4 is another member of the nucleoside transporter gene family and is known to has role for the uptake of purines. However, the functional mechanism has not yet been explained in detail. A detailed explanation of the mechanism may therefore offer advantages in the exploration of a new vaccine target.

2. THEORETICAL KNOWLEDGE

2.1 Malaria

Malaria is a parasitic disease caused by the bites of malaria infected female Anopheles mosquitos. *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi* are *Plasmodium* species that cause human infection [13]. In 2020, the World Health Organisation reported more than 240 million malaria cases around the world [1]. Sub-Saharan Africa, South America, South Asia, some parts of the Middle East, and Oceania are malaria transmission areas. Malaria cases were also recorded in Turkey between 1925 and 2010. Turkey is located between Asia, Europe, and Africa and thus in the middle of malaria-endemic areas. In addition, the back-and-forth movement of the population makes Turkey a high malaria risk country, so malaria has always been and will continue to be a public health problem in Turkey. Therefore, not only in our country but also in the world, malaria is a deadly parasitic disease that needs urgent and permanent treatment [14, 15]

2.2 Plasmodium Life Cycle

Plasmodium parasites have a complex life cycle between vertebrate hosts and mosquito vectors. Malaria-infected mosquitoes transfer sporozoites into the host's bloodstream with saliva flow during the blood meal. The sporozoites escape the host's immune system until they reach the liver. After arriving at the liver, the sporozoites break through the sinusoidal barrier and invade hepatocytes. Upon invasion of hepatocytes, the parasite utilises a portion of the host membrane and envelop itself with the membrane called the parasitophorous vacuole. Differentiation and replication of the parasite progresses within a few days. At the end of the liver stage, multinucleate exo-erythrocytic schizonts rupture and release merozoites. These merozoites invade red blood cells and begin asexual multiplication, which is accompanied by clinical symptoms such as fever, chills, headache, muscle pain, and fatigue. While some infected red blood cells develop asexual reproduction, others develop gametocytes. A large proportion of blood stage parasites maintain the asexual cycle, while a small

proportion of asexual parasites differentiate to the sexual stage. Gametocyte development begins in red blood cells, but the formation of gametogenesis and meiosis can only develop in the mosquito vector. Mature gametocytes circulate in the peripheral blood for several days. After another bite by a female Anopheles mosquito, the ingested gametocytes enter the lumen of the midgut, are activated and fertilised, and then differentiate into ookinetes and oocysts. The oocysts settle between epithelial cells and hemocoel and produce sporozoites in the midgut. Eventually, the sporozoites escape from the oocyst and migrate to the salivary gland of the female Anopheles mosquito in the hemocoel to infect another vertebrate host again [13, 16, 17].

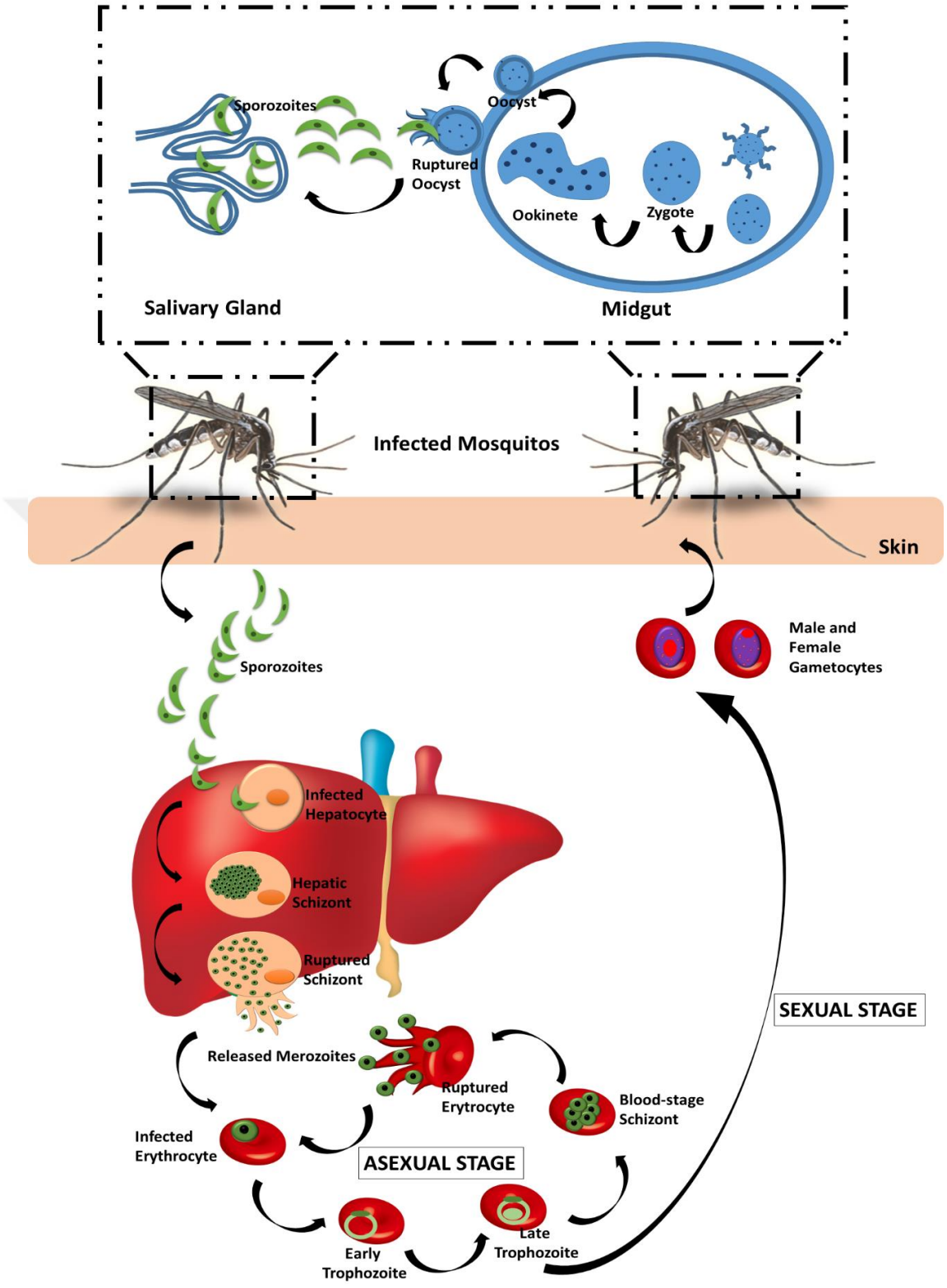

Figure 2. 1: *Plasmodium* Life Cycle.

2.3 Immune Responses in Malaria

After the mosquito bite, sporozoites enter the bloodstream and encounter members of the innate immunity. Studies on rodent malaria parasites have shown that only a small amount of sporozoites enter the bloodstream and the rest remain in the skin. In one study, transmigration knockout *P. berghei* parasites were cleared by CD11b+ phagocytic cells. In addition, parasites that had lodged in the skin were drained by lymphatic circulation, and most of them were killed by CD11c+ dendritic cells in the lymph nodes. Sporozoites escaping immune cells must cross the sinusoidal barrier of the liver. Stellate cells are located between parenchymal hepatocytes and the sinusoidal lining. These cells secrete highly sulfated heparan sulfate proteoglycans (HSPGs), which are required for binding of the circumsporozoite protein. At this point, phagocytic cells (dendritic cells, etc.) can deliver antigens to lymphocytes to activate the adaptive immune system. Sporozoites can escape from the effectors of the immune system and settle in the liver. When the sporozoites reach the liver parancyma, they produce parasitophorous vacuole instead of invading new hepatocytes. The mechanism of vacuole entry is not well understood, but *in vivo* and *in vitro* studies have shown that hepatocyte surface molecules, the tetraspanin CD81 and the receptor BI, are responsible for invasion. However, sporozoites, unable to hide in the preerytrocytic stage, awaken the liver to induce naive CD8+ T cells. Hepatic dendritic cells, hepatocytes, Kuppfer cells, and stellate cells present antigens to T cells. *Plasmodium* cell surface proteins are mainly responsible for the induction of immunity in the blood stage. A variety of surface proteins are also involved in cytoadherence of infected erythrocytes to endothelial cells. IgG antibodies against to surface proteins of sporozoites and merozoites block invasion of hepatocytes and erythrocytes [13, 18].

2.4 Malaria Control and Elimination

2.4.1 Vector control

In malaria-endemic areas, vector control is an approach to prevent mosquito transmission through the use of insecticide-treated nets (ITNs) and indoor residual sprays (IRSs). Insecticide-treated nets provide a physical barrier for preventing mosquito entry and killing them. Pyrethroids and pyrroles are insecticides primarily used on insecticide-treated bed nets made of polyester, polyethylene, or

polypropylene. Insecticide on bed nets needs to be renewed every six or twelve months. Alternatively, long-lasting bed nets have been developed that can be used for three years [19]. Another vector control approach is IRS. In this method, walls and other surfaces of the house are coated with an insecticide to kill mosquitoes. Pyrethroids, carbamates, and organophosphates are commonly used pesticides for IRS. ITNs and IRS are the most common method of malaria control, but outdoor mosquito bites limit this protection. Also, long-term insecticide using leads to increased pesticide resistance for mosquitoes [20]. The World Health Organisation (WHO) reported that insecticide resistance emerged in 79 malaria-endemic countries in 2010, rising to 91 in 2016 [21]. Therefore, the limitations of vector control have highlighted the need for a new method to eliminate malaria.

2.4.2 Chemoprotection

Malaria is treated with various drugs such as chloroquine, artemisinin derivatives, pyrimethamine, cycloguanil, amodiaquine, piperaquine, pyronaridine, mefloquine etc. Drug treatment is based on three strategies. The first is chemoprevention, which is used in asymptomatic patients. The goal is to inhibit the liver stage of malaria and the development of schizogony. The likelihood of drug resistance is low. The second is chemotherapy. This strategy is intended for symptomatic patients and eliminates the asexual stage of the parasite. However, the possibility of drug resistance is very high here. The last strategy aims to interrupt the transmission cycle by inhibiting gametocytes [1, 22]. The most well-known antimalarial drug, Artemisinin, was discovered by Chinese scientist Tu Youyu in 1972. This drug was extracted by the wormwood plant (*Artemisia annua*). The highly effective and fast-acting drug artemisinin gained popularity all over the world [23]. However, *Plasmodium falciparum* species in Africa developed resistance to artemisinin especially in young children and pregnant women. Therefore, a combination strategy was introduced. Artemisinin-based combination therapy (artesunate-amodiaquine, dihydroartemisinin-piperaquine, and artemether-lumefantrine) is the initial therapy recommended by WHO in endemic regions worldwide. The mechanism of artemisinin combination therapy focuses on damaging the asexual and sexual blood stage of the parasites [24]. According to the Center for Disease Control and Prevention in the United States, chloroquine derivatives were also accepted as the first quinine-based therapy. The mechanism of quinine was known as heme detoxification. Quinoline-based

combination therapy (atovaquone-proguanil) is used in chloroquine resistance and this therapy acts on cytochrome bc1 and dihydrofolate reductase. Quinoline-based combination therapy targets the liver stage and asexual blood stages. Despite the discovery of new drugs or combination strategies, *Plasmodium* parasites continue to develop drug resistance. Mutations in the chloroquine resistance transporter and the multidrug resistance transporter 1 (MDR1) mediate drug resistance in malaria parasites (CRT). In Southeast Asia, the Kelch13 mutation results in resistance to artemisinin combination therapy. For the proteasome inhibition of blood-stage parasites, aminoacyl-tRNA synthetases and translation elongation factor 2 (EEF2) are novel potential therapeutic targets. However, toxicity to the host was observed in the study. The mitochondrial electron transport chain (mETC) was another drug target for the liver and blood stages but rapidly development of drug resistance limits to use new drug target. The development of a highly effective vaccine is essential due to the limited treatment options with antimalarial drugs [22].

2.4.3 Vaccination

Vaccines, along with sanitation, clean drinking water, and waste disposal, are one of the most effective and cost-effective strategy for preventing infectious diseases worldwide. Smallpox is an infectious disease that was eradicated thanks to a successful vaccination strategy. The eradication of smallpox also offers hope for other infectious diseases. Therefore, vaccination programs for all infectious diseases have been promoted by public health organizations, governments, and donor agencies [25]. The promising results of eliminating infectious diseases through vaccination and the limitations of vector control and antimalarial drugs have led malaria researchers to work on development of an effective vaccine. The recombinant protein-based vaccine RTS, S/AS01 is the first malaria vaccine to be licensed from WHO in October 2021 [26]. When RTS, S/AS01 was used with chemoprevention, vaccine protection was increased approximately 70% [27]. Nevertheless, new studies are underway to improve the efficacy of RTS, S/AS01E, and other novel candidates for malaria prevention. This involves the use of antigens in monovalent and multivalent formulations, either alone or with other agents, viral vectors, and/or vaccine adjuvants. The main interest in the development of vaccines is in the elimination of malaria, targeting parasites in the pre-erythrocytic and blood stages, and blocking transmission [28].

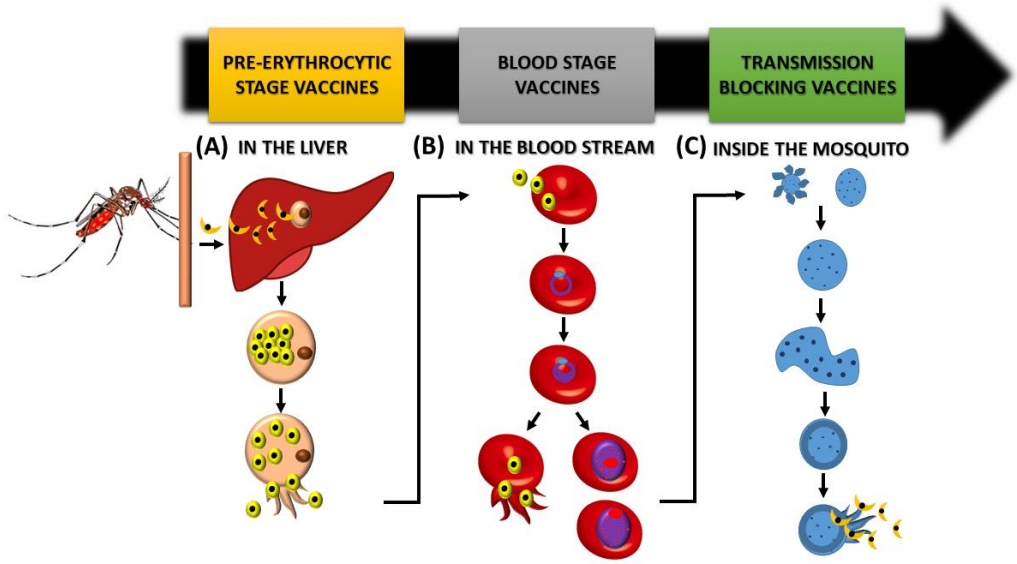

Figure 2. 2: Malaria vaccine aproaches the based-on malaria life cycle.

2.4.3.1 Pre-erythrocytic stage vaccines

Sporozoites and infected hepatocytes are the main targets for pre-erythrocytic stage vaccination. Antibodies against surface antigens of sporozoites and infected hepatocytes were induced by the pre-erythrocytic stage parasites. These antibodies envelop sporozoites for opsonization or inhibit sporozoite morbidity. In another mechanism, induced T cells (*Plasmodium*-specific CD8 T cells) attack the infected hepatocytes. T cell responses provide sterile immunity[29]. Main target antigen for the pre-erythrocytic vaccine is circumsporozoite protein (CSP). CSP responsible for sporozoite motility, attachment and entry into the hepatocyte. Anti-CSP antibodies and T cells block sporozoite invasion. The *P. falciparum* circumsporozoite protein (CS) has 412 amino acids. CSP consists of a central NANP repeat region and non-repeat regions. The central NANP repeats and the C-terminal region of CSP is displayed on the hepatitis B surface to develop an RTS, S vaccine. The vaccine proteins are expressed in yeast. The immunogenicity of the vaccine was enhanced by mixing the synthesized polypeptides with lipoproteins containing monophosphoryl lipid A, a detoxified lipopolysaccharide derivative, and QS -21 Stimulon®, a saponin molecule. Despite the acceptable protective efficacy of the RTS, S vaccine, *Plasmodium* parasites have different alleles of the circumsporozoite protein and all CSP alleles cannot be selected by vaccination. As a result, vaccine resistance may occur [30]. The vaccine R21, an improved version of RTS, S/AS01, was developed to achieve higher immunogenicity. Thus, the C-terminus of *Pf*CSP was bound to the N-terminus of

hepatitis B surface antigen without three-fold molar excess to increase the displayed antigen on the vaccine surface. The results demonstrated that vaccine induced humoral immunity. The adjuvants Abisco-100, Matrix-M, and AS01 were tried to increase immunity in the R21 strategy. This vaccine is currently being tested in phase 1/2a [31]. In another strategy, antigens of the pre-erythrocytic stage were presented by viral vectors. Viral vector vaccines elicit the cell-mediated immune response. For example, Fowlpox virus 9 and modified vaccinia virus Ankara were selected to present ME-TRAP antigens representing a fusion of 20 pre-erythrocytic stage epitopes of *P. falciparum* (ME) and thrombospondin-related adhesion protein (TRAP). However, because of the low protective efficacy of vaccines based on fowlpox virus 9 and modified vaccinia virus Ankara (2 of 5 vaccinations), another type of virus was needed for antigen delivery, e.g., chimpanzee adenovirus 63 (ChAd63). MVA ME-TRAP vaccine combined with ChAd63 virus. ChAd63/ MVA ME-TRAP vaccines highly induced CD4 and CD8 T-cell responses. However, in 2b clinical trials, 67% protection was achieved in Kenyan, whereas no protection was observed in Senegalese. Therefore, it was not accepted as a reliable vaccine. The MVA/ChAd63 virus-vector combination also tried with different antigens such as circumsporozoite protein and liver stage-associated protein 2, but no significant immune response was observed [32].

