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A plasmodium falciparum sporozoite's journey: through organs and across CD8+ T-cell challenges

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Chapter 1

General introduction

General introduction

Malaria is a mosquito-borne infectious disease caused by parasites, affecting millions worldwide. In 2023, there were approximately 263 million cases and 597,000 deaths attributed to malaria, with a staggering 94% of the burden occurring in Sub-Saharan Africa [1]. The predominant cause of malaria is *Plasmodium falciparum* (*Pf*), responsible for about 90% of cases [2]. Over the years, several preventive measures have been implemented to combat mosquitoes, including bed nets and repellents [3, 4, 5]. In addition, various drugs have been developed to prevent deaths and reduce the transmission of malaria parasites [6, 7, 8, 9]. While these strategies have led to some decline in malaria's burden, progress has stalled since 2015 and remains insufficient for eradicating the disease [10]. Furthermore, resistance to treatments is increasing among both the parasite and mosquitoes [11, 12, 13]. In response to this ongoing challenge, the WHO has established specific goals in its Global Technical Strategy (GTS). The primary goals are to reduce global malaria incidence and mortality rates by at least 90%, to achieve elimination in 35 countries by 2030, and the development of new tools, including vaccines, drugs, and vector control measures [14]. To achieve these goals a better understanding of how malaria parasites interact with the human immune system is required.

Malaria life cycle

Pf sporozoites (SPZ) are carried by female *Anopheles* mosquitoes. When an infected mosquito takes a blood meal, it injects approximately 20 to 100 SPZ into the human skin [15]. Around 20% of these SPZ manage to enter a blood vessel and are transported through the bloodstream to the liver [16]. Once in the liver, the SPZ infect hepatocytes and develop into liver schizonts, undergoing replication. This pre-erythrocytic liver stage takes about 6 to 7 days [16, 17]. After this period, the schizont ruptures, releasing merozoites into the bloodstream, marking the beginning of the blood stage of the infection. Each merozoite can invade a red blood cell (RBC) and develop from a ring stage into a trophozoite and then into a schizont [18, 19]. A schizont will multiply into 10 to 20 merozoites, which, upon rupture, can infect new RBCs [20]. After several cycles of asexual reproduction, some rings develop into male or female gametocytes, entering the sexual blood stage. These gametocytes can then be ingested by an *Anopheles* mosquito when it takes a blood meal. Inside the mosquito's midgut, the male and female gametocytes combine to form an oocyst, which ruptures after 10 to 14 days, releasing numerous SPZ that migrate to the salivary gland [21, 22, 23]. The malaria-infected *Anopheles* mosquito is then primed to infect another human, restarting the life cycle [24, 25].

Malaria vaccines

Malaria has existed for centuries, with evidence of malaria antigens found in Egyptian remains dating back to 3200 BC [26]. However, the term "malaria" was coined later. On the 5th of July in 1740, Horace Walpole wrote in a letter about "a horrid thing called the malaria (mal'aria meaning 'bad air') that comes to Rome every summer and kills one". This description led to the disease being named malaria [26]. The *Plasmodium* parasite responsible for malaria was discovered in 1880 by Alphonse Laveran, a French army doctor [27], and in 1897, Sir Ronald Ross demonstrated that the parasite is transmitted by *Anopheles* mosquitoes [28]. With the advancement of research techniques in the 1950s, scientists began studying malaria immunity with the goal of creating a vaccine [29]. Today, the malaria life cycle is well understood, and researchers are actively working on developing effective vaccines that target each stage of this cycle. The aim is to produce a highly effective malaria vaccine, with current candidates focusing on combinations with adjuvants that enhance vaccine effectiveness and longevity, like methods used in vaccines for viruses and bacteria. There are several approaches to vaccine development (Fig. 1):

