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

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# Chapter 8

## Summarizing discussion

## How will our study contribute to the field

In this thesis, we have enhanced the understanding of which potential antigens *Plasmodium falciparum* (*Pf*)-infected hepatocytes can present and where and how $CD8^+$ T-cell priming and activation occurs. In addition, we have shown the immunological differences between intradermal (ID) and intravenous (IV) immunization of late-arresting genetically attenuated parasite (LA-GAP) sporozoites (SPZ). Furthermore, we have explored how the age of *Pf* SPZ influences host immune responses. Finally, we have demonstrated that a single immunization with LA-GAP SPZ can protect against malaria in a human challenge model.

**Our work has introduced the following novel concepts:**

- Activated *Pf* SPZ-specific $CD8^+$ T-cells accumulate in mice's lungs after LA-GAP immunization **(Chapter 2)**. This chapter shows that the lungs may have a critical role in $CD8^+$ T-cell priming. Although further investigations of the $CD8^+$ T-cell functionality in the lungs are required to firmly establish their role in protection.
- *In vitro* co-culture of naïve human $CD8^+$ T-cells with antigen-presenting cells (APC) stimulated with *Pf* SPZ leads to the differentiation into *Pf* SPZ-specific memory $CD8^+$ T-cells **(Chapter 3).** This model can be used to further investigate which antigens are recognized by *Pf* SPZ memory $CD8^+$ T-cells. This information may help improve vaccines that focus on inducing $CD8^+$ T-cell responses.
- *Pf*-infected human hepatocytes can present the circumsporozoite protein (CSP) epitope YLNKIQNSL, which can be recognized and killed by YLNKIQNSL-memory $CD8^+$ T-cells (**Chapter 4**). This model can be used to identify additional epitopes by testing the killing capacity of different memory $CD8^+$ T-cells (like cultured in **Chapter 3**), which could enhance current vaccines.
- The age of the *Pf* SPZ influences host responses. Younger SPZ are more infectious but possess greater immune regulatory potential for $CD8^+$ T-cell activation in an *in vitro* human co-culture system (**Chapter 5**).
- Intradermal (ID) LA-GAP SPZ immunization compared with intravenous (IV) LA-GAP SPZ immunization in mice leads to decreased activation of $CD4^+$ and $CD8^+$ T-cells in the liver. This likely caused by a regulatory phenotype of myeloid cells in the skin-draining lymph nodes (DLN) following ID LA-GAP SPZ immunization (**Chapter 6**). These findings emphasize the importance of the age of the SPZ and the route of administration for optimizing protection after LA-GAP immunization.

- Finally, we have shown that a single immunization is sufficient to achieve 90% protection during a homologous controlled human malaria infection (**Chapter 7**). This was shown in a single-dose immunization using the LA-GAP with *Pf* SPZ-GA2 via mosquito bite in naïve Dutch volunteers. This is the first study to show protection after single-dose immunization and highlights the strength of LA-GAP immunization.

In this final chapter, we will discuss our findings in the context of existing immunological data and current vaccine responses, focusing on prospects for malaria vaccine development (Fig. 1).