Cell-traversal protein for ookinetes and sporozoites (CelTOS) is another target for pre-erythrocytic stage vaccine development. Highly conserved CelTOS protein enables ookinetes to cross mosquito midgut and infect liver cells in human hosts. The immune response of CelTOS was supported with the adjuvant Montanide ISA 720 and both humoral and cellular immune responses were induced. Moreover, high-affinity antibodies against the CelTOS protein prevent the development of *Plasmodium* in the mosquito stage. Therefore, the *Pf*CelTOS vaccine has a potential to block not only the pre-erythrocytic stage but also the mosquito stage [33].

Whole sporozoites were used to stop the development of parasites in the liver stage as a pre-erythrocytic vaccine with protection between 35% and 100%. There are 2 main strategies for development of whole sporozoite vaccine: attenuated sporozoites (irradiation or genetically attenuated) and wild type sporozoites with chemoprophylaxis. For chemoprophylaxis with wild-type sporozoites, the host is infected with wild-type sporozoites and treated with antimalarial drugs immediately.

Mice experiments demonstrated induction of IFN+CD8 T-cell response after sporozoite immunization and chloroquine treatment. In addition, human clinical studies have shown that memory T cells increase the expression of IFN-γ, TNF, IL-2 and provide 100% protection against homologous *P. falciparum* species after vaccination with sporozites. However, limitations of the sporozoite vaccine are inefficient protection against heterologous species and the possibility of drug resistance [34].

In radiation attenuated vaccine, sporozoites are collected from salivary glands of infected mosquitoes and attenuated by gamma rays to block development of liver stage. Immune studies have shown that the induction of protective cellular and humoral immune responses is essential for strong protection. Moreover, type of vaccine administration is critical to achieve a high immune response. For example, subcutaneous and intradermal immunization increased protection 10-fold in the *P. yoelii* study. Clinical studies have also been demonstrated by mosquito bites. In 1970s, a group of humans immunized with radiation-attenuated *Plasmodium falciparum* sporozoites by mosquito bite and study resulted in sterile immunity. However, radiation attenuation may limit pre-erythrocytic stage vaccine efficacy and unattenuated sporozoites may cause malaria illness. Therefore, genetically attenuation is another trend for the development of whole sporozoite vaccine [35]. The UIS3 and UIS4 proteins are essential for sporozoite development in hepatocytes and these proteins are part of the parasitophorous vacuolar membrane (PVM). The encoded genes of these proteins were investigated as targets for genetically attenuated pre-erytrocytic vaccine development. Individually, *Pb*UIS3 and *Pb*UIS4 knockout parasites developed CD8 T cell response. However, *Pb*UIS4 knockout parasites could grow in blood stage. The P36, P36p (P52), and B9 genes were other targets for genetically attenuated vaccine development, but despite gene deletion, parasitemia was observed in humans for each target. In addition, the asparagine-rich protein 1 (SAP1) was studied for genetically attenuated sporozoite development. In that study, *Pysap1(-)* parasites failed to complete liver stage development, parasitemia was not observed, and long-lasting protection was achieved. The development of successful pre-erytrocytic stage vaccine requires new potent target and intense studies [36].

2.4.3.2 Transmision blocking vaccine

The transmission-blocking vaccine targets blood stage gametocytes or sporogony-specific antigens and stops the development of the parasite in the mosquito vector. A mosquito easily transmits malaria up to 100 meters after biting a malaria-infected patient. Therefore, this approach aims to reduce the infectivity of mosquitoes and prevent the spread of the disease. Pre-fertilization, and post-fertilization antigens are targeted for transmission-blocking. Surface antigens of gametocytes and gametes such as *Pf*s48/45, *Pf*s47, and *Pf*s230 are mainly examined in pre-fertilization. *Pf*s25 is the most studied post-fertilisation antigen and localize at ookinate and zygote surface because this antigen is highly immunogenic and has low polymorphism. Another antigen is studied for transmission blocking vaccine is anopheline alanyl aminopeptidase N1 which localizes mosquito midgut protein. Transmission blocking vaccine approach is mainly studied on *Plasmodium falciparum* and *Plasmodium vivax* species. Combination of *Pf*s25 antigen and Alhydrogel adjuvant and *Pf*s25-EPA: *Pf*s25 conjugation have been tested in adults in the United States. However, the functional activity of the induced anti-*Pf*s25 antibodies was modest, and antibody titers declined rapidly. To address the problem of low immunogenicity and stability, the *Pf*s230 antigen was used alone and in combination with the *Pf*s25 antigen. Studies with *Pf*s230D1M-EPA in rhesus monkeys showed a stronger immune response, but the combination of *Pf*s230D1M-EPA/Alum did not significantly increase serum functionality. Therefore, new targets are needed to develop an efficient transmission blocking vaccine [37].

2.4.3.3 Blood stage vaccine

Blood stage vaccine aims to damage asexual erythrocytic stage which is associated with symptoms and death. Parasites multiply until rupture of erythrocytes, large percentage of merozoites are released into the bloodstream and infect other erythrocytes. Each cycle is accompanied with fever, chills, headache, muscle aches, and fatigue. The duration of the cycle varies depending on the *Plasmodium* species. For example, the cycle duration is 1 day for *P. knowlesi*, 2 days for *P. falciparum*, *P. vivax*, and *P. ovale*, and 3 days for *P. malariae*. The blood stage is very attractive for vaccine development. Malaria infection induces IgG production. Therefore, in

previous study, IgG isolated from malaria immunized adults and injected into malaria infected children. Parasite clearance has been observed [10].

The conserved and low polymorphic protein *P. falciparum* reticulocyte-binding protein homolog 5 (*Pf*RH5) is responsible for red blood cell invasion. In initial clinical trials, *Pf*RH5 was administered through viral vectors and the results showed tremendous persistence of the neutralizing antibodies. Moreover, combination of *Pf*RH5 expressed viral vector and adjuvant were injected in the group of macaques and protective immunity against heterologous parasites was observed. However, studies in monkeys have shown very low levels of induced antibodies. This means, vaccine cannot protect for a long time. In addition, a very high amount of anti-*Pf*RH5 IgG is required for protection, but this could not be achieved by vaccination. Therefore, new strategies have been developed with *Pf*RH5 to eradicate these limitations, such as virus-like particles or *Pf*RH5 in Drosophila cells [38, 39].

Another blood-stage vaccine candidate is the AMA-RON2 complex, which is essential for erythrocyte invasion by merozoites. The highly antigenic AMA-RON2 complex induces a large amount of anti-invasion antibodies. Also, studies in monkeys showed protection against heterologous parasites. However, AMA1 exhibits extensive sequence variation. Therefore, new studies are needed to develop chimeric sequences. *P. falciparum* erythrocyte membrane protein 1 adheres erythrocyte membrane receptor and causes severe malaria symptoms. This antigen induces protective immunity, but it has highly polymorphic sequence, large size, and cysteine-rich conformational structure which complicate the using as target for blood-stage vaccine development. *P. falciparum* tropozoites express glutamic acid-rich protein to infect red blood cells [40]. Anti-*Pf*GARP antibodies induced apoptosis and killed trophozoite-infected erythrocytes in an *in vitro* culture. Furthermore, partial protection against *P. falciparum* was achieved in monkeys immunized with *Pf*GARP antigens. In a human experiment, Tanzanian children vaccinated with antibadies without anti-*Pf*GARP antibodies were at a 2.5-fold increased risk of developing malaria. Thus, combining *Pf*GARP proteins with other malaria targets can increase the potential efficacy of vaccination [41]. *Plasmodium* antigens have role not only for invasion and but also shizont rupture. For example, anti-*Pf*SEA1 antibodies binded *Pf*SEA1 and blocked shizont rupture of *P. falciparum* in an *in vitro* study. Reduced parasitemia was observed in mice immunized with recombinant *Pb*SEA1 protein [42]. A large number

of blood-stage-based subunit vaccine candidates have low efficacy, short-term protection, antigen polymorphism, and conformational dependence. This leads to safety concerns. As a dissapointing results of the blood-stage subunit vaccine forced scientists to find another approach and whole blood stage vaccine has gained importance as an alternative to subunit blood stage vaccine [10].

Whole blood stage vaccine

The positive effect of heat on mental health has been known for centuries. First, Hippocrates recognized that malaria could decrease epileptic symptoms as heat therapy. In the early 1900s, Julius Wagner Jauregg used malaria as a therapeutic agent, inoculating whole malaria parasites to treat dementia paralytica. He won Nobel Prize with that treatment. After malaria based neurological treatment, induced patients had protection against malaria [43]. This invention directed scientist to use low dose blood stage parasite for malaria immunization. As a result of this, several low-dose *P. falciparum* blood stage samples were injected into naive humans and then treated with antimalarial drugs. This study resulted up to 75% protection. The first approach to a whole blood stage vaccine was identified in the 1940s using animal models with ducks (immunized with *Plasmodium lophuraei*) and monkeys (immunized with *Plasmodium knowlesi*). Whole parasite vaccine delivers broad range of antigens to the immune system and, unlike a subunit vaccine, provides stronger protective immunity. Not only rodents, but also humans immunized with whole attenuated sporozoites produced by irradiation, genetic or chemical ways induced sterile protection against homologous wild type sporozoites. The main concern is the delivery of cryopreserved sporozoites under sterile and purified conditions. Because the sporozoites complete their development in the mosquito vector. The development of the whole blood stage parasite is a good alternative because of the well-explained mechanism and ease of production. Thus, many vaccines have been developed with killed or live attenuated blood stage parasites [44].

Whole killed blood stage vaccine

Killed blood-stage parasites were first tested in ducks and monkeys and protective immunity was observed. This approach has also been used to develop vaccines for humans. Low dose killed blood stage parasites and adjuvant combination elicited T-cell response. Killed blood-stage vaccines have several advantages. Killed blood-stage parasites cannot replicate in the host and have no virulence. However, immunity must

always be boosted with an adjuvant, and multiple vaccinations are required for protective immunity [9].

Whole radiation attenuated blood stage vacine

Radiation attenuated blood stage *P. berghei* parasites were used to immunize mice and protection was observed for both malaria and cerebral malaria. In another study, 100 kilorads of radiation were used for parasite attenuation. The high parasite dose protected against parasite development, severe symptoms and cerebral malaria, but multiple low parasite doses did not induce immunity [45]. In this approach, the main concern is determination optimal radiation dose, because all parasites may not be attenuated, and severe malaria infection might be observed. To address this issue, avirulent *Plasmodium* species are recommended for safe protective immunity. This approach was also used with human *Plasmodium* species, but avirulent parasites converted to the virulent version. Attenuation of radiation was aimed at molecular and cellular attenuation. However, normal growth of parasites was observed, which means that some parasites were not attenuated or attenuated parasites could repair themselves. Therefore, the mechanism of radiation attenuation should be understood in detail for vaccine development [46, 47].

Whole chemically attenuated blood stage vacine

Chemical attenuation approach combines the wild-type parasite induction and drug treatment. Centanamycin, tafuramycin-A, doxycycline, or azithromycin are most preferred drugs fo chemically attenuated blood stage vaccine development. In one study, mice infected with *Plasmodium* were treated with seco-cyclopropyl-pyrroloindole analogues and then challenged with sporozoites. Then protective immunity was subsequently observed. In that study, the poly-A tail of the parasite's DNA was alkylated and blocked. However, genotoxic activity of this drug has been demonstrated [48]. In another study, mice were infected with a low dose of blood stage *P. chabaudi* parasites and treated with 2M centanamycin or tafuramycin-A. These mice were protected against both homologous and heterologous parasites for up to 6 months. Induction of CD4+ T cells and CD8+ T cells was observed, but there was no antibody induction. No parasitemia developed in vaccinated mice, but parasite DNA was detected in blood sample. This means that there were live parasites or undetectable parasites in the bloodstream. The blood of the immunized mice was transferred to other naive mice, but no protection was observed after challange. Centanamycin or

tafuramycin-A were also used *P. falciparum* attenuation. Parasitemia did not observed in *in vitro* studies. *In vivo* studies focused on setting the effective dose for chemical attenuation. More data are needed to characterize the immune response and evaluate protective efficacy. Apicoplast is a key organel for lipid metabolism and was used as another target for the development of a chemically attenuated blood stage vaccine. Tetracyclines inhibit protein expression encoded by the apicoplast genome, and the parasite cannot produce the apicoplast. Until effect of the medication, *P. falciparum* could complete one cycle of replication and increased the vaccine immunogenicity. This approach has the potential to develop future clinical trials [49, 50].

However, cellular tropism and sequestration limits chemical attenuation-based treatment. *P. yoelii* and *P. vivax* primarily infect reticulocytes. Another difference is the mechanism of immunity. Chemically attenuated *P. chabaudi*, for example, induce T cells. Chemically attenuated *P. yoelii*, on the other hand, mainly induce the production of antibodies, which are important for long-term protection. Therefore, new studies are needed to develop an effective chemically attenuated blood stage vaccine [44].

Whole genetically attenuated blood stage vacine

Genetically attenuated parasites are developed based on deletion or inactivation of essential metabolic pathways [51]. The first study to produce a genetically attenuated blood stage vaccine targeted deletion of the *P. yoelii* nucleoside phosphorylase (PNP) gene responsible for recycling and recovery of purines. Mice were immunized with a single dose of *Pypnp(-)* parasites ($2x10^4$- $2x10^5$). Parasitemia was detected up to 30%, but the development of knockout parasites was slower than the control group [52]. Plasmepsin-4 is accepted as another target for genetically attenuated vaccine. Plasmepsin-4 expresses aspartic proteinase enzymes that digest hemoglobin. However, deletion of the plasmepsin-4 genes of *P. falciparum* and *P. berghei* slightly reduced blood stage parasite development compared to the control group. In another study, both the plasmepsin-4 and merozoite surface protein-7 genes were deleted and more successful attenuation was achieved compared with single gene deletion. Double gene deletion of parasites induced strong immunity to both homologous and heterologous parasites in a mouse study. These genes might be potential targets for vaccine development against human malaria parasites as well [53]. Erythrocyte membrane protein 1 is a virulence protein expressed in red blood cells during blood

stage development and exported to the erythrocyte surface. This protein binds infected erythrocytes to the endothelial blood vessel to hide from the spleen-dependent killing mechanism. However, *Pf*EMP1 protein is encoded by 60 var genes per genome and all genes could not be successfully deleted [54]. Therefore, another target protein, Knob Associated Histidine Rich Protein (KAHRP), was investigated as target for genetic attenuation. This protein is responsible for the formation of knob structures on red blood cells. The safety and infectivity of the *Pfkahrp(-)* parasite has been demonstrated in human studies. However, the protection and infectivity mechanism of the vaccine needs to explain in detail [44].

2.4.4 Nucleoside transporter family in *Plasmodium* life cycle

2.4.4.1 Nucleoside transporter gene family as a target to develop genetically attenuated blood stage parasites

Plasmodium species are purine auxotrophs like all other parasitic protozoas. Nucleoside transporters (NTs), a distinct class of proteins are essential for intake nucleosides from the host to the parasite [55]. *Leishmania* and *Trypanosoma* are most studied species for NTs, but also important genetic and molecular research have been done on *Plasmodium* and *Toxoplasma* [51]. *Leishmania donovani* has two different nucleoside transporters *Ld*NT1 and *Ld*NT2 (NTs). *Ld*NT1 transports adenosine and pyrimidine, while *Ld*NT2 transports inosine and guanosine nucleosides [55]. NT3 protein is expressed by *L. major* species, and only intakes purine nucleosides [56]. *Trypanosoma brucei* has *Tb*NT2-*Tb*NT10 proteins which are known to transport purine nucleosides and nucleobases [57]. Purine and pyrimidine are also necessary for the life cycle, growth, and cell replication of *Plasmodium* parasites. Purine and pyrimidine are vital for several critical processes, including DNA replication, protein synthesis, ATP and ADP synthesis, and cell signaling. Studies showed that *Plasmodium* parasites take up purines into the cell via a combination of endogenous transporters and novel permeation pathways. Subsequently, purines enter the parasitophorous vacuole via the non-selective large pores of the parasitophorous vacuole. Within the vacuole, the purines pass through the parasite plasma membrane. This process is described as a rapid, equilibrium, low-affinity process. The genome sequence of *Plasmodium* was compared with identified nucleoside transporter sequences of protozoa and mammals. This study demonstrated; nucleoside

transporters localize at the *Plasmodium* membrane surface [58]. Human malaria parasites encode four nucleoside transporter genes (NT1, NT2, NT3, and NT4) that are expressed in the blood stage of the parasite. However, rodent malaria parasites do not have nucleoside transporter 3. The mechanism of *Pf*NT1 was determined using the *Xenopus* oocyte expression system. *Pf*NT1 mRNA was transferred into oocytes and the uptake of adenosine and hypoxanthine increased [59]. Subsequently, immunoelectron microscopy was used to determine the localization of nucleoside transporter gene 1, and the results showed that all NT1-expressed proteins were specifically placed at the plasma membrane [60]. In another study, the *Pf*NT1 gene was deleted. NT1 knock out parasites failed to intake most of essential purine nucleosides and only survived in physiological purine-containing media. Therefore, purine salvage pathway has high potential target for the development of a genetically attenuated blood stage vaccine. *Pf*NT1 and *Py*NT1 share approximately 60% amino acid identity, and both have nine trans-membrane domains. Therefore, similar results were expected for nucleoside transporter gene 1 for both *Plasmodium* parasites. In 2010, this pathway was studied by Aly etal. to develop a genetically attenuated blood stage vaccine. In this study, the *Py*NT1 gene was deleted by homologous recombination. Parasitemia was not observed immunization with 50 blood-stage *Pynt1(-)* parasites into a group of BALB/c mice. In another grup of mice immunized with 5000 blood-stage *Pynt1(-)* parasites and only negligible amount of parasitemia was observed. Two different group of mice immunized with 1000 *Pynt1(-)* parasites and challenged with *Py*WT blood stage parasites and *Py*WT sporozoites, respectively. 100% sterile protection was observed for both studies. Subsequently, *Pynt1(-)* injected group of mice were challenged with *P. berghei* WT parasites, and partial protection was observed [4].