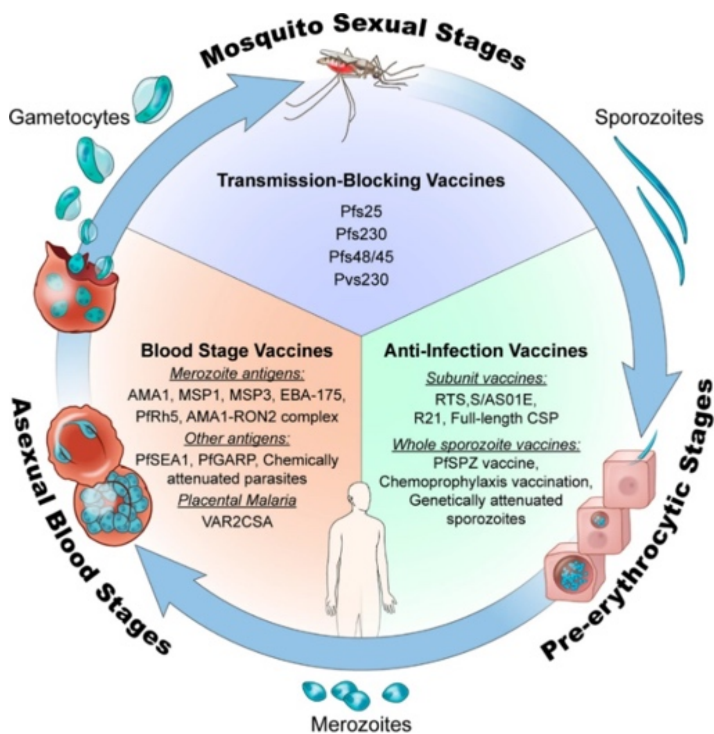

Figure 1. Malaria vaccine development against each stage of the parasite.
Illustration by Alan Hoofring, Medical Arts Design Section, NIH.

Blood-stage vaccines

Among the 37 blood-stage vaccines, 32 focus on merozoite proteins MSP1 and AMA1 [30]. While these vaccines have shown promise in laboratory studies, they have not provided sterile protection during human clinical trials or field studies [31]. This may be because merozoites are exposed for only a short duration outside the RBC, limiting the time available for antibodies to target them. Recently, a new blood-stage vaccine targeting the *Pf* reticulocyte-binding protein homologue 5 (RH5) which is required for red blood cell invasion demonstrated reduced parasite growth 3 to 4 months after administration of the RH5-AS01 vaccine and booster in humans [32]. With these encouraging results, the RH5-Matrix-M vaccine is now being tested in a phase II trial in West Africa, where it has demonstrated an efficacy of 55% [33]. This positive outcome is also associated with high levels of anti-RH5.1 serum IgG antibodies and inhibition of *Pf* growth *in vitro* [34].

Transmission-blocking vaccines

Transmission-blocking vaccines (TBV) aim to eliminate the parasite within the mosquito rather than within humans. They are designed to induce antibodies that target and kill the parasite in mosquitoes, a concept introduced in the 1970s [35]. One example, TBV Pfs25 targets the surface of the zygote/ookinete and has demonstrated efficacy in mice [36]. However, this level of success has not been achieved in humans due to suboptimal immune responses from antigen specific B cells, $CD4^+$ T cells, and T follicular helper (Tfh) cells [37, 38]. In studies involving TBV Pf230D1, which targets the surface of male and female gametes, researchers observed an increase in specific IgG antibody titres and inhibition of transmission *in vitro* after conducting standard membrane feeding assays with mosquitoes [39]. Recently, a human clinical trial combined Pfs230D1 with the adjuvant AS01, but whether this will lead to vaccine efficacy remains to be confirmed [40]. Additionally, new promising targets, such as Pfs47, a protein on the parasite surface that mediates *Plasmodium falciparum* evasion of the mosquito immune system, are currently being investigated [41].

Pre-erythrocytic vaccines

Vaccines targeting pre-erythrocytic stages of malaria are believed to be the most effective, as they address the parasite during the SPZ or liver stages, prior to the potentially deadly blood stage [42]. Vaccines against SPZ primarily aim to induce antibody production, while vaccines against the liver stage mainly aim to elicit T-cell responses [43, 44]. A significant milestone in the development of pre-erythrocytic vaccines was achieved with the World Health Organization's approval of the first-ever