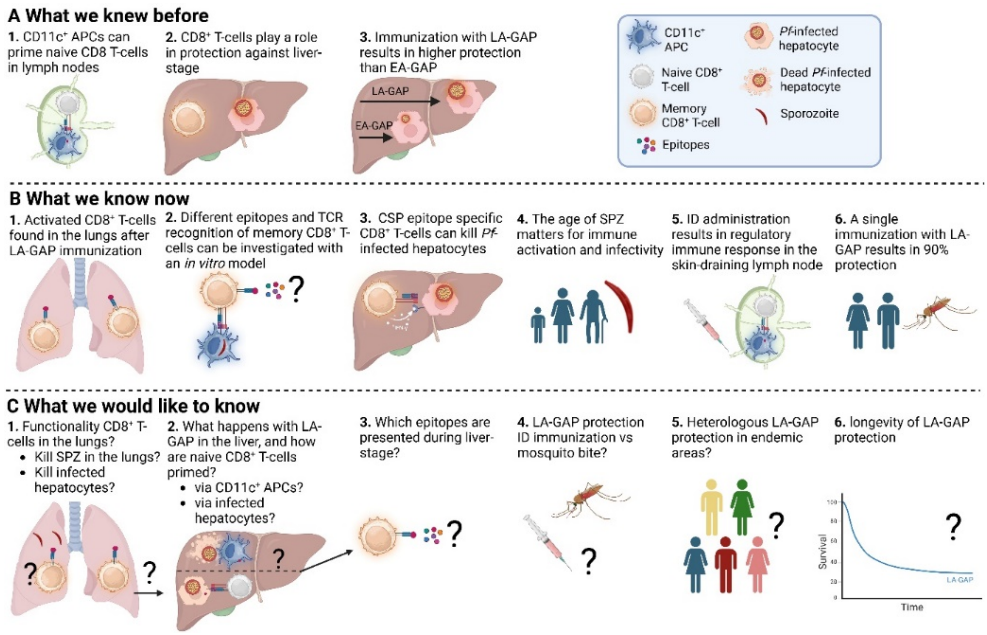


**Figure 1**. A Before we started the work in this thesis it was known that: 1. CD11c$^{+}$ APC can prime naïve CD8$^{+}$ T-cells in the lymph nodes. 2. CD8$^{+}$ T-cells play a crucial role in protection against malaria liver stage. 3. Late arresting genetically attenuated parasite (LA-GAP) immunization results in higher protection compared with early arresting genetically attenuated parasite (EA-GAP) immunization in human clinical trials. B With this thesis we have learned that 1. Activated CD8$^{+}$ T-cells are found in the lungs after SPZ LA-GAP immunization. 2. We set up a model to investigate which T-cell receptors (TCR) are present on CD8$^{+}$ T-cells primed with SPZ-stimulated APC. This can help investigate which epitopes can specifically be recognized by SPZ-primed CD8$^{+}$ T-cells. 3. We have shown that CSP

epitope YLNKIQNSL specific CD8$^+$ T-cells can kill Pf-infected hepatocytes in vitro. 4. The age of SPZ matters for immune activation and hepatocyte infectivity. 5. A more regulatory immune activation appears in the skin draining-lymph node (DLN) after LA-GAP intradermal (ID) administration. 6. And finally, we have shown that one single immunization with LA-GAP results in 90% protection in Dutch naïve volunteers. C The main follow-up questions that we would like to answer are: 1. Can primed CD8$^+$ T-cells in the lungs kill SPZ or can they kill infected hepatocytes and are a beneficial distraction of the parasite? 2. How are naïve CD8$^+$ T-cells primed after LA-GAP infection, via CD11c$^+$ APCs in the lymph node or infected hepatocytes? 3. Which epitopes are presented during the liver stage? 4. Investigate LA-GAP protection after ID immunization, with a heterologous challenge in endemic areas and longevity.

## Priming of immune cells

### SPZ-specific priming

In 1976, it was demonstrated that protection against malaria is possible following priming with SPZ. Heat-killed SPZ (HK SPZ) vaccination, which never reaches the liver, can result in protection ranging from 0% to 53% in mice. This protection is contingent upon a high and repeated immunization dose [1]. HK SPZ immunization leads to the formation of memory CD8$^+$ T-cells in the spleen and liver, as well as an increase in the number of CD8$^+$ tissue-resident memory T cells (Trm) in the liver during infection [2, 3]. This indicates that SPZ-specific immunization can indeed stimulate immunity against malaria. It has been established that the priming of SPZ-specific CD8$^+$ T-cells by antigen-presenting cells (APCs) occurs in the draining-lymph node (DLN) and spleen [4], primarily through CD8α$^+$ dendritic cells (DCs) in the skin-DLN and CD11c$^+$ APCs in the liver DLN [5, 6]. The activation of T-cells requires antigen presentation via major histocompatibility complex class I (MHC-I) by the APCs and subsequent recognition by the T-cell receptor (TCR). During this process, an interaction between CD80/CD86 on the APC and CD28 on the T-cell is required [7]. While initial priming of SPZ-specific CD8$^+$ T-cells occurs in the skin-DLN and spleen, our detection of activated CD8$^+$ T-cells in the lungs suggests that SPZ pass through the lungs, become trapped in small capillaries and are presented by APCs to naïve CD8$^+$ T-cells in the lungs. Alternatively, CD8$^+$ T-cells activated by APC in systemic lymphoid organs may be recruited to the lungs (**Chapter 2**). It remains uncertain whether these activated SPZ-specific memory CD8$^+$ T-cells can directly eliminate SPZ and thus prevent hepatocyte invasion, or if their priming and activation in the lungs serve merely as a distraction for the parasite during liver-stage. Knowing in which organs the priming of naïve CD8$^+$ T-cells happens and what their functionality is can help target organs specifically which can induce CD8$^+$ T-cell responses and protection. Moreover, each SPZ-specific memory CD8$^+$ T-cell can