Nucleoside transporter 2 has the greatest sequencing similarity to nucleoside transporter gene 1. However, immunofluorescence and immunoelectron microscopy results show that the protein expressed by *Pf*NT2 is localized in the endoplasmic reticulum. In addition, yeast studies have shown that *Pf*NT2 has intracellular localization and is responsible for nucleoside/nucleobase transport [60]. Nucleoside transporter 3 is expressed by human malaria parasites however function and mechanism of this gene is not clear. The mechanism of nucleoside transporter 4 was explained based on the oocyte expression system of *Xenopus laevis* using radiolabeled

substrates. The substrates adenine and 2'-deoxyadenosine are transported into the cell by nucleoside transporter 4. In addition, hypoxanthine, which is known to be a substrate of the purine-salvage pathway, and AMP did not accumulate in the oocytes. In addition, *Pf*NT4 and *Pv*NT4 were inhibited cytokinin compounds and dipyridamole. Thus, nucleoside transporter 4 was explained as purine transporter with excellent substrate and inhibitor specificity. However, the physiology of the parasite was not explained in detail [61].

Development of genetically attenuted malaria vaccine by CRISPR-Cas9 system

Bacteria and archea developed several defence system to protect themselves against foreign nucleic acids such as viral genomes and plasmids. These defence systems are prevention of phage adsorption, blocking of phage DNA injection, phage abortive infection. The CRISPR-Cas9 system stores immunological knowledge of bacteria for future invasion by viruses. The CRISPR-Cas9 system was declared 90% for archaea and 50% for bacteria [62]. The CRISPR loci was discovered as a palindromic repeat in the iap gene of *E. coli*. Then the CRISPR system was screened in other bacterial and archaeal species such as *Mycobacterium tuberculosis*, *Streptococcus pyogenes*, *Methanocaldococcus jannaschii*, *Haloferax mediterranei*, *Haloferax volcanii*, *Thermotoga maritima*. Invasion by the same bacteriophage helped to evaluate Cas proteins and guide RNAs with different composition, structure, and nucleic acid targets that are genetically engineered and used for gene deletion, tag insertion, transcriptional activation/repression, and epigenetic modification [63]. Genome editing technologies such as ZFNs, TALENs, and CRISPR-Cas9 create double-strand breaks at specific sites in DNA. Genetic engineering has modified this technology for cell repair. Cas9 or Cas12a are the preferred endonucleases to generate specific gene deletions in cells using CRISPR technology. Moreover, ~20 nucleotide target-specific sgRNA is another essential element of CRISPR. The target sequence should be unique in the genome and downstream of the target should have Protospacer Adjacent Motif (PAM) site. PAM provides a type of binding signal for Cas9, but the exact digestion sequence is determined by the type of selected Cas9. gRNA and Cas9 interact on the gRNA scaffold to form a positively charged, groove-shaped ribonucleoprotein complex. Binding of gRNA activates Cas9 to bind DNA. The spacer region of gRNA is free to bind to the target DNA. The gRNA directs Cas9 to bind precisely to the target site on the DNA, and annealing begins at 8-10 bases at the 3' end of the gRNA in a 3'

to 5' direction. Cas9 then undergoes a conformational change (RuvC and HNH) to break the complementary sequence of the target DNA at ~3-4 nucleotides upstream of the PAM site. As a result, a double-strand break of the DNA occurs at the target site. There are two main pathways for repairing double-strand breaks in eukaryotic cells. These are non-homologous end joining (NHEJ) and homology directed repair (HDR). Non-homologous end joining is the major repair mechanism, but it causes nucleotide insertions or deletions, which in turn lead to amino acid deletions, insertions, or frameshift mutations. Homology-directed repair has relatively low efficiency and is observed mainly in phases S and G2 of the cell cycle [64].

Many organisms repair the breaks in their genome through the error-prone mechanism of non-homologous end joining, which leads to gene disruption. However, *Plasmodium* species do not have a non-homologous end joining mechanism. Gene disruption is limited in *Plasmodium*, but off-target effects also decrease. Bioinformatics was used to identify many Cas9 endonuclease. Among them, *Streptococcus pyogenes* Cas9 is the most preferred endonuclease, especially for *Plasmodium* studies. For the success of the homology-guided repair pathway, the CRISPR-Cas9 system needs to deliver the three components Cas9 endonuclease, sgRNA and donor plasmid into the *Plasmodium* in different strategies. A potential gRNA consists of 20 nucleotides and is located upstream of the protospacer-adjacent motif (PAM site) (-NGG). Online tools such as Benchling, Protospacer, CHOPCHOP, and EuPaGDT are used to determine the PAM site and off-target mutations. *Plasmodium* species have an A-T -rich genome, so the possibility of active targeting is lower. As a consequence of the AT -rich genome, poly-T segments could lead to premature termination by RNA pol III and T7 RNAP and should be avoided. To minimize the off-target effect, the gRNA should be selected as close to the target site as possible. For a single point mutation, the gRNA should be 100-200 bps away from the target. The efficiency of the gRNA can be increased by using multiple gRNA in a single cell. The design of donor plasmids depends on the intended uses, such as gene disruption, modification of single nucleotides, tagging, or marker-free insertion of fluorescent reporters. In addition, positive or negative selection markers on the donor plasmid or CRISPR plasmid can be designed to determine gene-edited cells. Moreover, episomes can be eliminated upon transfection with a linearized donor plasmid [12].

The first CRISPR-based *Plasmodium* study was conducted by Ghorbal et al. and Wagner et al. In this study, the CRISPR element and the donor plasmid were inserted separately into *Plasmodium falciparum*. The most important problem is the onset of gRNA expression from the first nucleotide. This problem was solved by using the U6snRNA promoter or a T7 phage promoter. Secondly, CRISPR was used to increase transfection efficiency and achieve genetic modifications of *P. knowlesi*. In addition, the functional analysis of some genes of *P. knowlesi* was explained and the results were compared with those of *P. yoelii* to determine similarities in evaluation. The positive selection marker dihydrofolate reductase (DHFR) is widely used for CRISPR technology, either in the form of a single plasmid or in the form of two plasmids. SpCas9 and the DHFR selection marker successfully edited the genome of *P. yoelii*, but elimination of the plasmid was difficult. So, negative selection marker, the bifunctional yeast fusion cytosine deaminase/uracil phosphoribosyl transferase (yFCU) gene, ensured elimination of the gene and activated sensitivity to anti-folate drugs for re-editing. To increase the efficiency of gene deletion and tagging RNA polymerase II promoter transcribed ribozyme-guide ribozyme system in *P. yoelii*. This study has shown that it is possible to use multiple gRNA for gene replacement [12].

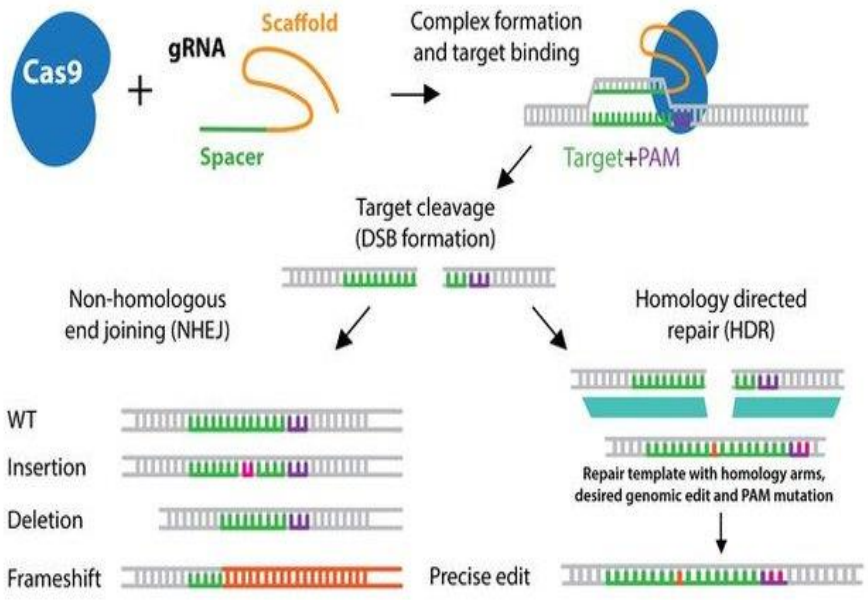

Figure 2. 3: Gene editing mechanism by CRISPR-Cas9 Technology [65].

3. MATERIAL AND METHODS

3.1 Ethics Statement

The Animal Ethics Committee at Bezmialem Vakif University, Istanbul, Turkey (2020/141), authorized all animal care and handling practices. Every effort was made to minimise animal suffering.

3.2 Animals, Parasites and Mosquitos

6-8 week-old female BALB/c, C57BL/6 or CD1 mice were purchased from Bezmialem Vakif University, Experimental Animal Research Centre. BALB/c, CD1, C57BL/6, and female Wistar rats were infected with *P. yoelii* 17X- NL (*Py*WT), *P. berghei* ANKA (*Pb*WT), *Pbnt1(-),* and *Pynt1(-).* The life cycle of the *Pbnt4(-)* mutant and *P. berghei* ANKA (PbWT) was studied in CD1 mice and Anopheles stephensi mosquito vectors. Mosquito cages contained ~500 mosquitoes/cage and infected mosquitoes were maintained in sugar water at 24°C and 70% humidity.

3.3 Chemicals, Enzymes and Kits

RPMI, FBS, Giemsa, Accudenz, PBS, Heparin, Pyrimethamine, Alsevier, saponin, dNTP, ethidium bromide, agarose, ampicilin, Tris-HCl, KCl, Tris-base, disodium EDTA, glacial acetic acid, DMSO, gentamycin, glycerol, ethanol, mehanol, SacII, BamHI, HindIII, KpnI, BbsI, EcorV, mytaq red mix, Q5 DNA polymerase, Ligase, Ligase Buffer, Cutsmart Buffer, QIAprep Spin Miniprep Kit, QIAquick Gel Extraction Kit, Qiagen DNeasy Blood&Tissue kit have been used in this study.

3.4 Buffers, Broths and Stock Solutions

<u>*Luria-Bertani (LB) Broth:*</u> 25 g LB Broth powder was dissolved in 1 L ddH_2O. The mixture was autoclaved for 121 °C, 15 min.

Terrific Broth: 47.6 g Terrific Broth powder was dissolved in 1 L ddH_2O. The mixture was autoclaved for 121 °C, 15 min.

Luria-Bertani (LB) Agar: 1 LB agar capsule was dissolved in 1 L ddH_2O. The mixture was autoclaved for 121 °C, 15 min. ~ 25 mL of sterile agar was poured into a 90 mm Petri dish.

Ampicillin stocks: 100 mg/mL ampicillin stocks were prepared and sterilized using 0.22 µm syringe filters.

50X TAE buffer (Tris-acetate-EDTA): Tris-base (242 g), disodium EDTA (18.61 g), and glacial acetic acid (57.1 mL) were dissolved in ddH_2O (total volume: 1 L).

Accudenz density gradient buffer for parasite culture: 5 mM Tris-HCl pH 7.5, 3 mM KCl and 0.3 mM EDTA were dissolved in ddH_2O to prepare a stock solution. Accudenz powder (27.6 g) was dissolved in 100 mL stock solution. The buffer was autoclaved at 121°C for 15 minutes and stored at 4°C.

Parasite culture media: RPMI 1640 (141.25 mL), FCS (35 mL), and gentamycin (70 µL) were mixed and sterilized using a 0.22 µL vacuum filter.

Pyrimethamine solution for parasite selection: 95 mg pyrimethamine was dissolved in 10 mL DMSO using a vortex mixer. The solution was mixed with 490 mL of deionized water and the pH was adjusted between 4 and 4.6.

Giemsa staining solution: Giemsa stain is diluted to 10% with ddH_2O and used for staining thin blood smears.

Freezing Solution: 5 ml glycerol was dissolved in 45 ml Alsever's solution using a vortex mixer.

3.5 Devices

Class II Biological Safety Cabinet (Thermo Scientific Maxisafe 2020), CO_2 Incubator (Thermo Scientific™ Forma™ Steri-Cycle™ i160 CO_2 165 L Incubator, UV/VIS Spectrophotometer (Nanodrop), pH Meter (Hanna Edge), Water Purification System (Sartorius), Thermal Cycler (Biorad T100), Agarose Gel Electrophoresis (Biorad), Gel Imaging System (Fusion S), Vortex (Fisher Scientific), Water Bath (Thermo Scientific, Precision GP 15D), Microbalance (Ohaus), Incubator Shaker (Infors HT Ecotron), Incubator (Thermo Scientific Heratherm), Magnetic Stirrer (Biorad),

Refrigerated Centrifuge (Beckman Coulter, Allegra X-30R, Thermo Scientific MicroCL 21R), Ice Maker (Scotsman AF80), Phase-contrast microscopy (Leica DM 2500 LED), Confocal Microscopy (Leica SP8Flow Cytometer (Biorad S3e).

3.6 Plasmids

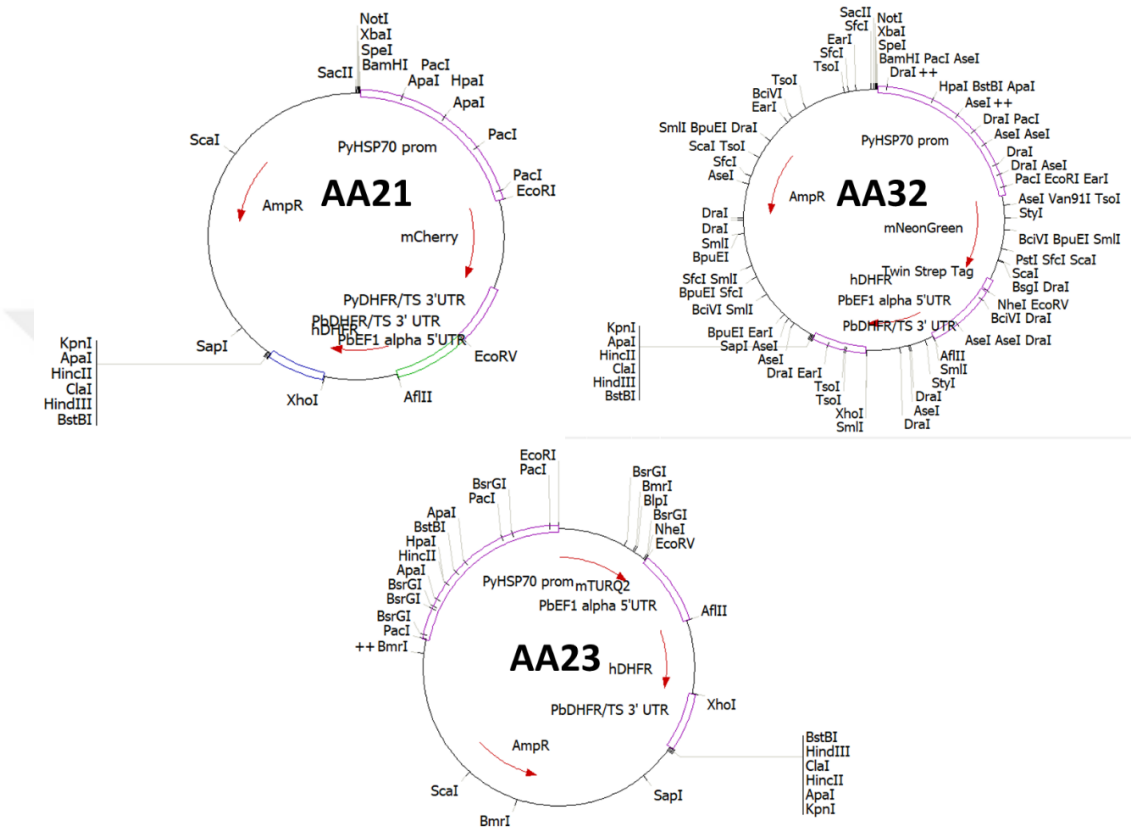

Figure 3. 1: Maps of vectors used as templates for *Pb*NT1, *Py*NT1, *Pb*NT4 donor plasmids and CRISPR plasmid.

3.7 Nucleoside Transporter Gene 1

3.7.1 Generation of donor plasmids for *P. berghei* and *P. yoelii*

3.7.1.1 Genomic DNA isolation for *P. berghei* and *P. yoelii*

BALB/c donor mice were infected intraperitonally with frozen *Py*WT infected blood sample using a 27-gauge needle. The same step was used for *Pb*WT infection of the CD1 donor mice. Parasitemia of the donor mice was detected every two days by Giemsa staining of thin blood smears. Infected blood samples were collected by cardiac puncture when parasitemia was between 1-5%. Leukocytes and platelets and were removed from each blood sample by passing them through a cellulose column

run separately with PBS buffer. Infected erythrocytes were pelleted by centrifugation (1500 rpm, 8 min, RT, Acc:6, Decc:2). The supernatant was removed and infected erythrocytes were lysed with 0.1% saponin solution. Parasites were pelleted by centrifugation (3600 rpm, 8 min, acc:6, decc:2) and parasite genomic DNA was extracted using the Qiagen DNeasy Blood&Tissue Kit protocol.

3.7.1.2 Generation of PCR Products from Isolated genomic DNAs

The coding and regulatory sequences of the nucleoside transporter 1 (NT1) genes of *P. berghei* and nucleoside transporter 1 gene of *P. yoelii* were obtained from PlasmoDB (Plasmodium database resource). The left and right arms (5' UTR and 3' UTR of NT1) of the inserts which is responsible for double homologous recombination were amplified. Primers were designed for amplification of the left and right arms from genomic DNA of *P. berghei* and *P. yoelii* by PCR (Table 3.1).

Table 3. 1: Details of primer sequences used to prepare left and right homology arms in *Py*NT1 and *Pb*NT1.

Primer	**Sequence (5'-3')**
Construct Preperation	
Pynt1L-F	GGCCGCGGTTTCCATTTATGCATGTAATATGGTTTTACCTTG
Pynt1L-R	TCCGGATCCTATACCACTAGATCATTCTTTAATTTTACTCAT
Pynt1R-F	TGCCAAGCTTTACAAATATAGTTCAGCAATGCATTTGTATGGCT
Pynt1R-R	TCCGGTACCTACGTATATAACATACATCTCGAATGGCTATTGC
Pbnt1L-F	GGCCGCGGACATGACTATATTCCCAAAATAATAATAACACG
Pbnt1L-R	TCCGGATCCTATACCACTAGATGACTATTTAATTTTACTCAT
Pbnt1R-F	TGCCAAGCTTTCCTACGTTTATTATTCATCCCATGGTTTGTATT
Pbnt1R-R	TCCGGTACCTCTCGAATGGCTATTGCTGCTGATAGCCAATGGT

3.7.1.3 Cloning

Amplified left and right arms of *Pb*NT1 cloned into the AA21 plasmid vector flanked by mCherry protein and hDHFR (Figure 3.1). Amplified left and right arms of *Py*NT1 cloned into the AA32 vector flanked by neon green protein and hDHFR (Figure 3.1). For both species left arm was inserted into the plasmid between SacII-BamHI, right arm was inserted into the plasmid between HindIII-KpnI restriction enzyme sites. The targeted *Pb*NT1 and *Py*NT1 fragments were designed not to affect the coding sequences of upstream or downstream neighboring genes. The cloned plasmids were verified by a Sanger sequencing commertially [4].