parasitic vaccines: two subunit vaccines targeting SPZ: RTS,S/AS01 in 2021 and R21/Matrix-M in 2023 [45, 46]. This progress came 34 years after the RTS,S antigen was patented, highlighting the complexities involved in vaccine development and approval [47]. Both RTS,S and R21 vaccines combine the same fragment of the circumsporozoite (CSP) with a hepatitis B surface antigen to generate virus-like particles [48]. CSP, which is expressed on the outside of SPZ and during liver-stage, plays a key role in motility and the invasion of hepatocytes [49]. The antibodies generated by RTS,S/AS01 and R21/Matrix-M vaccinations can, with a sufficient antibody titre, prevent hepatocyte invasion by inhibiting SPZ motility [50, 51]. A phase III trial demonstrated that four doses of RTS,S/AS01 provided 56% efficacy in children aged 5-17 months living in endemic areas, although efficacy declined after 18 months [52]. A WHO-led phase IV trial confirmed RTS,S's safety and its potential to reduce all-cause childhood mortality by 13% [53]. The R21/Matrix-M vaccine, in a phase III study conducted in endemic areas with a vaccination schedule of 0-1-2 months, showed a similar 75% reduction in malaria incidence in areas with seasonal infections and administration and a 68% reduction with standard administration in children aged 5-36 months over 12 months [54]. However, long-term protection afforded by this vaccine remains to be evaluated. While these vaccines represent a promising first step toward lowering malaria cases, they will not eradicate malaria. Moreover, the short duration of protection and the necessity for multiple doses make these vaccines potentially expensive.

Consequently, other pre-erythrocytic vaccines are being explored, including those using whole SPZ, such as radiation attenuated SPZ (RAS), as well as SPZ immunization following parasitic treatments and genetically modified SPZ. The vaccine using RAS, can infect hepatocytes but fail to replicate DNA, thus arrest early in the liver [55]. RAS was the first vaccine to demonstrate sterile protection in naïve adults through immunization using RAS infected mosquitoes. The company Sanaria developed a method to purify and freeze SPZ, leading to the creation of the PfSPZ Vaccine [56]. This innovation improved dose control and logistical feasibility in regions where malaria is endemic. In 2013, a controlled human malaria infection (CHMI) trial using the PfSPZ Vaccine demonstrated 100% efficacy after administering five doses of 1.35×10^5 SPZ intravenously. Notably, the study observed a dose-related antibody and cellular response [57]. However, subsequent field studies revealed that the PfSPZ Vaccine achieved only 50% efficacy among Malian adults, highlighting challenges with its effectiveness and cost of production [58].

Interestingly, the development of SPZ within hepatocytes appears critical for inducing immunity. Administration of live *Pf* SPZ followed by chloroquine chemoprophylaxis (PfSPZ-CVac) provided 100% protection against challenges with SPZ during controlled human malaria infection (CHMI) [59]. Chloroquine is effective against parasites in the blood stage, allowing for complete development in the liver stage. However, challenge with *Pf* blood-stage parasites after three doses of PfSPZ-CVac resulted in 0% protection, indicating that while PfSPZ-CVac induces immunity against SPZ and/or liver stages, it does not eliminate existing blood-stage infections [60]. Furthermore, the efficacy of these vaccines can vary, and the use of chloroquine treatment raises safety concerns.

Researchers are therefore developing genetically attenuated parasites (GAP), with specific essential genes knocked out to impede development in the liver stage, which makes chemoprophylaxis unnecessary. There are two types of GAP: early-arresting genetically attenuated parasites (EA-GAP, *Pf*Δ*b9*Δ*slarp*), such as GA1, and late-arresting genetically attenuated parasites (LA-GAP, *Pf*Δ*mei2*), such as GA2. GA1 represents a double knock-out of the *B9* protein, a member of the Plasmodium 6-Cys family that plays a crucial role in forming the parasitophorous vacuole and the *SLARP* protein, expressed in both SPZ and early liver stages, is involved in transcription regulation [61]. This EA-GAP variant expresses a variety of antigens but lacks those produced during late liver-stage development and hereby limits protection [62].

The first LA-GAP was developed by deleting a gene that encodes a protein involved in fatty acid synthesis (Fabb/f) [63]. In mouse models, this LA-GAP demonstrated stronger protective effects than the EA-GAP. However, the deletion of fatty acids led to an arrest in parasite growth within the mosquito, which hindered the formation of SPZ [64] and thus presents challenges for further research and development. Consequently, scientists investigated other genes and identified the *Mei2* gene. The *Mei2* protein is part of a family of RNA-binding proteins that contain an RNA recognition motif [65]. Knocking out the *Mei2*-like RNA gene (GA2, *Pf*Δ*mei2*) leads to arrest during late liver-stage development. Importantly, the arrest occurs after the expression of both early and late liver-stage antigens, as well as some blood-stage antigens, which are typically expressed during the late liver stage [65]. This cross-stage antigen expression provides an opportunity for cross-stage immunity and increases the likelihood of protection [66]. Interestingly, GA2 provided 89% protection, while GA1 resulted in only 13% protection in CHMI in naïve Dutch volunteers [67]. These findings suggest that the immune system plays a crucial role in protection during the late liver-stage development of malaria; however, the underlying mechanisms remain unclear.