recognize different antigens that are presented by APCs [8]. Along with determining the exact location of T-cell priming, knowing which antigens are presented by APCs that lead to protective $CD8^+$ T-cell responses is essential to improve $CD8^+$ T-cell-inducing vaccines. We have demonstrated that SPZ-stimulated APCs can induce naïve $CD8^+$ T-cells to differentiate into a memory response *in vitro* (**Chapter 3**). Using TCR sequencing on these SPZ-specific memory $CD8^+$ T cells, we identified several clusters that warrant further investigation for epitope-specific responses. This technique could facilitate the establishment of a library for TCR and epitope recognition, aiding research into epitope-based $CD8^+$ T-cell vaccines.

**Liver-stage specific priming**

We show that a single immunization with LA-GAP *Pf*SPZ-GA2 results in remarkable protection of 90% (**Chapter 7**), further indicating that both SPZ-specific priming and late liver stage-mediated priming play a crucial role in effective $CD8^+$ T-cell activation. Hepatocytes express MHC-I and intracellular adhesion molecule 1 (ICAM-1), which have been documented to interact with naïve $CD8^+$ T-cells [9]. This interaction implies that naïve $CD8^+$ T cell priming via infected hepatocytes could be feasible. However, studies using murine hepatitis models suggest that hepatocytes are poor APCs for naïve $CD8^+$ T-cells [10]. Both hepatitis- and malaria-infected hepatocytes require intracellular processing of antigens and presentation via MHC-I, which makes it unlikely for malaria-infected hepatocytes to prime naïve $CD8^+$ T-cells. Moreover, it is hypothesized that hepatic $CD11c^+$ APCs can take up antigens from dead infected hepatocytes and present them in the liver-DLN to naïve $CD8^+$ T-cells [11]. However, the whole SPZ immunization with chemoprophylaxis treatment (CPS) model shows 100% protection, even when the parasite fully develops in the liver and subsequently transits through it [12]. This suggests that either infected hepatocytes function as APCs or that hepatocytes are cleared by hepatic $CD11c^+$ APCs following liver schizont rupture. The development of LA-GAP halts during late liver-stage, but it remains unknown if the hepatocyte dies once the development halts and presentation occurs via $CD11c^+$ APCs or if it stays alive and primes naïve $CD8^+$ T-cells directly. To date, this remains uninvestigated due to a lack of a human *in vitro* liver-stage model which can follow the liver-stage development until after liver-schizont rupture. Understanding how priming occurs during the liver stage is crucial, as priming via $CD11c^+$ APCs might present different epitopes than those processed and presented by infected hepatocytes. This could lead to the formation of distinct $CD8^+$ T-cell pools and potential mismatches with infected hepatocytes after natural infection.

Various epitopes have been identified where $CD8^+$ T-cell responses, isolated from the blood, target for example circumsporozoite protein (CSP, involved in SPZ motility and hepatocyte invasion) and liver-stage antigen 1 (LSA-1, expressed during liver stage) following human immunization with whole SPZ vaccine radiation attenuated sporozoites (RAS) [13]. The finding of LSA-1-specific $CD8^+$ T-cells suggests that priming can occur during liver-stage, when this protein is exclusively expressed. In conclusion, priming of naïve $CD8^+$ T-cells may occur before liver-stage and possibly during liver-stage via hepatocytes or $CD11c^+$ APCs. Understanding when and how $CD8^+$ T-cell priming occurs is essential for identifying the presented antigens, which could ultimately enhance current vaccine strategies.

**What is being presented during the liver stage?**

As of today, the details of what occurs during the *Pf* liver-stage remain largely unknown due to the intricacies of the parasite's development and the challenges involved in culturing human *Pf*-infected liver cells. Once *Pf* SPZ invades and infects hepatocytes, they create a parasitophorous vacuole membrane (PVM), which separates the parasite from the host cytoplasm. Interestingly, the parasite modifies the PVM to allow small molecules from the hepatocyte cytoplasm to pass through, which nourishes the developing liver-stage parasite. However, for antigens to be presented via MHC-I, they must pass in the reverse direction through the PVM into the cytoplasm, a process that is considered highly complex [14].