3.7.2 Design and synthesis of CRISPR-Cas 9 elements

3.7.2.1 CRISPR plasmid construction

5'UTR to drive expression of the coding high-fidelity Cas9 sequence flanked at both ends by a 3X FLAG tag (to confirm Cas9 localization) and an NLS (nuclear localization signal), followed by a 3'UTR. In the sgRNA cassette, the 5'RNA U6 promoter is followed by a gRNA adaptor site directly attached to the scaffold sequence (Cas9 attachment site) and immediately followed by a 3' terminator sequence. Sequences were designed and codon-optimized using Clone Manager software. A gene synthesis company supplied the desired sequences with a custom enzyme interface that incorporated the synthetic sequences into the efficient plasmid. The individual cloning steps are detailed below. The Cas9 sequence was arranged in two fragments and cloned into laboratory-modified plasmid AA21 (Figure 3.1) with restriction enzyme sites EcorI-EcorV and KasI-NheI, respectively. Then the sgRNA cassette was amplified and cloned into the CRISPR plasmid using the restriction enzyme XbaI-BamHI. Another cassette cloned into the CRISPR plasmid is hDHFR-T2A-mTurquoise with AflII-XhoI.

3.7.2.2 Design of sgRNA for nucleoside transporter 1 editing

*Pb*NT1 and *Py*NT1 genes are genetically 95.23% identical. Therefore, the same CRISPR element was designed for both *P. berghei* and *P. yoelii* species. The coding sequences of *Pb*NT1 and *Py*NT1 were manually searched to find the AGG or TGG PAM sites. The off-target binding efficiency of the sgRNA was analyzed using the Benchling online tool. The forward and reverse primers (complementary to each other)

of the determined sgRNA (20 nucleotides) were synthesized with specific overhangs for cloning into the CRISPR plasmid BbsI restriction enzyme site from Sentebiolab Biotechnology. The forward and reverse primers of the sgRNAs were phosphorylated and annealed by PCR as in the following protocol:

1 µl sgRNA Forward (100 µM)

1 µl sgRNA Reverse (100 µM)

1 µl 10X T4 ligation buffer (NEB)

6.5 µl ddH_2O 0.5 ul T4 PNK (NEB)

10 µl total

PCR parameters:37 °C 30 min; 95 °C 5 min and then cooling to 25 °C at 5 °C/min.

The CRISPR plasmid was digested with BbsI and the annealed sgRNA was cloned into the backbone CRISPR plasmid. Positive colonies were selected and verified by test digestion and sequencing [66].

Table 3. 2: *Py*NT1 and *Pb*NT1 sgRNA Forward and Reverse Primers

Primer	Sequence
ny+b NT1 gRNA+F	TATTTCTGCAGGTATTGGAATGTC
ny+b NT1 gRNA+R	AAACGACATTCCAATACCTGCAGA

3.7.3 Parasite cell culture and transfection

3.7.3.1 *In vitro* parasite culture

For *P. yoelii* culture 6 female BALB/c mice of 6-8 weeks were infected with frozen *P. yoelii* 17X-NL G0 stocks (G0: Blood stage parasites from sporozoite induced blood stage culture). 1-2 blood drops were collected from the tail of the mouse and parasitemia was calculated during 1 week. Approximately 1 ml of infected blood was collected from each mouse with parasitemia of 1-3% by cardiac puncture using a 1 ml 26-gauge syringe. 5-6 ml of the collected blood was transferred to compatible medium (RPMI, FBS, gentamycin, heparin). The cells were pelleted by centrifugation (300 g, 8 min, RT, Acc:3, Decc:1). The supernatant was discarded from the cell pellet. Cells were resuspended in 150 ml of complete culture medium in a 500 ml Erlenmayer flask. The flask was agitated for 12 hours in an incubator with a gas mixture of 5% CO_2 and

5% O_2 on a shaker (45 rpm). For *P. berghei* culture, 6 female CD1 mice of 6-8 weeks were infected with frozen *P. berghei* ANKA G0 stocks and same protocol was followed mentioned for *P. yoelii* mentioned above. *P. berghei* culture was incubated 16-17 hours in an incubator with a gase mixture of 5% CO_2 and 10% O_2 on a shaker (45 rpm) [4].

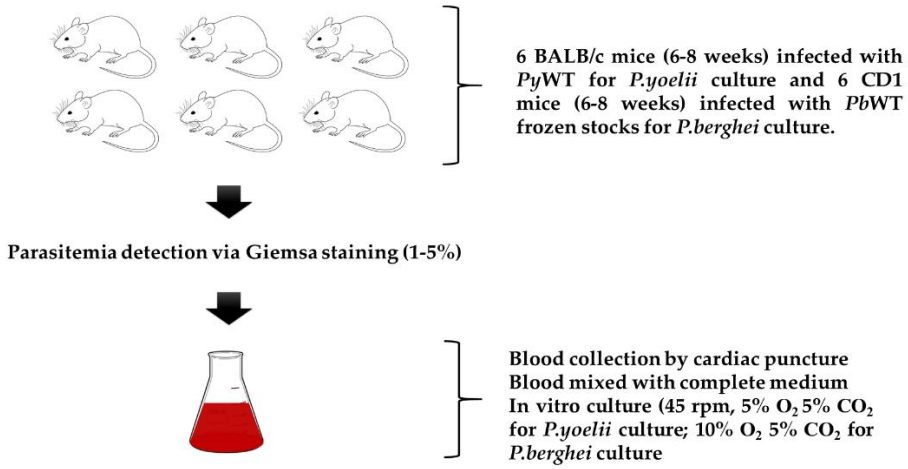

Figure 3. 2: *In vitro* parasite culture for *Py*WT and *Pb*WT.

3.7.3.2 Purification of the mature shizonts

Accudenz/PBS (55% for *P. berghei* shizont purification; 60% for *P. yoelii* shizont purification) solution was prepared for density gradient separation. The culture suspension containing the shizonts was transferred to four 50 ml tubes (35 ml/tube). 10 ml of the Accudenz solution was carefully added to each tube under the culture suspension using a 10 ml pipette. The culture tubes were centrifuged in a swing-out rotor at 300 g, 25 min, RT, Acc:0, Decc:0. The brown layer at the interface between the two suspensions was carefully collected. 20 ml of culture medium was added to the shizont and pelleted by centrifugation 300 g, 10 min, RT, Acc:0, Decc:0. The supernatant was discarded (Figure 3.3A) [4].

3.7.3.3 Electroporation of schizonts with donor and CRISPR plasmids

The *P. yoelii* shizont pellet was resuspended in 400 µl of culture medium. 25 µl of the shizont pellet was transferred to 1.5 ml microcentrifuge tubes. 100 µl Human T-cell Nucleofector solution buffer, linerized *Py*NT1 donor plasmid (5 µl, 5 µg) and CRISPR plasmid (5 µl, 5 µg) were added to the shizont pellet. Donor plasmids were lineraized with SacII-KpnI to minimize unexpected recombination and allow efficient integration

of the donor plasmid. Transfection was performed using the Lonza Nucleofector 2b. After transfection, 50 µl of culture medium was immediately added to the electroporation cuvette. 150 µl of the transfected parasite suspension was injected into the tail veins of the mice using 27-gauge insulin syringes. The same steps were followed for *Pb*NT1 donor plasmids with transfection into *P. berghei* shizont pellet (Figure 3.3B) [4, 67].

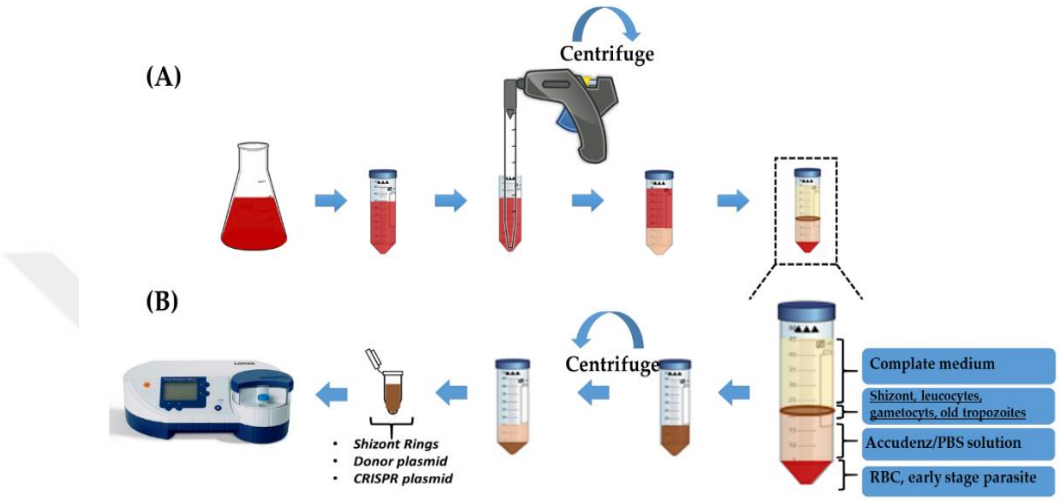

Figure 3. 3: The schematic diagram for shizont purification (A) and electroporation (B).

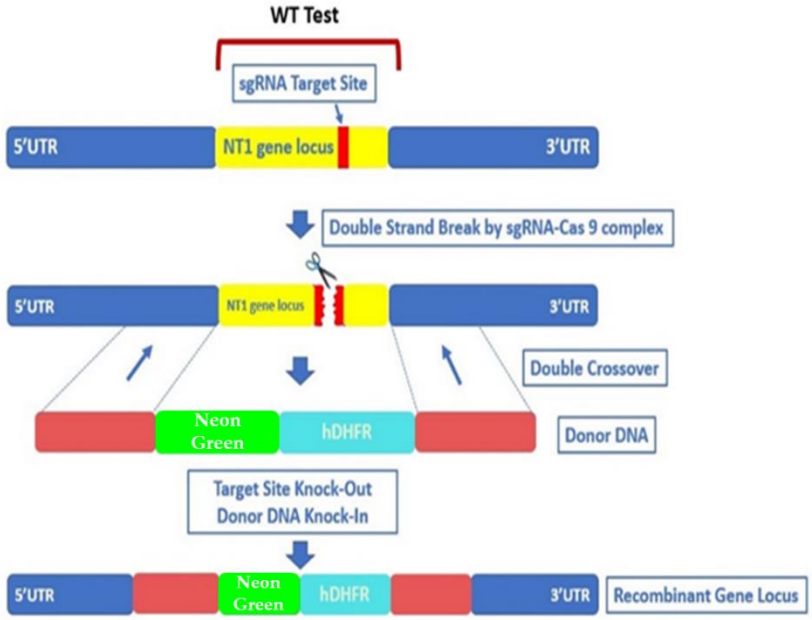

Figure 3. 4: *P. yoelii* Nucleoside transporter 1 deletion by CRISPR-Cas9 Technology. Same steps were followed by *P.berghei* Nucleoside Transporter 1 deletion with donor plasmid flanking by mCherry casette.

3.7.4 Determination of knockout nucleoside transporter 1 parasites for *P. yoelii* and *P. berghei* species

3.7.4.1 Drug selection for transfected parasites

Transfected groups of mice were treated with pyrimethamine in drinking water for 72 hours, a day later infection. Parasitemia was calculated each day after transfection by Giemsa staining. The parasitemia of the drug-treated mice decreased rapidly. After 10 days, parasitemia began to increase, and when a parasitemia of 0.1% was reached, FACS sorting was started.

3.7.4.2 FACS sorting for transfected parasites

The neon green protein was excited at wavelength of 488 nm and the mCherry protein was excited at wavelength of 585 nm. These were sorted based on their fluorescence intensity (FI) and forward scattering (FSC). The neon green and mCherry proteins were detected using the FL2 filter and the FL4 filter respectively. The sorting rate was 10,000 events/sec, and 50,000 cells were collected, stored, and analysed using the Biorad S3e instrument. A parasitemia of 13.26% for *Pynt1(-)* and 23.8% for *Pbnt1(-)* was detected. The sorted parasites were injected into the tail veins of another naive mouse using a 27 insulin syringe, respectively.

3.7.4.3 Genotype analysis of knockout parasites and preparation of cryopreserved blood samples

At approximately 1-5% parasitemia, ~1 ml of blood of *Pynt1(-)* parasite-infected mouse was collected by cardiac puncture. The infected blood sample was passed through the cellulose column. The eluate was collected in 15 ml PBS. Infected erythrocytes were pelleted by centrifugation (1500 rpm, 8 min, RT, Acc:6, Decc:2). The supernatant was removed, and infected red blood cells were lysed with 0.1% saponin solution. Parasites were pelleted by centrifugation (3600 rpm, 8 min, acc:6, decc:2). Parasite genomic DNA was extracted using the Qiagen DNeasy Blood&Tissue Kit protocol. The coding and regulatory sequences of the nucleoside transporter 1 (NT1) gene of *P. yoelii* and *P. berghei* were retrieved from PlasmoDB (Plasmodium database resource). Primers were designed for amplification of the nucleoside transporter gene sequence of *P. yoelii* and *P. berghei* (Table 3.3). The rest of the blood after cardiac puncture was mixed with a freezing solution and stored at

-80 °C. 2 µl of infected blood were analyzed by confocal microscopy for *Pynt1(-)* and *Pbnt1(-)* parasites [4].

Table 3. 3: Details of primer sequences used to prepare diagnostic PCR analysis for the deletion in *Py*NT1 and *Pb*NT1.

Primer	**Sequence**
yNT1_KOtest5F	ACTTAAAAATTGTATGCAATGTAGAATACCCCA
yNT1_KOtest3R	GGTATTGTGTTTTACGCATTAAAGATAACTAAA
bNT1_KOtest5F	CTAAAAACATATCTAAATTTTTGATAC
bNT1_KOtest3R	TTCGATATATAAACATATGTATAGATA

3.7.4.4 Growth analysis of mixed nucleoside transporter 1 deficient *P. yoelii* and *P. berghei* parasites

Pbnt1(-) and *Pynt1(-)* parasites were mixed and injected into a group of mice for analysis of blood stage development and compared with same route of wild-type injected into a group of mice. The infectivity of mixed doses of NT1 knockout *P. yoelii* and *P. berghei* species was studied in four different groups (n=4) of mice explained below.

The first group: mixed 100 *Pbnt1(-)* and 100 *Pynt1(-)* dose; intravenous injection

The second group: mixed 1000 *Pbnt1(-)* and 1000 *Pynt1(-)* dose; subcutaneous injection

The third group: mixed 100 *Pb*WT and 100 *Py*WT dose; intravenous injection

The fourth group: mixed 1000 *Pb*WT and 1000 *Py*WT dose; subcutaneous injection

3.7.4.5 Vaccination and challenge study:

Prepared *Pynt1(-)* and *Pbnt1(-)* frozen stocks were injected into two naive mice, respectively. Parasitemia reached levels above 1% in two of the samples within three days. Then, infected erytrocytes were collected from both mice by cardiac puncture. Fresh vaccine doses were prepared in RPMI and frozen vaccine doses were prepared in RPMI and freezing solution as shown in the Figure 3.4. The number of asexual BS

parasites per microliter of infected donor blood was determined by Giemsa staining thin blood smears and the number of erythrocytes in the blood were counted to prepare the immunization doses. Same steps were followed for *Py*WT and *Pb*WT frozen stock injected donor mice for preperation of challange doses. The efficacy of the mixed *Pbnt1(-)* and *Pynt1(-)* whole blood parasite vaccine was tested in six-to eight-weeks-old female BALB/c and C57BL/6 mice. Mice were immunized with the fresh and frozen blood-stage *Pynt1(-)* and *Pbnt1(-)* parasites. The fresh parasite immunization strategy used 1000 and 2000 infected erythrocytes, whereas 10,000 infected erythrocytes were used to immunize mice as frozen stock. The fresh infected erythrocytes were used only in immunization against a single species (*Pbnt1(-)* only or *Pynt1(-)* only), whereas the frozen stocks were used in both immunization against a single species and immunization against mixed species. Frozen stocks (24 hours old) were used for the immunization study. Live attenuated parasites for fresh vaccine dosese were prepared using RPMI. Frozen vaccine doses were prepared using freeze-thaw freezing solution and RPMI. Therefore, RPMI (n= 5) and freezing solution (n= 5) were injected subcutaneously as negative control groups. In addition, one group (n= 5) of naive mice was injected as a positive control group for both species in the challenge study. Eight weeks after immunization, mice were challenged intravenously (IV) with 10,000 *P. berghei* WT ANKA or *P. yoelii* WT 17X- NL infected erythrocytes in separate groups. All immunization and challenge doses were prepared in a total volume of 150 µl using incomplete RPMI medium. After parasite challenge, infection was monitored for three weeks by Giemsa staining of thin blood smears [4].