The role of CD8$^+$ T-cells

Vaccination strategies can elicit both humoral and cellular immune responses, depending on the nature of the antigen and the vaccine platform [68]. In malaria, pre-erythrocytic vaccines have thus far demonstrated the greatest potential in achieving protective efficacy. Next to antibodies targeting the CSP antigen primarily, these vaccines induce cellular immune responses, with CD8$^+$ T cells playing a central role in mediating protection during the liver stage [69]. It has been shown in mice that antigen-specific CD8$^+$ T cells are primed in secondary lymphoid organs upon RAS vaccination and then trafficked to the liver, where they recognize and eliminate infected hepatocytes [70, 71]. Depletion of CD8$^+$ T cells resulted in a significant loss of vaccine-induced immunity, indicating that CD8$^+$ T cells are essential for protection [72]. These findings underscore the critical importance of cellular immunity, and specifically CD8$^+$ T-cell-mediated mechanisms, in the context of pre-erythrocytic malaria vaccine efficacy.

Priming of CD8$^+$ T-cells

For naïve CD8$^+$ T-cells to recognize malaria antigens during the liver stage, three conditions must be met: 1) antigen priming through peptides bound to major histocompatibility complex I (MHC-I) molecules on antigen-presenting cells (APCs), which bind to the T-cell receptor (TCR) on CD8$^+$ T-cells; 2) co-stimulation through molecules such as CD28, CD40, 4-1BB, CD27, ICOS, and/or OX40; and 3) inflammatory cytokine stimulation, particularly IL-2 and IFNγ [73]. These stimuli enable naïve CD8$^+$ T-cells to proliferate, gain effector functions (such as the expression of cytokines like IFNγ and TNF), and produce cytolytic molecules like perforin and granzyme [74]. Furthermore, they differentiate into memory CD8$^+$ T-cells [75]. Memory CD8$^+$ T-cells can be categorized into subsets based on the expression of markers such as CD45RA, CD45RO, CD28, CD62L, CD69, and CCR7. These subsets include follicular helper T-cells (Tfh), effector memory re-expressing CD45RA (Temra), central memory T-cells (Tcm), effector memory T-cells (Tem), and tissue resident memory T-cells (Trm) [76]. Studies in mice indicate that Trm are crucial for liver protection against malaria [77, 78]. Therefore, the priming and differentiation of CD8$^+$ T-cells are vital for protection. However, it remains unclear how and where liver CD8$^+$ Trm are primed, whether antigens are presented during the liver stage, and if CD8$^+$ T-cells can specifically recognize and eliminate malaria-infected hepatocytes in humans.

Where does CD8⁺ T-cell priming take place?

When SPZ enter the body through the skin, they encounter dermal dendritic cells (DCs), which then travel to the skin draining lymph nodes (DLN) to present antigens directly to naïve CD8⁺ T-cells. In the DLN, DCs can present antigens to naïve CD8⁺ T-cells after encountering SPZ. Once activated, these CD8⁺ T-cells migrate to other organs, such as the spleen and liver [72]. Although the removal of the DLN resulted in a 60% reduction of activated CD8⁺ T-cells in the liver, it did not affect the frequency of primed CD8⁺ T-cells in the spleen [79]. This aligns with findings that CD8α⁺ DCs in the spleen can prime CD8⁺ T-cells, as can monocyte-derived CD11c⁺ cells in the liver DLN [80]. The removal of both the DLN and spleen drastically reduced the activated T-cell pool in the liver, but it did not eliminate it completely, suggesting that priming occurs simultaneously in multiple organs [79]. In addition to priming along the routes taken by SPZ, it has also been suggested that CD8⁺ T-cell priming can occur in the liver during the traversal and infection of hepatocytes [81]. DCs, with the help of natural killer T-cells (NKT), may present antigens in the liver after uptake from viable infected hepatocytes [79]. This is consistent with findings that depleting CD11c⁺ DCs fails to induce CD8⁺ T-cell responses during *Plasmodium yoelii* infection [82]. Moreover, studies in mice have demonstrated that traversed and infected hepatocytes can present CSP antigens directly, reactivating CSP-specific CD8⁺ T-cells and inducing protection [83]. *In vitro* studies with purified primary murine hepatocytes were able to activate and proliferate antigen-specific naïve CD8⁺ T-cells even in the absence of added cytokines, CD4⁺ T-cells, and the costimulatory molecules CD80 and CD86, which are typically involved in the priming of naïve CD8⁺ T-cells via antigen-presenting cells (APCs) [84]. This suggests that infected hepatocytes present antigens externally, indicating that Plasmodium antigens must enter the hepatocyte cytosol to be accessible for processing and presentation by MHC-I. Furthermore, immunization with LA-GAP suggests that unique epitopes are expressed during the development of parasites in the liver. Therefore, it seems likely that hepatocytes function as APCs and present antigens that can prime or activate CD8⁺ T-cells. However, it remains unclear whether and which antigens might be presented in humans.