The most studied and abundant antigen during the pre-erythrocytic stage of *Pf* is CSP, which is expressed by SPZ during hepatocyte invasion and liver-stage [15]. CSP contains several epitopes for $CD8^+$ T-cells, with the epitope YLNKIQNSL being the most well-characterized in humans [16]. In mice, *Plasmodium berghei Pb*-infected and *Pb*-traversed hepatocytes can present CSP epitopes, triggering interferon-gamma (IFNγ) responses [17, 18]. Additionally, *Pb* CSP-specific $CD8^+$ T-cells have been shown to effectively kill *Pb*-infected hepatocytes *in vitro*, confirming that CSP is indeed presented during liver-stage [6, 19]. Moreover, liver-specific $CD8^+$ Trms have been found to control liver-stage infections in mice [20, 21]. In humans, there has been one study that involved taking small liver biopsies from *Pf*-infected volunteers, which identified liver-specific $CD8^+$ Trms [22]. This finding suggests that in humans, $CD8^+$ T-cells may play a similar role in differentiating into Trms as observed in mouse models. However, it remains unclear what antigens human *Pf*-infected hepatocytes present and whether memory $CD8^+$ T-cells can recognize them.

Our research has demonstrated that CSP epitope sequence YLNKIQNSL-specific CD8$^+$ T-cells can recognize and kill *Pf*-infected HC-04.j7 cells *in vitro* (**Chapter 4**). This evidence shows that *Pf* epitopes can be processed and presented via MHC-I by *Pf*-infected hepatocytes and can be recognized and eliminated by memory CD8$^+$ T-cells. Nevertheless, more peptides, besides CSP, have been found during liver-stage which makes it likely that, next to CSP epitope sequence YLNKIQNSL, other epitopes are presented during the liver stage [23]. To identify which epitope memory CD8$^+$ T-cells can recognize after LA-GAP immunization, *Pf*-infected hepatocyte-specific CD8$^+$ T-cells could be cultured *in vitro* using the same method employed for culturing SPZ-specific CD8$^+$ T-cells (**Chapter 3**). Subsequently, T-cell receptor (TCR) sequencing and epitope specific re-stimulation would provide insight into the diversity of TCRs primed by *Pf*-infected hepatocytes.

How can memory CD8$^+$ T-cells kill *Pf*-infected hepatocytes?

In addition to understanding where and how CD8$^+$ T-cell priming occurs and what hepatocytes present, it is crucial to know how memory CD8$^+$ T-cells can effectively kill malaria-infected hepatocytes. After priming and boosting, CD8$^+$ T-cells undergo proliferation and acquire effector functions, allowing them to kill infected hepatocytes. This process occurs by MHC-I-mediated direct antigen presentation and TCR recognition. The mechanisms through which memory CD8$^+$ T-cells kill include direct cytolysis via perforin/granzymes and Fas/FasL interactions, as well as the activation of immune responses through the secretion of cytokines such as IFNγ and TNF [24]. The protective effect of the multiple epitope-thrombospondin-related anonymous protein (ME-TRAP, expressed on the surface of SPZ and crucial for hepatocyte invasion) vaccine has been linked to the presence of IFNγ and TNF-secreting CD8$^+$ T-cells, which correlates with a reduction in parasitic load during *Pf* liver stage *in vitro* [25]. Memory CD8$^+$ T-cells can expand, with a subset persisting as long-term memory cells capable of responding when the same epitope is presented again [26]. In addition to the direct antigen presentation that activates memory CD8$^+$ T-cells, indirect activation may also occur. For example, memory CD8$^+$ T-cells unrelated to the hepatitis A virus (HAV) can kill HAV-infected hepatocytes after they produce IL-15 *in vitro*. This occurs through the interaction between the NKG2DL (found on HAV-infected hepatocytes) and NKG2D (found on memory CD8$^+$ T-cells) complex and happens non-specifically via perforin/granzyme mechanisms [27]. This phenomenon, known as "bystander activation", complicates *in vitro* research on memory-specific CD8$^+$ T-cell killing of infected hepatocytes, requiring controls for non-specific memory CD8$^+$ T-cell responses (**Chapter 4**). Additionally, for CD8$^+$ T-cell activation following direct antigen presentation, memory CD8$^+$ T-cells must recognize the presented epitope. Given the

low infection rate during the liver stage and the limited time for immune cells to respond, it is essential to have a diverse pool of $CD8^+$ T-cells in high numbers ready to respond.