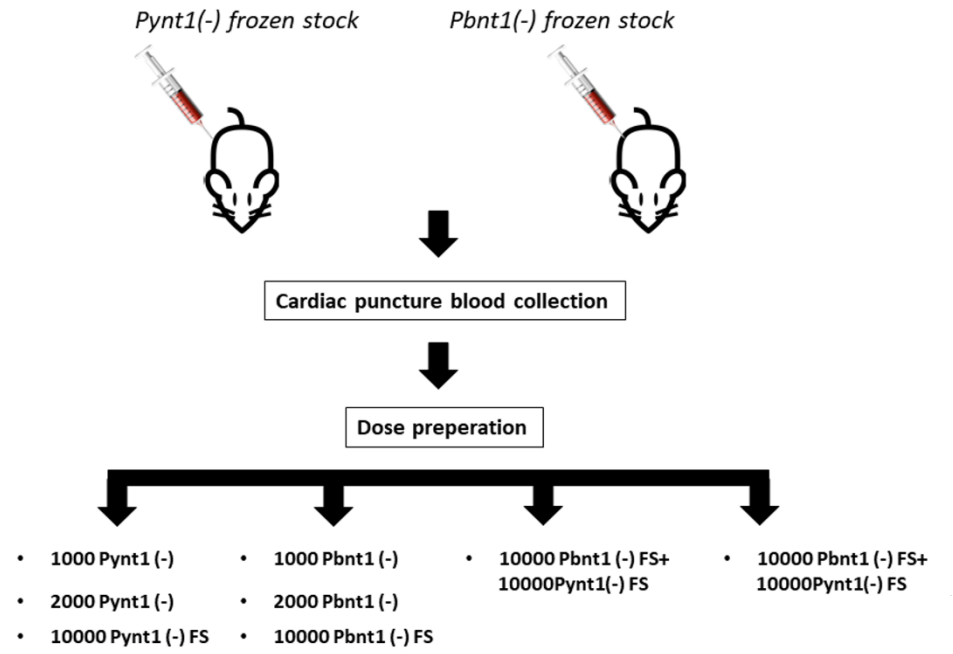

Figure 3. 5: Preperation of fresh and frozen *Pbnt1(-)* and *Pynt1(-)* vaccine doses.

3.8 Nucleoside Transporter 4

3.8.1 Preperation of NT4 knockout *P. berghei* parasites

The coding and regulatory sequences of the nucleoside transporter 4 (NT4) gene of *P. berghei* were taken from PlasmoDB (Plasmodium database resource). Primers were designed for amplification of the NT4 left and right arms from *P. berghei* genomic DNA. Left and right homology arms (5' UTR and 3' UTR of NT4) of the nucleoside transporter 4 gene were cloned into efficiency plasmid AA23 (Figure 3.1) flanking by the mTurq2 and hDHFR casette, respectively. Left homology arm is cloned into restriction enzyme site SacII-BamHI, and right homology arm is cloned into restriction enzyme site HindIII-KpnI. The final donor plasmids were analyzed by a company using Sanger sequencing. Gene deletion of *P. berghei* ANKA Nucleoside Transporter 4 was carried out by double crossover homologous recombination with a modified donor plasmid flanking the fluorescent protein mTurquoise2 and the human DHFR cassette. Donor plasmid was linearized with SacII-KpnI enzymes and transfected into the *P. berghei* ANKA parasites. Transfection positive parasites were selected with fluorescent protein and drug resistance marker. Same transfection steps were followed in section 3.7.3. Genomic integration of the recombinant construct was confirmed by diagnostic PCR with integration-specific primers. The sequences of primer pairs for

donor DNA plasmid preparation and integration-specific diagnostic PCR are listed in Table 3.4 [4].

Table 3. 4: Details of primer sequences used to prepare left and right homology arms and in diagnostic PCR analysis for the deletion in *Pb*NT4.

Primer	Sequence (5'-3')
Construct Preperation	
Pbnt4L-F	GGCCGCGGGATAACAGTAGAATAATGCATAAATGGCATATG
Pbnt4L-R	TCCGGATCCTTCTTCACTCTTTTACAACTTATTATATTCCAT
Pbnt4R-F	TGCCAAGCTTGCAGTTGTAAATTTATGAGGCACTAAAAATTGC
Pbnt4R-R	TCCGGTACCCTATATAAACTACAGCAATTAGATCGTTTACTC
Diagnostic Analysis	
5' Integration-F	GTGTATGAATAAGGTATTTATTTAAGG
5' Integration-R	CACTAGCAAAGTATGTACATACGTATATTTTCC
3' Integration-F	GGTACTAGTATGGTGAGCAAGGGCGAAGAACTATTTACTGGA
3' Integration-R	CTCTTTAAGTCCTTATTTCATTTTTCC
WT Test-F	TGGGGTTATTGCTAACACTTTTATGTGT
WT Test-R	GTAACTAAAATAGAAAAACTTATGGGTA

3.8.2 Determination role of nucleoside transporter 4 in blood stage development and male gamete exflagellation

Pbnt4(-) frozen stock and *Pb*WT frozen stock were injected subcutaneously into different CD1 donor mice, respectively. Parasitemia was detected by Giemsa staining thin blood smear. When parasitemia reach between 1-5%, *Pbnt4(-)* and *Pb*WT infected mice bled and blood samples were collected by cardiac puncture. For both *Pb*WT and *Pbnt4(-)* parasites, 20,000 infected erythrocyte doses were prepared with RPMI (injection volume: 150 µl). A group of mice (n= 3) were infected intravenously with

each *Pbnt4(-)* samples and same route dose of *Pb*WT injected another group of mice. Parasitemia was detected 10 days after infection by Giemsa staining of thin blood smears. At least 50 fields of the slides were counted for both WT and knockout parasites using phage contrast microscopy at 1000× magnification. Exflagellation of male gametes and development of ookinetes was demonstrated for *Pbnt4(-)* and compared with *Pb*WT. Frozen stocks for *Pbnt4(-)* and *Pb*WT were injected into donor mice [16, 68].

Mice were bled at an optimal parasitemia between 1-5%, and 10 million parasites dose was prepared and injected into group of mice (n= 5) for each genotype. After three days, 2 µl of blood was collected from the tail of each mouse and mixed with 98µl of ookinate culture medium (RPMI, sodium bicarbonate, hypoxanthine, pen/strep solution, xanthurenic acid, fetal bovine serum). 10 minutes later, the prepared blood sample was placed in a hemocytometer and counted by phage contrast microscopy at room temperature at 400X magnification. On that time, male and female gametocytes were determined by Giemsa staining thin blood smear for *Pbnt4(-)* and *Pb*WT samples [16, 68].

3.8.3 *In vitro* ookinate culture

After the exflagillation study, the highly exflagillated mice were anesthetized and used for blood meal of Anopheles stephensi mosquito. Then, mice were used for mosquito feeding were bled and by cardiac puncture. 500 µl of infected *Pb*WT and *Pbnt4(-)* blood samples were transferred into a 6-well plate seperately. 4.5 ml ookinate culture medium was added in each well and mixed with infected blood samples. The ookinate culture was incubated overnight at 24 °C. After incubation, the cultures were transferred into 50 ml tubes and spun at 300g, 5 min, 6 acc., 3 dec., 4 °C. The supernatant was removed and lysis buffer was added into the each tube and incubated on ice for 8 minutes. Lysed erythrocytes released ookines for wild-type and knockout parasites and purification steps were started. First, samples were centrifuged at 300 g, 5 min, 6 acc., 3 dec., 4 °C. The supernatant was removed and parasite pellets were washed with 1x PBS and centrifuged at 5 min, 6 acc., 3 dec., 4 °C. The purification steps were repeated 3 times. Parasites were resuspended and counted in a hemocytometer by phase contrast microscopy at 400 X [69].

3.8.4 Determination role of nucleoside transporter 4 in mosquito stage via Oocysts, oocyst sporozoites, hemolymph sporozoites and salivary gland sporozoites quantification

Mosquitos were fed with *Pb*WT and *Pbnt4(-)* infected mice *were* dissected and midguts were collected. These midguts were placed on glass slide in RPMI and covered with glass slide. The number of oocysts were counted on day 10, 14, 16, 18, and 21 after blood feeding using phage contrast microscopy at 400X. Minimum 20 mosquitoes were dissected for each count and experiment is repeared 3 times. After midgut dissection, oocysts (day 14, 16, 18 and 21) and salivary gland (day 16, 18 and 21) sporozoites were collected in RPMI containing 3% BSA for both *Pbnt4(-)* and *Pb*WT parasites by crushing with pestles. After each crushing, sporozoite samples were centrifuged at 3000 rpm for 2 min and this step was repeated 2 times. On day 18 and 21, sporozoites were also collected from the hemolymph for both knockout and WT parasites, respectively. All sporozoite samples were loaded into a hemocytometer and counted at 400x magnification using phage contrast microscopy [69].

3.8.5 Analysis of NT4 knockout sporozoite infectivity to the rodent host

Pbnt4(-) hemolymph and salivary gland sporozoites were injected intravenously into a different group of CD1 mice. The results were compared with *Pb*WT sporozoites injected by the same route. Therefore, on day 18, *Pb*WT and *Pbnt4(-)* salivary gland sporozoites were collected and injected into different groups of mice at a dose of 10000 sporozoites. On day 21, both hemolyph and salivary gland sporozoites were collected from *Pbnt4(-)* and *Pb*WT parasites. 10000 salivary gland sporozoites and 100000 hemolymph sporozoites were injected intravenously into different groups of mice for both *Pbnt4(-)* and *Pb*WT (n= 3) on day 21. Parasitemia was determined during 16 days by Giemsa staining thin blood smear.

3.8.6 Statistical analysis

All data statistically analyzed with One-way ANOVA and student t-test by through Graph Pad prism 5. Data were presented as mean ± S.D. of at least triplicate determinations. P-values of <0.05 were considered as statistically significant. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

4. RESULTS AND DISCUSSION

4.1 Nucleoside Transporter 1

4.1.1 Targeted deletion of *Py*NT1 and *Pb*NT1 by CRISPR gene editing technology

4.1.1.1 Generation of *Py*NT1 and *Pb*NT1 Donor Plasmids

Left and right homology arms of *Py*NT1 was cloned plasmid AA21 and Left and right homology arms of *Pb*NT1 was AA32 plasmid. First, the left and right homology arms for *Py*NT1 and *Pb*NT1 were amplified by PCR using the designated forward and reverse primers. The bands were consistent with the expected PCR product size. Left homology arm of *Py*NT1 is at 1123 bps, the right homology arm iof *Py*NT1 is at 1192 bps, the left homology arm of *Pb*NT1 at 1211 bps, and the left homology arm of *Pb*NT1 at 1081 bps. (Figure 4.1)

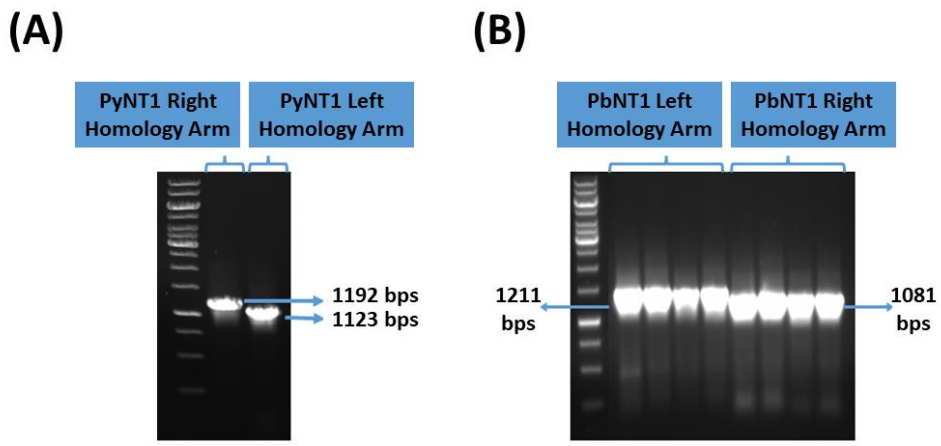

Figure 4. 1: Amplification of left and right homology arms amplification from A) *P.yoelii* genomic DNA and from B) *P.berghei* genomic DNA (Thermo 1 kb DNA ladder has been used for analysis).

Cloning of the homology arms into the plasmids was verified by digestion with the restriction enzymes Sac2-BamH1 and Hind3-Kpn1, respectively (Figure 4.2). In addition, the final donor plasmids were analyzed by a company using Sanger

sequencing, and the results matched the left and right arms for both *Py*NT1 and *Pb*NT1.

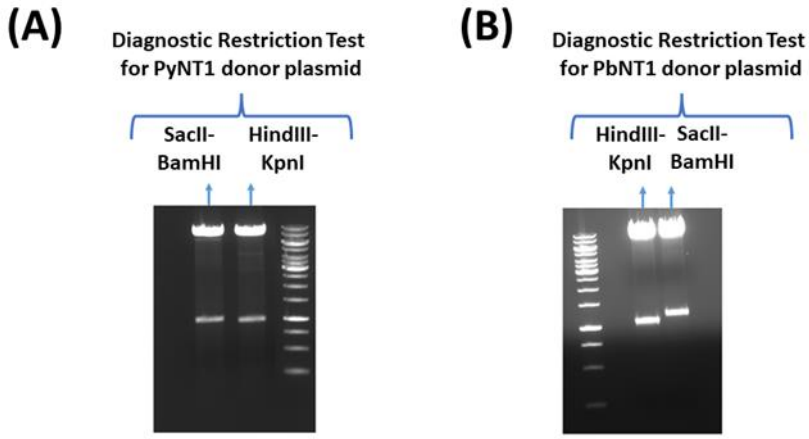

Figure 4. 2: Test digession after cloning of left homology arm by SacII-BamHI and right homology arm by HindIII-KpnI into the efficiency plasmid A) for *P.yoelii* NT1 donor plasmid and B) for *P.berghei* NT1 donor plasmid (Thermo 1 kb DNA ladder has been used for analysis).

4.1.1.2 Generation of CRISPR-Cas9 Construct

Since Cas9 itself could easily be generated under a stage-specific promoter to enable rapid genome editing, the U6 5'UTR used to express the gRNA is constitutively active. Homology-directed repair (HDR) is the only repair mechanism that interfaces in the current CRISPR/Cas9 system in parasites. To stabilize the parasites, the CRISPR element and the donor template are introduced separately. In this thesis, *Pynt1(-)* and *Pbnt1(-)* were generated using CRISPR technology and both knockout species were used to develop a mixed growth-attenuated blood-stage vaccine. Therefore, we developed the novel high-fidelity CRISPR Cas9 elements (a newer version of the Cas9 system that reduces off-target detection by Cas9) and introduced them into highly efficient transfection vectors. Establishment of this technology tested by targeted deletion of nucleoside transporter 1 for the species *P.berghei* and *P.yoelii*. The codon-optimised Cas9 endonuclease and gRNA sequences were designed and then ordered from a gene synthesis company. The synthesised sequences were introduced into the AA21 laboratory-modified vector (a series of impovent transection plasmids with reporting genes developed by Dr. Aly at Tulane University). Donor DNA for homologous recombination for targeted gene deletion is also introduced into another improved transfection plasmid. The separate plasmids for the CRISPR-Cas9 elements and for the donor DNA both contain state-of-the-art fluorescent protein cassettes as

positive selection markers by cell sorter (Biorad s3e cell corter). hDHFR (human dihydrofolate reductase) is one of the preferred markers for drug selection.

CRISPR-Cas9 plasmid constructed into the efficiency plasmid (AA21) containing Cas9 sequence, sgRNA scaffold, hDHFR-T2A-mTurquase genes. 5'UTR to drive expression of the coding high-fidelity Cas9. In the sgRNA cassette, the 5' RNA U6 promoter is followed by the gRNA adaptor site, which is directly attached to the scaffold sequence (Cas9 attachment site), and is immediately followed by the 3' terminator sequence. The plasmid size is 12.1 kb after cloning all CRISPR elements into the efficiency plasmid (AA21) (Figure 4.3). After cloning, a test digestion was performed to verify whether the cloning was correct or not. For this purpose, the CRISPR plasmid was digested with restriction enzymes EcorV-Hind III. The obtained band sizes of 2.5 kb and 9.6 kb confirm our results (Figure 4.4). Py+bNT1 sgRNA primers were designed for both *Pb*NT1 and *Py*NT1 genes. Designed forward and reverse primers were ordered commercially (Table 3.2). These primers were aligned and cloned into the sgRNA cassette containing the restriction enzyme site BbsI. An important test for sgRNA cloning is to control for the existence of BbsI. This is because the BbsI restriction enzyme site disappears after sgRNA cloning. Therefore, Py+bNT1 sgRNA was cloned into the CRISPR plasmid and the CRISPR plasmid was digested with BbsI. The uncut CRISPR plasmid served as a control (Figure 4.5). The Py+b NT1 sgRNA cloned into the CRISPR plasmid appears to be an uncut CRISPR plasmid. This result confirms that the cloning was successfully completed and the restriction enzyme site Bbs1 was removed. Another control of cloning is sequencing. The final plasmid was analyzed by a company using Sanger sequencing and the results were consistent with our commercially available Py+b NT1 sgRNA primers (Figure 4.6)

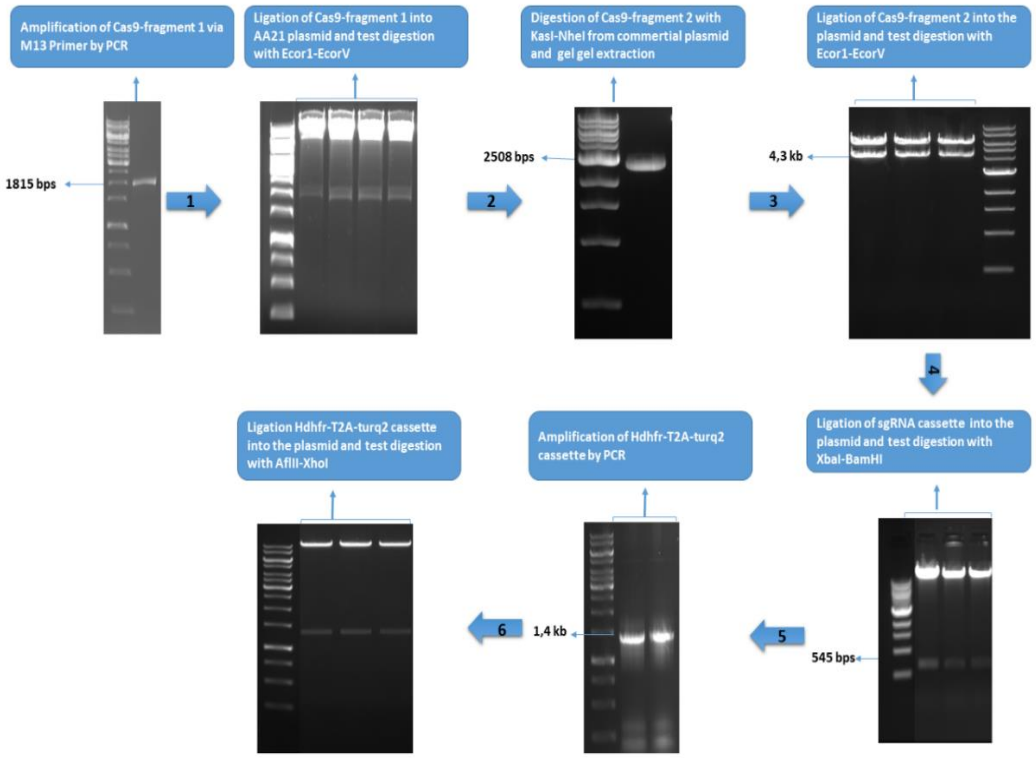

Figure 4. 3: Preperation of CRISPR-Cas9 Plasmid (Thermo 1 kb DNA ladder has been used for analysis.