Long-term immunity is required

Achieving long-term protection is essential for malaria elimination worldwide, especially given the high prevalence of the parasite. This requires the presence of long-lived memory CD8⁺ T-cells. It is now understood that CD8⁺ T-cell priming by APCs is necessary. However, other immune cells also play a crucial role in acquiring long-lived memory CD8⁺ T-cells. Importantly, IL-4 secreted by CD4⁺ T-cells is essential for the full

development of $CD8^+$ T-cell responses [85]. For long-term survival, the cytokines IL-7 and IL-15, along with the chemokine CXCR6 expressed by $CD8^+$ tissue-resident memory (Trm) cells, are vital [86]. In contrast, type I interferon (IFN) signalling, activated by exoerythrocytic forms (EEF) of the parasite in hepatocytes, inhibits the generation of liver $CD8^+$ Trm cells [72, 77]. Given the low infectivity levels of the parasite in the liver (1 in 10^9 in humans and 1 in 10^6 in mice [87]) and its limited time in hepatocytes (6-7 days in humans, 2-3 days in mice [88, 89]), a high number and diverse pool of antigen-specific Plasmodium-specific $CD8^+$ Trm cells are needed for immunity.

Why the route of administration/infection matters

Because different organs and immune cells are involved in T-cell priming and differentiation, the route of administration for whole SPZ vaccines is key to inducing the right response in the right organ. The differences between intradermal (ID), intramuscular (IM), subcutaneous (SC), mosquito bite administration, and intravenous (IV) routes, where IV injection bypasses the skin and skin draining lymph nodes, significantly impacts (long-term) protection. For instance, studies have shown that ID administration of RAS does not lead to inflammatory responses or parasite antigen expression in the liver, unlike IV administration [90]. This indicates that IV immunization is more likely to induce sterile protection compared to ID or SC immunization. Additionally, vaccine dosage strategies should also be considered. Recent data suggest that protection against malaria diminishes with an increasing number of booster vaccinations [91]. This indicates that T-cell exhaustion may occur, leading researchers to hypothesize that fewer, more effective immunizations could generate long-lasting immunity. In the framework of this thesis, we aim to gain a deeper understanding of how and where sporozoite-specific $CD8^+$ T-cell priming occurs and what these cells can recognize. In this thesis we investigate the role of $CD8^+$ T-cells during human liver-stage infection and whether they can specifically kill *Pf*-infected hepatocytes. Furthermore, we explore the impact of the age of SPZ in induction of immunity and and compare IV with ID administration in mice. Finally, we aim to determine if we can minimize the common booster vaccine strategy in controlled human malaria infection (CHMI) to reduce the likelihood of T-cell exhaustion. Our goal is to better understand where $CD8^+$ T-cell priming occurs, whether these cells can effectively kill malaria, and the optimal administration method for LA-GAP immunization. This information is crucial for current vaccine development strategies and will contribute to the fight against malaria.

Thesis outline

The first part of this thesis provides a comprehensive insight into the location of CD8$^+$ T-cell priming and the specific SPZ antigens that are processed by APCs and recognized by CD8$^+$ T-cells. In **Chapter 2**, we demonstrate that SPZ-specific CD8$^+$ T-cell activation occurs in the lungs. This happens earlier and to a greater extent in the lungs than in the liver. **Chapter 3** reveals that naïve CD8$^+$ T-cells, after being primed multiple times by SPZ-stimulated APCs, develop a memory response for SPZ and can recognize specific epitopes. **Chapter 4** shows that CD8$^+$ T-cells specific to the CSP epitope sequence YLNKIQNSL can recognize and kill *Pf*-infected hepatocytes *in vitro*. These data provide initial evidence that antigens are presented by human hepatocytes and are recognized by memory CD8$^+$ T-cells.

With the rise of effective LA-GAP vaccines, there is an urgent need for further understanding of optimal vaccine strategies. In **Chapter 5**, we demonstrate that the age of SPZ used during LA-GAP immunization may influence the outcome. Younger SPZ, specifically those collected 14 days post blood meal, appear to be more infectious and activate regulatory CD8$^+$ T-cells compared to older SPZ collected on day 17 and beyond. Furthermore, the route of administration plays a crucial role. In **Chapter 6**, we show that there is no activation of CD4$^+$ and CD8$^+$ T-cells in the skin after SPZ ID administration when compared with salivary gland extract (SGE). This results in lower immune activation of double-negative (DN) and CD8$^+$ T-cells in the lungs, spleen, and liver after ID administration compared to IV administration. In **Chapter 7**, we illustrate that a single low dose of LA-GAP can achieve up to 90% protection in controlled human malaria infections (CHMI). This suggests that LA-GAP immunization effectively primes CD8$^+$ T-cells, minimizes the risk of T-cell exhaustion, and simplifies the vaccination process.

Finally, **Chapter 8** summarizes the main findings presented in this thesis. It discusses the variation in vaccine efficacy across different regions, as well as considerations regarding immunization dose and route of administration. This final chapter concludes with recommendations for the next experimental steps aimed at designing an effective vaccine to eradicate malaria.

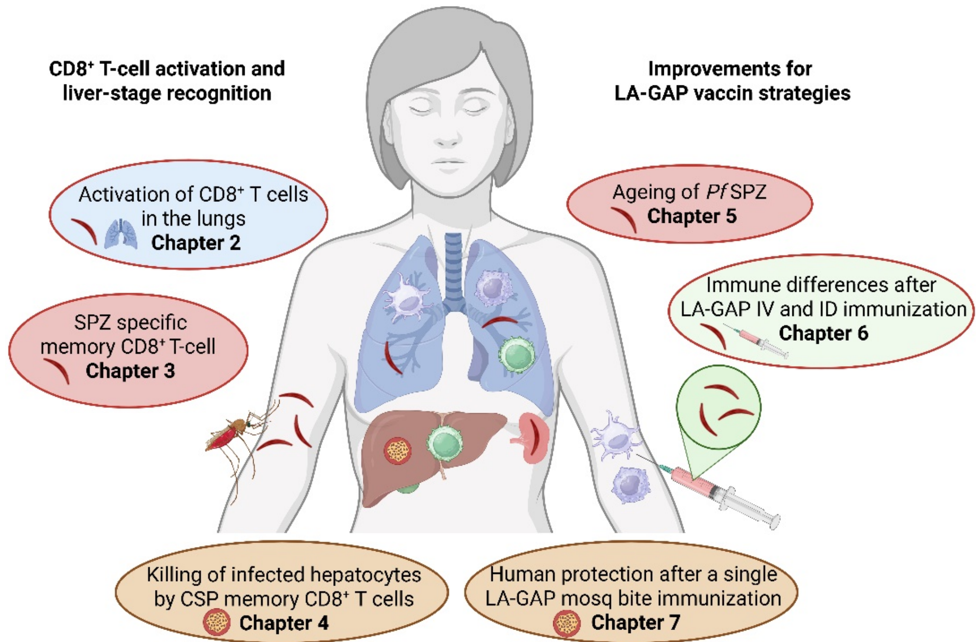

Figure 2. Overview of the outline of this thesis. Left part shows chapters focusing on CD8$^+$ T-cells priming and liver-stage recognition and the right shows chapters focusing on improvement for late-arresting genetically attenuated parasites (LA-GAP) strategies. Red filled circled chapters focusing on sporozoites (SPZ) and immune responses. Red outlined circled chapters focusing on SPZ but in context of CD8$^+$ T-cells priming in the lungs (blue filled circle) or different routes of administration (green filled circle). The chapters shown in brown filled circles describe recognition and protection against liver-stage malaria.

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