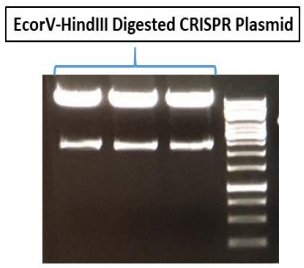

Figure 4. 4: Test Digestion of final CRISPR Plasmid (Thermo 1 kb DNA ladder has been used for analysis**.**

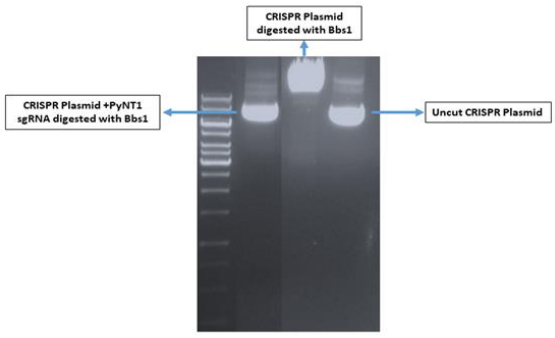

Figure 4. 5: Test Digestion for Pb+y NT1sgRNA inserted CRISPR Plasmid (Thermo 1 kb DNA ladder has been used for analysis).

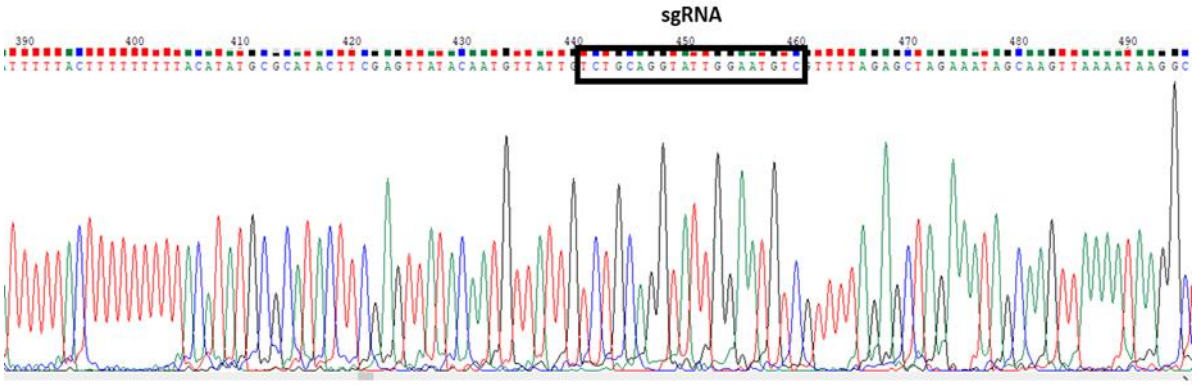

Figure 4. 6: Sanger sequencing result of Pb+y NT1sgRNA inserted CRISPR plasmid.

4.1.1.3 Verifying of *Pynt1(-)* and *Pbnt1(-)* Parasites

Targeted deletion of nucleoside transporter gene 1 in *P.yoelii* 17X- NL and *P.berghei* ANKA parasites was confirmed by PCR. Forward and reverse primers were developed and tested to determine the deletion of the open reading frame for both *Py*WT and *Pb*WT. The PCR products for both *Pynt1(-)* and *Pbnt (-)* mutant parasites did not show any bands. However, the PCR products of *Py*WT and *Pb*WT showed bands arounf 550 bps. In addition, mCherry protein sequences were integrated into *Pbnt1 (-)* and neongreen protein sequences were integrated into *Pynt1(-)* parasites. Distinct florescent signals were detected in both nucleoside transporter gene 1 knockout species by confocal microscopy.

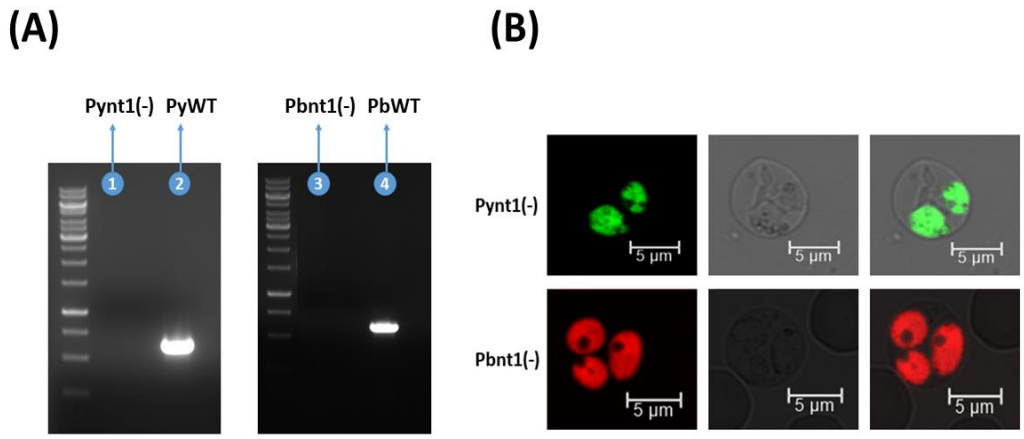

Figure 4. 7: Targeted deletion of *Py*NT1 and *Pb*NT1 was achieved by double crossover homologous recombination with CRISPR-Cas9 Technology. A) Integration of the gene replacement construct confirmed by deletion of nucleoside transporter1 deletion by genomic PCR for *Pynt1(-)* and *Pbnt1(-)* parasites. B) Neon green expressed *Pynt1(-)* and mCherry expressed *Pbnt1(-)* parasites were analyzed by confocal microscopy.

4.1.1.4 NT1 knockout parasites are safe vaccine candidates

In this thesis, genetically attenuated parasite strains of *P. berghei* ANKA and *P. yoelii* 17X- NL with deficient nucleoside transporter 1 gene were developed as candidates for blood stage whole parasite vaccine. Vaccine safety in whole-parasite vaccines is the main concern, as safety has been the most important parameter that has hindered the success of many vaccines [8]. Therefore, the first and most important step was to verify the infectivity of NT1-deficient vaccine candidates in the blood stage. After intravenous and subcutaneous injection of mixed live NT1 knockout parasites, parasitemia was not observed in any of the mice injected with NT1-deficient parasites 36 days after infection (Figure 4.8). However, the mice injected with WT parasites developed parasitemia on day 5 postinfection (IV) and day 7 postinfection (SC). (Figure 4.8). Parasitemia detected by Giemsa-stained thin blood smear. These results indicate that NT1-deficient parasites are highly attenuated and can be safely developed as a genetically attenuated whole-parasite blood-stage vaccine.

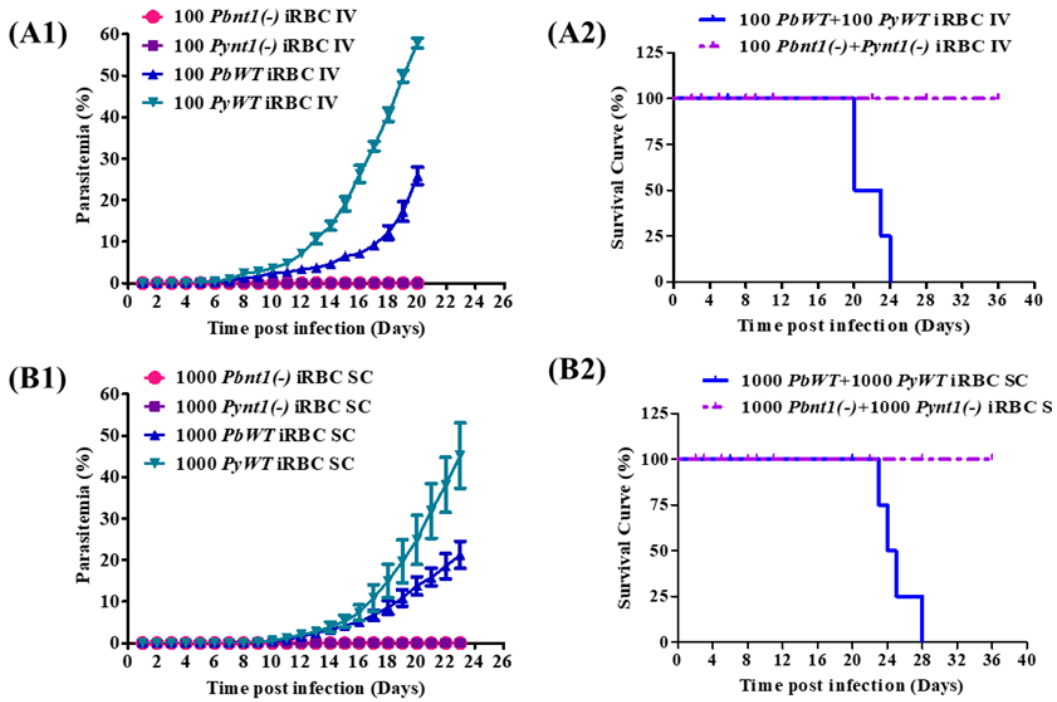

Figure 4. 8: Mixed *Pynt1(-)* and *Pbnt1(-)* parasite infected erythrocytes does not cause infection. (A) In one group, 100 *Pbnt1(-)* and 100 *Pynt1(-)* parasite-infected erythrocytes were mixed and injected intravenously. (B) In one group, 1000 *Pbnt1(-)* and 1000 *Pynt1(-)* parasite infected erythrocytes were mixed and injected subcutaneously. Both groups were compared same size mouse groups with similar dose and routes of mixed *Py*WT and *Pb*WT parasites. Whereas no parasites were detected in giemsa-stained thin blood smears of the groups infected with mixed genetically attenuated parasites.

4.1.1.5 A Single dose of genetically-attenuated malaria blood-stage parasites protects against two Plasmodium species infections

Immunization was performed with NT1 knockout P.berghei and P.yoelii parasites, which show sterile protection against infectious parasites at the intravenous blood stage parasite challenge. Mice were immunized with single and mixed parasites, fresh and frozen stocks of NT1-deficient parasites via subcutaneous immunization. (Table 4.1). All immunized mice were fully protected after 8 weeks challenges with 10,000 P. yoelii 17X- NL and 10,000 P. berghei ANKA IV injected blood-stage parasites. For all groups of immunized mice, there were strain- and age-matched naive control groups, all of which developed parasitemia as detected by Giemsa-stained thin blood smears (Table 4.1).

Table 4. 1: Single-dose subcutaneous immunizations with *Pynt1(-)* and *Pbnt1(-)* live growth-attenuated blood stages confer sterile immunity against infection challenges by two different malaria parasite species**.**

Blood-stages Immunization Dose	Blood-stages patency	Intravenous Blood-stages Challenge Dose	Challenge Period (weeks)	Protected/ Challenged
1000 *Pynt1(-)*	0/5	10.000 *PyWT* 17X-NL	8	5/5
2000 *Pynt1(-)*	0/5	10.000 *PyWT* 17X-NL	8	5/5
10.000 *Pynt1(-)* FS	0/5	10.000 *PyWT* 17X-NL	8	5/5
1000 *Pbnt1(-)*	0/5	10.000 *PbWT* ANKA	8	5/5
2000 *Pbnt1(-)*	0/5	10.000 *PbWT* ANKA	8	5/5
10.000 *Pbnt1(-)* FS	0/5	10.000 *PbWT* ANKA	8	5/5
10.000 *Pbnt1(-)*FS 10.000 *Pynt1(-)*FS	0/5	10.000 *PyWT* 17X-NL	8	5/5
10.000 *Pbnt1(-)*FS 10.000 *Pynt1(-)*FS	0/5	10.000 *PbWT* ANKA	8	5/5
RPMI 1640 Negative Control	0/5	10.000 *PyWT* 17X-NL	8	0/5
RPMI 1640 Negative Control	0/5	10.000 *PbWT* ANKA	8	0/5
Freezing Solution Negative Control	0/5	10.000 *PyWT* 17X-NL	8	0/5
Frezing Solution Negative Control	0/5	10.000 *PbWT* ANKA	8	0/5

4.2 Nucleoside Transporter 4

4.2.1 Generation of PbNT4 Donor Plasmids

Upstream and downstream sequences of *Pb*NT4 gene were cloned into AA23 vector. First, the left and right homology arms for *Pb*NT4 were amplified by PCR using the designated forward and reverse primers. Cloning of the homology arms into plasmid AA23 was controlled by Sac2-BamH1, Hind3-Kpn1 diagnostic digestion. The band size of the left arm of *Pb*NT4 is 898 bps, and that of the right arm of *Pb*NT4 is 849 bps. Agarose gel results confirmed expected band sizes (Figure 4.9). Final donor plasmids is analyzed via Sanger sequencing by a company and results matched up with the *P.berghei* NT4 left and right arm.

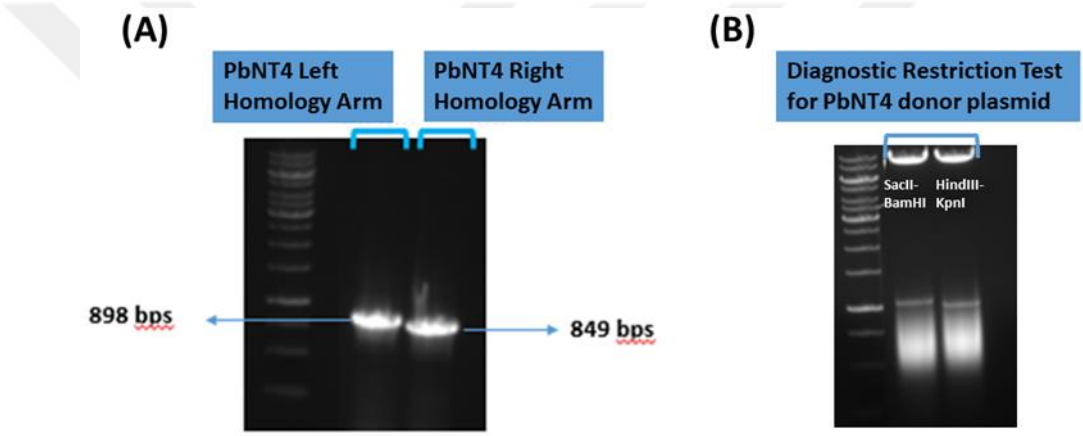

Figure 4. 9: A) Amplification of NT4 left and right homology arms from *P.berghei* genomic DNA and B) Verifying of *Pb*NT4 donor plasmid by test digession with SacII-BamHI and HindIII-KpnI enzymes (Thermo 1 kb DNA ladder has been used for analysis).

4.2.2 Phylogenetic analysis of nucleoside transporters family

Based on the sequences from PlasmoDB, TriTrypDB, TrichDB, CryptoDB, and ToxoDB, the phylogenetic tree of all nucleoside transporters in parasitic protozoa was constructed using the evolutionary gene analysis program (MEGA) (Figure 4.10). A star was placed next to unannotated proteins in the phylogenetic tree. According to the phylogenetic study, parasitic protozoa have conserved members of the ENT family, and the Plasmodium species contains four different NTs (Figure 4.10). While NT1 and NT2 are more closely related and highly conserved in all plasmodial parasites, NT3 is exclusively present in human malaria parasites, and NT4 is also conserved in all plasmodial parasites but is more distant from NT1 and NT2 and closely related to NT3

(Figure 4.10). Phylogenetic trees are shown using the protein IDs of the nucleoside transporter gene family. Below are the ID numbers [70].

T.brucei AT1 (Tb927.5.286b), *T.brucei* AT-A (Tb927.9.15980), *T.brucei* AT-E (Tb927.3.590), *T.brucei* NT2 (Tb927.2.6150), *T.brucei* NT3 (Tb927.2.6200), *T.brucei* NT4 (Tb927.2.6220), *T.brucei* NT5 (Tb927.2.6240), *T.brucei* NT6 (Tb927.2.6320), *T.brucei* NT7 (Tb927.2.6280) ,*T.brucei* NT8.1 (Tb927.11.3610), *T.brucei* NT8.2 (Tb927.11.3620), *T.brucei* NT8.3 (Tb927.11.3630), *T.brucei* NT9 (Tb927.6.220), *T.brucei* NT10 (Tb927.9.7470), *T.vaginalis* NT (TVAG_271570), *T.vaginalis* NT (*1) (TVAG_192810), *T.vaginalis* NT (*2) (TVAG_271560), *T.vaginalis* NT (*3) (TVAG_053320), *T.vaginalis* NT (*4) (TVAG_166380), *T.vaginalis* NT (*5)(TVAG_483030), *T.vaginalis* NT (*6)(TVAG_441760), *T.vaginalis* NT (*7) (TVAG_341290), *T.vaginalis* NT (*8) (TVAG_101510), *C.parvum* ENT (cgd2_310), *T.gondi* AT1 (TGME49_244440), *T.gondi* NT (TGVEG_359630), *T.gondi* NT(*) (TGME49_233130), *T.gondi* NT(**) (TGME49_288540), *L.major* NT1 (LmjF.15.1230), *L.major* NT2 (LmjF.36.1940), *L.major* NT3 (LmjF.13.1210), *L.major* NT4 (LmjF.11.0550), *L.donovani* NT1 (LdBPK_151230.1), *L.donovani* NT2 (LdBPK_362040.1), *L.donovani* NT3 (LdBPK_131110.1), *L.donovani* NT4 (LdBPK_110520.1), *E.histolytica* (EHI_110730), *E.histolytica* (*)(EHI_169580), *E.histolytica* (**) (EHI_017040), *Hepatocytis* NT1 (HEP_00329700), *Hepatocytis* NT2 (HEP_00036700), *Hepatocytis* NT3 (HEP_00306500), *Hepatocytis* NT4 (HEP_00067300), *Hepatocytis* NT4 (HEP_00067400), *P.berghei* NT1 (PBANKA_1360100), *P.berghei* NT2 (PBANKA_0706200), *P.berghei* NT4 (PBANKA_0209900), *P.falciparum* NT1 (PF3D7_0824400), *P.falciparum* NT2 (PF3D7_0824400), *P.falciparum* NT3 (PF3D7_1469400), *P.falciparum* NT4 (PF3D7_0103200), *P.vivax* NT1 (PVP01_1207600), *P.vivax* NT2 (PVP01_0509900), P.vivax NT3 (PVP01_1237400), *P.vivax* NT4 (PVP01_0211500), *P.yoelii* NT1 (PY17X_1365800), *P.yoelii* NT2 (PY17X_0706500), *P.yoelii* NT4 (PY17X_0211300), *P. cauboudi* NT1 (PCHAS_1364700), *P. cauboudi* NT2 (PCHAS_0715500), *P. cauboudi* NT4 (PCHAS_0208300), *P. knowlesi* NT1 (PKNH_1253700), *P. knowlesi* NT2 (PKNH_1318400), *P. knowlesi* NT3 (PKNH_1211600), *P. knowlesi* NT4 (PKNH_0210300), *P.ovale* NT1 (PocGH01_12015900), *P.ovale* NT2 (PocGH01_05016500), *P.ovale* NT4 (PocGH01_02018700), *P.malariae* NT1 (PmUG01_12017700), *P.malariae* NT2

(PmUG01_05021600), *P.malariae* NT3 (PmUG01_12047500), *P.malariae* NT4 (PmUG01_02020400), *P.vinckei* NT1 (PVVCY_1306320), *P.vinckei* NT2 (PVVCY_0700610), *P.vinckei* NT4 (PVVCY_0200970)

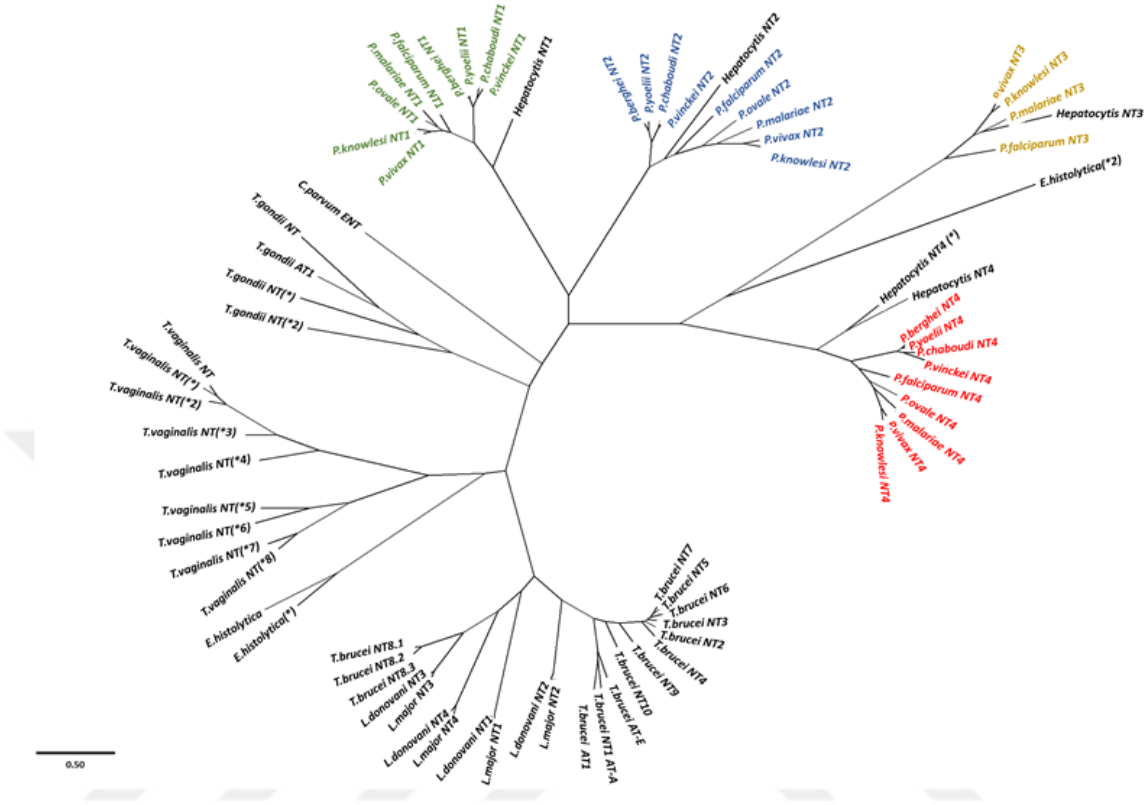

Figure 4. 10: Phylogenetic analysis of the nucleoside transporter gene family for parasitic protozoa. Molecular evolution gene analysis (MEGA) was used to study the phylogenetic tree. Unannotated genes were indicated with an asterisk (*), and the gene ID was mentioned above.

Using the online Clustal Omega Alignment tool, the percent identity of NT4 in *Plasmodium* spp. and *Hepatitis* spp (Figure 4.11). The database PlasmoDB contained domains that were recognized. According to the alignment results, *P. berghei* NT4 parasites have similarity to rodent malaria parasites more than 80% (Figure 4.11) [70].

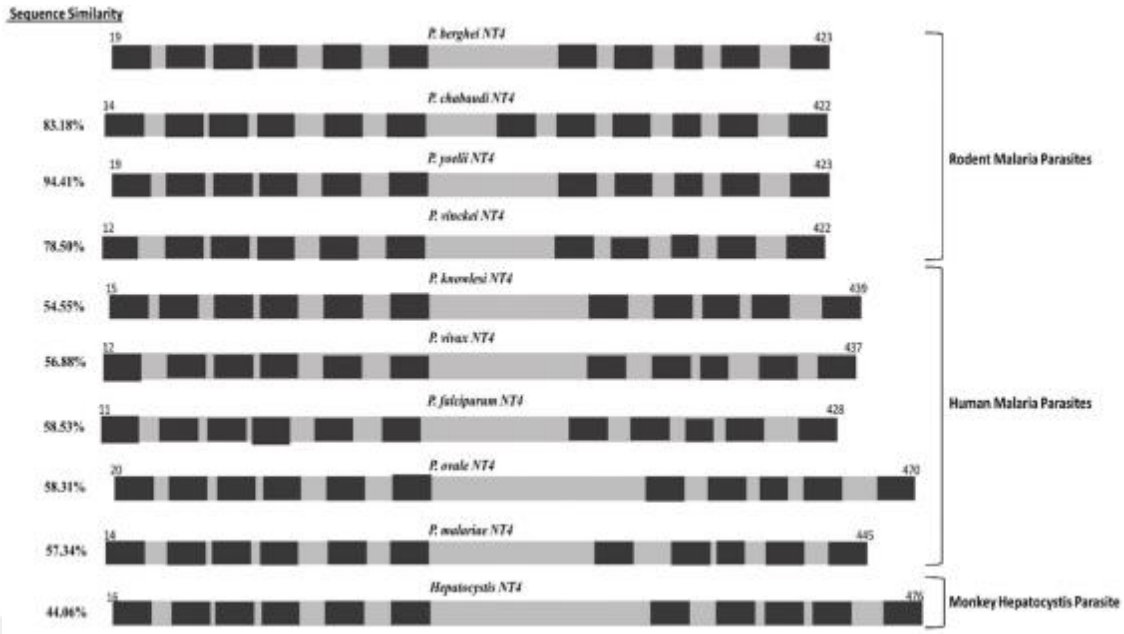

Figure 4. 11: Amino acid identities and the NT4 transmembrane domains in *Plasmodium* species and *Hepatocytis* are shown schematically. The transmembrane domains are shown as boxes in dark gray. Similar amino acids are displayed on the left side.

4.2.3 Preperation and verification of *Pb*NT4 donor plasmid

The role of NT4 in the parasite life cycle as been determined by deletion of nucleoside transporter gene 4 using homologous recombination strategy in *Pb*WT. Upstream and downstream of nucleoside transporter gene 4 were clonned into a donor plasmid AA23 flanking by the mTurq2 fluorescent protein cassette and the human DHFR drug selection marker. Gene deletion of NT4 and genomic integration of selection markers was analyzed by diagnostic PCR. Spesific primers have been designed to test 5' and 3' integration sites of nucleoside transporter 4. Mechanism of preperation NT4 knockout parasite and genomic integration have been shown at Figure 4.12 [70].

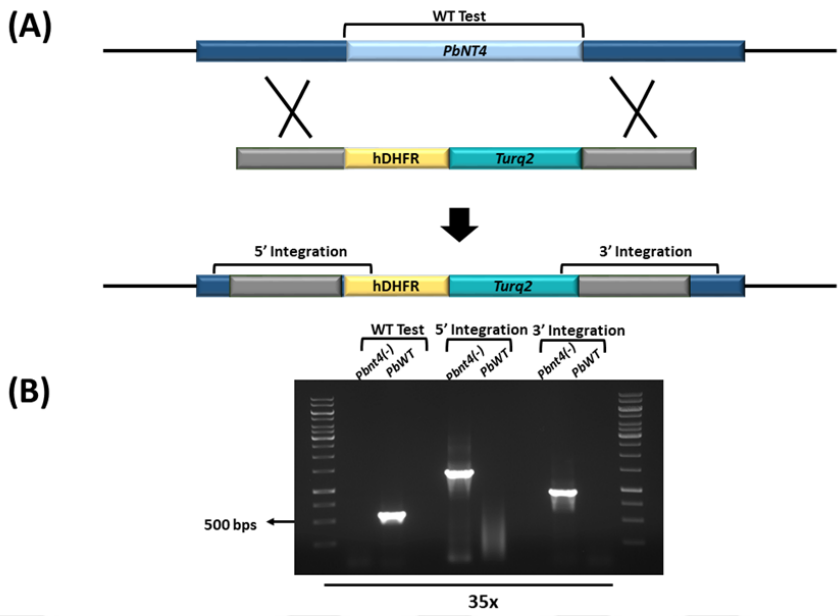

Figure 4. 12: Preperation of *Pbnt4(-)* parasites by homologous recombination. Schematical representation of NT4 gene deletion is shown in (A). Diagnostic PCR was used to validate genomic integration in (B). The integration of recombinant loci was verified using gene-specific primer pairs that can only amplify from the 5' and 3' integration sites and WT deletion in recombinant loci.

4.2.4 Knockout NT4 *P.berghei* parasites do not have role for blood stage development in rodents

Role of nucleoside transporter 4 has been studied in blood stage development. For this purpose, 20,000 *Pbnt4(-)* and 20,000 *Pb*WT blood stage parasites were intavenously injected into a group of CD1 mice (n= 4 mice per genotype). Parasitemia was evaluated using a Giemsa-stained thin blood smear over a 2-week period beginning on the day after injection. No appreciable difference was detected between the *Pb*NT4 knockout parasites and the *Pb*WT parasites injected mice for the development of the asexual blood stage. This result showed that *Pb*NT4 does not play a critical role in blood stage development (Figure 4.13a) [70].

4.2.5 *Pb*NT4 is not essential for gametocytogenesis, male gamete exflagellation, and ookinete development

The role of the NT4 gene in the sexual blood stage was explained in terms of gametocyte formation, maturation, and gametogenesis compared with *Pb*WT. In addition, the role of NT4 in ookinetes development was investigated in an *in vitro* experiment. Here, $1x10^7$ *Pbnt4(-)* and $1x10^7$ *Pb*WT parasites were injected intravenously into a group of mice (n= 5). On day 3 post-infection, the exflagration

centers for *Pbnt4(-)* and *Pb*WT injected parasites were determined and no significant differences were found. (Figure 4.13b). The ratio of male and female gametocytes was also calculated for WT and knockout parasites. These results indicate that the NT4 gene is not essential for blood sexual stage *Pbnt4(-)* and *Pb*WT. Injected mice were bled to *in vitro* ookinete culture started. The collected blood was cultured in complete ookinetes medium (containing RPMI, sodium bicarbonate, hypoxanthine, and xanthurenic acid) at 24°C for 24 hours. The developed ookinetes were collected by centrifugation using a hemocytometer. There are no significant differences between the number of *Pbnt4(-)* and PbWT parasites in ookinetes development (Figure 4.13c) [70].

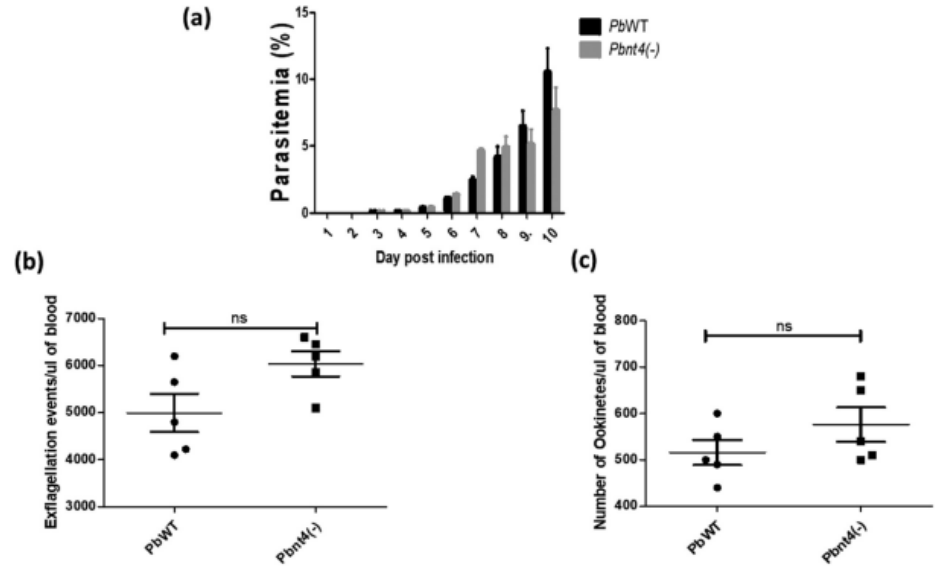

Figure 4. 13: Ookinete development, male gamete exflagellation events, and blood stage development are verified for *Pbnt4(-)* and *Pb*WT. (a) A group of three mice that were intravenously infected with 20,000 blood stage parasites. No obvious difference was found. (b) Number of male gamete exflagellation centers per µL of mouse blood determined by a hemocytometer (1:50 dilution) for *Pbnt4(-)* and *Pb*WT. (c) After overnight incubation, the number of ookinetes of the parasites *Pbnt4(-)* and *Pb*WT was determined in an *in vitro* culture. P value of 0.05 was used to determine statistical significance for all one-way analyses of variance (ANOVA).

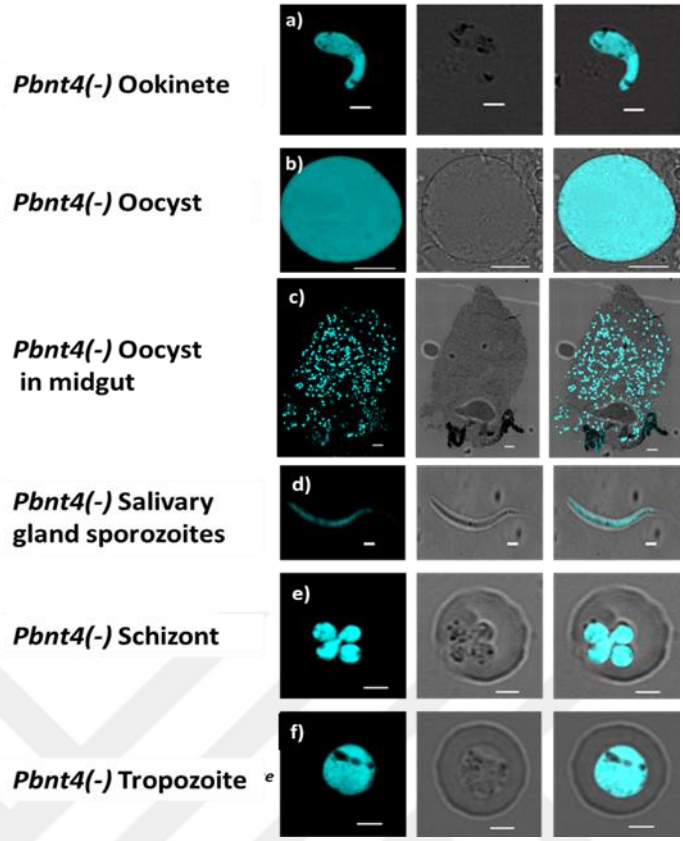

Figure 4. 14: Confocal microscopy images of the *Pbnt4(-)* life cycle in host and mosquito vector.

4.2.6 NT4 plays an important role in oocyst egress and colonization of the salivary gland of *P. berghei*

Female Anopheles stephensi mosquitoes were fed with highly exflagellated *Pbnt4(-)* and *Pb*WT infected mice. The development of oocysts, oocyst sporozoites, and salivary gland sporozoites were determined for both the knockout parasite and WT parasite. The number of oocysts was counted on the 10th, 14th, 16th, 18th, and 21st day after feeding by mosquitoes. On day 10, 14 and 16, there is no significant differences between *Pbnt4(-)* and *Pb*WT oocyst development however, on day 18 and 21, the number of oocysts was less in *Pb*WT compared to *Pbnt4(-)* parasites. (Figure 4.15a). Oocyst sporozoites were counted on the 14th, 16th, 18th and 21st day after feeding. On day 21, the number of oocyst sporozoites increased in *Pbnt4(-)* compared to PbWT, while the other days were similar. (Figure 4.15b) [70].

To explain the differences between oocysts and oocyst sporozoites, the number of sporozoites in hemolymph and salivary gland was determined for *Pbnt4(-)* and *Pb*WT parasites. On day 18 and 21 post feeding, sporozoites were counted in the hemolymph

to understand the migration of sporozoites from the midgut to the salivary gland. On day 18, the number of *Pb*WT sporozoites is higher than *Pbnt4(-)* sporozoites, and on day 21, the number of *Pbnt4(-)* hemolymph sporozoites is higher than *Pb*WT hemolymph sporozoites. (Figure 4.15c). In addition, salivary gland sporozoites were counted for both *Pbnt4(-)* and *Pb*WT to clearly explain the role of the NT4 gene. On the 16th, 18th and 21st day after feeding, the number of PbWT sporozoites increased rapidly, whereas *Pbnt4(-)* salivary gland sporozoites numver increased slightly (Figure 4.15d) [70].

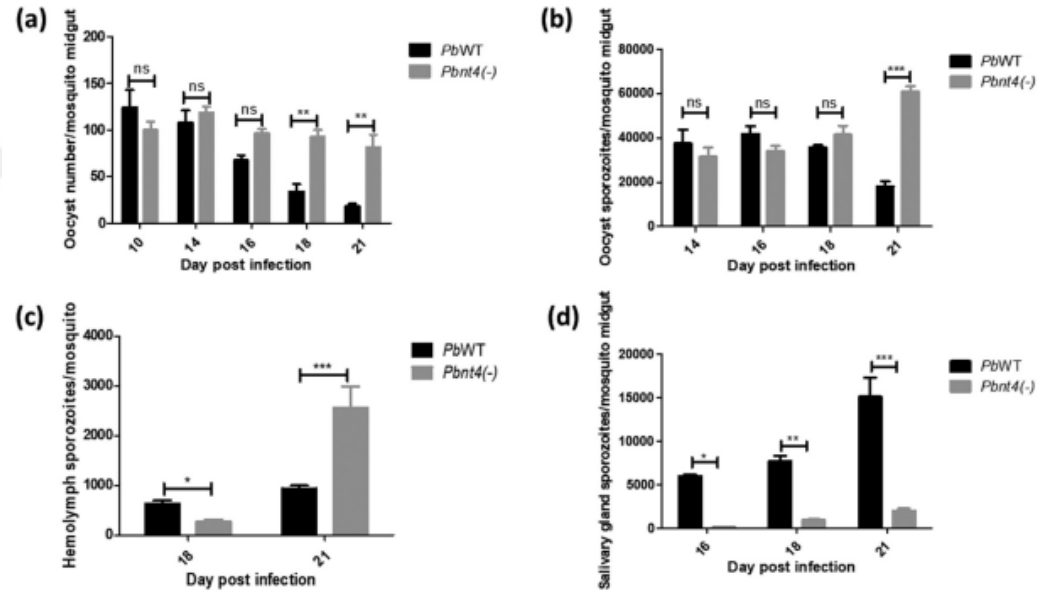

Figure 4. 15: Determination role of NT4 in mosquito stage development (a) There was no discernible difference in the number of oocysts till day 18 post-infection but on day 18 and day 21 number of *Pb*WT oocyst decreased. (b) *Pbnt4(-)* oocyst sporozoite numbers did not change significantly but *Pb*WT oocyst sporozoite number steadily decreased until day 21 postinfection. (c) Although *Pb*WT oocyst sporozoites egress regularly from oocysts as shown in (b), they did not accumulate significantly in hemolymph as indicated in (c), and PbWT sporozoites could easily localize in salivary glands unlike *Pbnt4(-)* (d).

As a result of experiments with mosquito vectors, we found that nucleoside transporter 4 does not play an essential role in oocyst development, but this gene is responsible for sporozoite exit from oocysts. The presence of *Pbnt4(-)* salivary gland sporozoites and hemolymph sporozoites indicates that oocyst egress is not completely blocked, but the high number of oocyst sporozoites in the late midgut indicates the importance of the NT4 gene for oocyst egress. In addition, the rapid increase in the number of hemolymph sporozoites from day 18 to 21 in *Pbnt4(-)* parasites and the slight increase

in salivary gland sporozoites indicate that the NT4 gene plays an essential role in migration from the midgut to the salivary gland [70].

4.2.7 NT4 is essential for the infectivity of sporozoites in the mammalian host

The infectivity of nucleoside transporter-4-deficient hemolymph and salivary gland sporozoites was declared for mammalian hosts. Therefore, on day 18, *Pb*WT and *Pbnt4(-)* salivary gland sporozoites were collected and injected at a dose of 10000 sporozoites into different groups of mice. On day 21, both hemolyph and salivary gland sporozoites were collected from *Pbnt4(-)* and *Pb*WT parasites. 10000 salivary gland sporozoites and 100000 hemolymph sporozoites were injected intravenously into different groups of mice for both *Pbnt4(-)* and *Pb*WT (n= 3). Parasitemia was observed in salivary glands (day 18 and day 21) and hemolymph sporozoites on day 4 and day 7 after infection with PbWT, respectively. In contrast, NT4 knockout parasites did not develop parasitemia at any time point (Table 4.2). These results indicate that NT4 plays a critical role in the infectivity of *P. berghei* sporozoites in the mammalian host [70].

Table 4. 2: The contribution of NT4 to sporozoite infectivity in mice infected with *Pbnt4(-)* and PbWT parasites via salivary gland and hemolymph sporozoites.

Group (n)	Injection Route	Infection Dose	Blood stage infection	Prepatent period
Day 18 sgspz *Pbnt4(-*) (3)	Intravenous	10000	0/3	-
Day 18 sgspz *Pb*WT (3)	Intravenous	10000	3/3	4
Day 21 hmspz *Pbnt4(-*) (3)	Intravenous	100000	0/3	-
Day 21 hmspz *Pb*WT (3)	Intravenous	100000	3/3	7
Day 21 sgspz *Pbnt4(-*) (3)	Intravenous	10000	0/3	-
Day 21 sgspz *Pb*WT (3)	Intravenous	10000	3/3	4

In this thesis we aimed to explain the following statements.

1. Development of nucleoside transporter 1 gene defficient parasites by CRISPR-Cas9 technology

2. Generation of a promising live attenuated vaccine strain that can be used as a mixed live attenuated vaccine against blood stage malaria.

3. Identification of possibility of a new target for malaria vaccine: nucleoside transporter 4.

Plasmodium species are purine-auxotrophic parasites, meaning that intracellular malaria pathogens lack *de novo* purine synthesis pathways [61]. Human malaria parasites (NT1-NT4) have four nucleoside transporter proteins (NT) [71] One of these NTs transporters, nucleoside transporter-1, has been shown in previous studies to be critical for parasite growth at physiological purine concentrations. Studies on the deletion of the NT1 gene of the *Plasmodium* parasite show that NT1-deficient parasites have a significant developmental defect [4, 72, 73]. Among the four NTs, NT1 is the most studied, but little is known about the other three nucleoside transporter proteins in *Plasmodium*. While human malaria parasites have all nucleoside transporter genes (NT1-4), rodents have only NT1, NT2, and NT4. In the previous study about NT1-deficient *P. yoelii* parasite, it was discovered that parasites missing NT1 had significantly limited growth and are unable to complete development at the mosquito stage [4]. To overcome the limitations of some genetic approaches, CRISPR-Cas9 technology has been used to produce genetically attenuated live vaccines. Herein, the CRISPR/Cas9 nuclease system was combined with donor plasmid for NT1 deletion. Florescent proteins and hDHFR selection markers were used for the determination of nucleoside transporter gene-1 efficient parasites. Neon green protein expressed *Pynt1(-)* and mCherry protein expressed *Pbnt1(-)* were successfully produced by CRISPR gene editing technology. These NT1 knockout parasites are reported as a highly effective live attenuated malaria vaccine. As a result of our study, mixed *Pynt1(-)* and *Pbnt1(-)* parasites, provided protection against two different malaria species. Whole-parasite vaccination is a more effective and preferable method to achieve long-lasting sterile immunity against malaria because it overcomes many of the antigenicity and longevity limitations of subunit vaccines [74, 75]. In addition to developing an attenuated vaccine based on CRISPR-Cas 9 technology against the entire parasite blood- stage vaccine, a mixed immunization strategy for protection against two different malaria species was used here as an extension of the study.

Combined immunization may provide immunity against both malaria species in areas where *P. falciparum* and *P. vivax* are common in the population, such as Oceania [76]. Mixed infections have also been observed in India [77]. There are cases from Southeast Asian areas in which *P. falciparum* infection is followed by *P. vivax* infection, although malaria transmission in this region is low, irregular, and seasonal [78]. These reports show that cross-species vaccination does not provide protection efficiently [8]. Therefore, a combined immunization strategy could provide the basis for protection against multiple malaria species. The results presented here support the idea of combination vaccination and call for further research to identify vaccine candidates that can offer defense against multiple *Plasmodium* species.

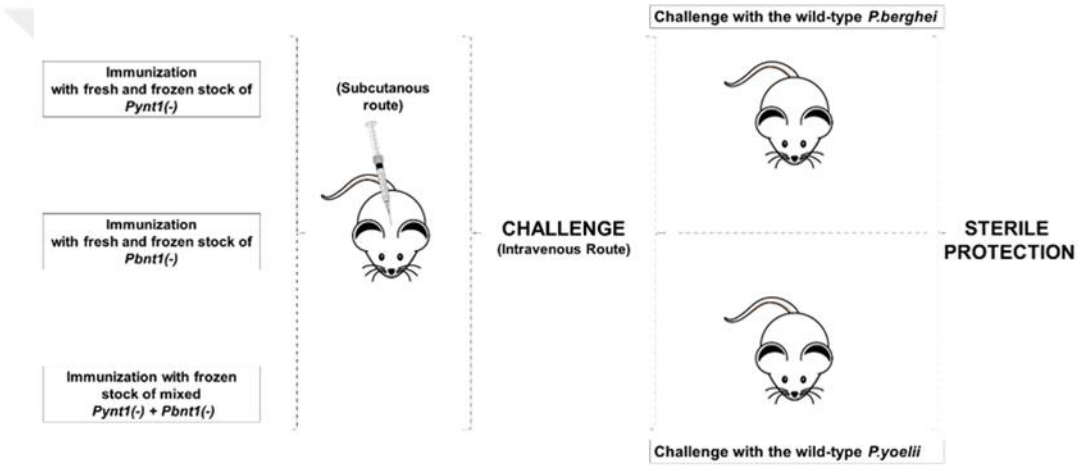

Figure 5. 1: Vaccination strategy for immunization with mixed *Pynt1(-)* and *Pbnt1(-) parasites.*

Third, we focused on the rodent malaria parasite *P. berghei* ANKA nucleoside transporter 4 to highlight the possibility of new malaria vaccine targets. To investigate the importance of NT4 at different stages of the parasite life cycle, we generated an NT4-deficient strain of the *P. berghei* ANKA parasite. Examination of the *Pb*NT4 gene revealed that this gene is not required for *P. berghei* to develop to the blood stage. Investigation of NT4 role on male gamete exflagellation also revealed that this gene had no appreciable impact on the process (Figure 4.15b). A substantial decrease in gametocytogenesis and a large delay in exflagellation of male gametes were seen in our previous study of NT1 deletion in *P. yoelii* [4]. Complete suppression of NT1-deficient parasite transmission by mosquitoes was observed [4]. Oocyst formation was normal in NT4-deficient parasites compared with wild-type parasites, indicating that deletion of NT4 does not affect transmission of the parasite to the mosquito (Figure

4.15a). On days 14, 16, and 18, both NT4-deficient and WT parasites had the same amount of oocyst sporozoites. However, on day 21, a substantial decrease in PbWT oocyst sporozoites was observed. However, there was an upward trend in the number of *Pbnt4(-)* oocyst sporozoites (Figure 4.15b). This might be because the oocysts of the *Pb*WT parasite are fully mature by day 18 and release sporozoites that move into the mosquito's salivary gland [77]. However, in parasites lacking NT4, oocyst formation can be problematic, resulting in an abundance of sporozoites on day 21. When comparing the amount of sporozoites in the salivary glands at days 16, 18, and 21 post-infection, it can be seen that *Pbnt4(-)* and *Pb*WT had significantly different numbers of sporozoites (Figure 4.15d). When compared to NT4-deficient parasites, the amount of PbWT sporozoites in salivary glands dramatically increased between days 16 and 21. Kenthirapalan and colleagues also observed that NT4-deficient parasites have a low amount of salivary gland sporozoites [78]. Although in small quantities, the presence of sporozoites in the salivary glands of NT4-deficient parasites suggests that oocyst discharge is not entirely repressed, contrary to what has previously been observed with cysteine protease-deficient parasites [79]. We next looked at the quantity of sporozoites in the hemolymph as a result of this finding and conducted a through regional and temporal analysis of sporozoite distribution in female Anopheles mosquitoes. As a result of this, nucleoside transporter 4 cannot used as target for blood stage. But this gene might have potential for transmission blocking vaccine [80].

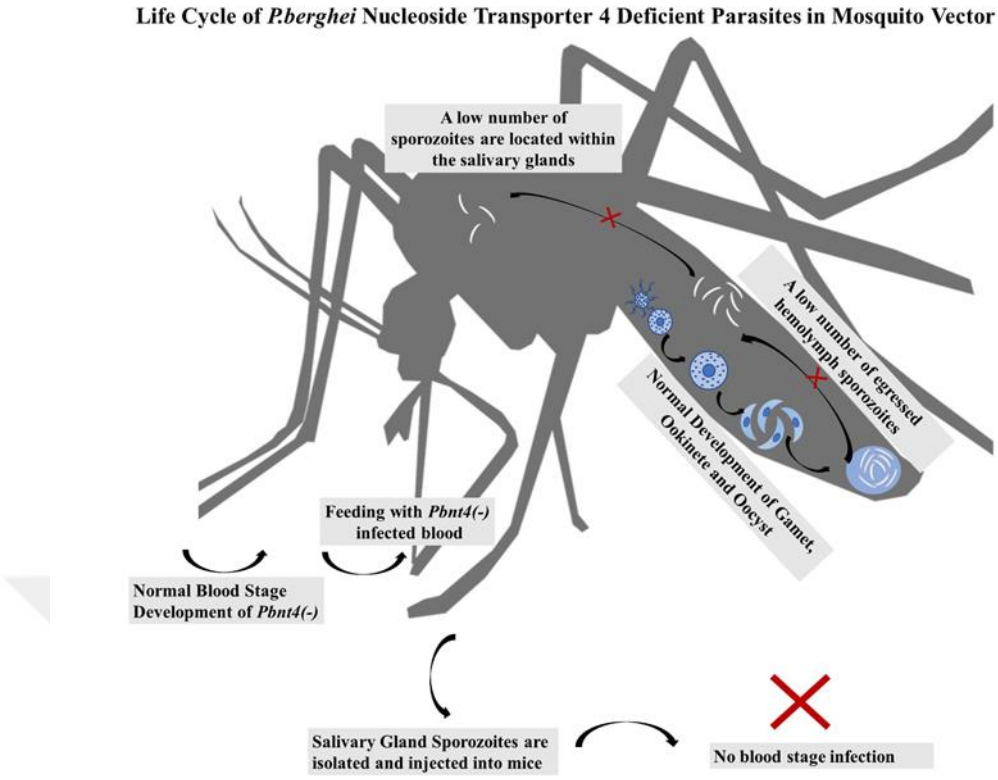

Figure 5. 2: Life cycle of nucleoside transporter 4 deficient parasites in mosquito vector.

5. CONCLUSIONS AND RECOMMENDATIONS

This thesis indicates that NT1 deficient parasites could successfully produced by CRISPR gene editing technology. Secondly, single subcutaneous sub-patent low-dose of two equally mixed species of genetically-growth-attenuated parasites, can protect mice against infectious IV challenge with WT parasites of *P. yoelii* 17X-NL and *P. berghei* ANKA strains. This suggests that genetically-attenuated parasites could be tested in clinical trials designed to protect humans against infection from two human *Plasmodium* spp. The genetically-attenuated parasites could be propagated at cultures with supra-physiological concentrations of purines, and the doses can be shipped to endemic areas as frozen stocks in liquid nitrogen.

Moreover, NT4 is not essential for blood stage growth of the parasite and is easily accessible for genetic modification. We conclude that NT4 is crucial for infectivity sporozoite functions. The results presented here in this study could be helpful in designing of future studies to evaluate the importance of NT4 in transportation of nutrients across the plasma membrane and develop it as a potential anti-plasmodial drug target. It will also be interesting to explore the cross talk between NT4 and other important nucleoside/nucleobase salvage pathways that will help in designing of more precise strategies in the development of antiparasitic chemotherapy or transmission blocking vaccine.

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APPENDICES

APPENDIX A: Ethics committee approval

APPENDIX A

CURRICULUM VITAE

Name SURNAME **:** Gözde Deveci

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E-mail **:**

EDUCATION:

- **BSc:** 2015, Yildiz Technical University, Faculty of Chemical and Metallurgical Engineering, Department of Bioengineering
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  TUBITAK 2211-C National Priority Fields PhD Scholarship Program

PUBLICATIONS, PRESENTATIONS AND PATENTS PRODUCED FROM THE THESIS:

- **Deveci, G.**, Temel, B.A., Aly, A.S.I. (2021) Single Dose Mixed Species Malaria Vaccinations by Genetically Attenuated Blood Stage Malaria Parasites Induce Sterile Immunity Against Mixed Species Malaria Infection.International Biotechnology Congress (BioTurkiye). (Presentation)
- **Deveci, G.**, Kamil, M., Kina, U., Temel, B. A. and Aly, A. S. I. (2022). Genetic disruption of nucleoside transporter 4 reveals its critical roles in malaria parasite sporozoite functions. Pathog Glob Health, 1-9. (Article)

OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS:

- 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,
- 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.

- Kamil, M., Kina, U. Y., **Deveci, G**., Akyuz, S. N., Yilmaz, I. and Aly, A. S. I. (2022). Mitochondrial Spermidine Synthase is Essential for Blood-stage growth of the Malaria Parasite. Microbiological research, 265, 127181.
- Aly, A. S. I., **Deveci, G**., Yilmaz, I., Abraham, A., Golshan, A. and Hart, R. J. (2019). Phenotypic Analysis of Rodent Malaria Parasite Asexual and Sexual Blood Stages and Mosquito Stages. Journal of visualized experiments : JoVE, (147).
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