Which epitopes can be recognized by $CD8^+$ T-cells depends on the human leukocyte antigen (HLA) type. MHC-I molecules are categorized into HLA-A, B, or C, while MHC-II molecules fall into HLA-DQ, DP, or DR categories [28]. Each MHC-I molecule consists of an α chain, where the α1 and α2 domains interact with the peptide, and the α3 domain interacts with the TCR [28]. Every nucleated cell expresses three different MHC-I molecules on its surface. The high polymorphism of HLA genes results in over 25,000 unique HLA alleles for MHC-I [29]. For HLA-A alone, there are more than 7,000 different alleles, each capable of binding a distinct peptide combination. This genetic diversity means it is rare for two individuals to possess the same MHC-I molecules, leading to variations in the peptides presented by MHC-I antigen-presenting cells (APCs) among individuals. Consequently, some populations may respond more effectively to specific pathogens than others. For instance, HLA-B*53 is known to be associated with resistance to severe forms of malaria [30]. Thus, considering HLA type is crucial when developing $CD8^+$ T-cell-inducing vaccines. Furthermore, regional variations in HLA types could contribute to discrepancies in malaria protection between Europeans and Africans, complicating controlled human malaria infection vaccine trials conducted outside Africa. Interestingly, there are HLA supertypes (such as HLA-A*01, -A*02, -A*03, -A*24, -A*26, -B*07, -B*08, -B*39, -B*44, -B*58, and -B*62) that can bind to the same peptide with similar affinity. Three supertypes, HLA-A*02, -A*03, and -B*07, are found at high frequencies in diverse ethnic populations [31, 32]. These HLA types may be promising candidates for developing vaccines aiming at inducing $CD8^+$ T-cell responses. In our research, we investigated HLA-A*02 positive cells as approximately 50% of the Europeans express the HLA-A*02 allele (**Chapters 3, 4, and 5**) [33]. It would be interesting to explore recognition and activation in the context of African HLA population-specific types, such as HLA-A*30, HLA-B*15, HLA-C*07 [34], as well as the other HLA supertypes HLA-A*03 and HLA-B*07.

### How to improve LA-GAP vaccine

In this thesis, we emphasize the critical role of $CD8^+$ T-cells during LA-GAP vaccinations and highlight the significance of identifying which epitopes are presented to precisely target infected hepatocytes (**Chapters 2-7**). Notably, a single immunization with LA-GAP provides 90% homologous protection in naïve Dutch volunteers (**Chapter 7**). This remarkable achievement demonstrates the vaccine's high efficacy in inducing protective immunity. The rapid and potent priming of memory $CD8^+$ T-cells, along with

their ability to specifically recognize and kill *Pf*-infected human hepatocytes illustrates the potential of LA-GAP vaccines as transformative tools in malaria eradication efforts. However, despite the high level of protection afforded by a single LA-GAP immunization in naïve Dutch volunteers, we anticipate that protection in endemic regions will be significantly lower. This is partly due to the variety of different HLA types and the multitude of genetically distinct parasite strains that can co-exist in a single region [35]. Therefore, an improved LA-GAP vaccine strategy is urgently needed. Interestingly, it appears that several factors significantly influence the efficacy of malaria vaccines. These factors include the age of the administered SPZ (**Chapter 5**), the vaccination dose (**Chapters 2, 5, 6, and 7**), the timing of vaccination, and the route of administration (**Chapter 6**).

**LA-GAP vaccination dose and SPZ age**

Our findings indicate that the age of *Pf* SPZ is a critical determinant of both infection and immune response (**Chapter 5**). *Pf* SPZ that are 14 days old, are more infectious but induce a regulatory immune activation profile *in vitro*. This regulatory response could dampen the overall efficacy of the immune response. This highlights the need for further investigation into SPZ age in LA-GAP vaccines to achieve a balance between infectivity and immunogenicity. Moreover, the administration dose plays an essential role in this balance, where the dose seems to play an important factor in activating $CD8^+$ T-cells in the lungs (**Chapter 2**). If activated $CD8^+$ T-cells can directly target SPZ in the lungs, a vaccine should focus on administering a high dose of SPZ to induce $CD8^+$ T-cell responses in the lungs. However, a recent study in mice showed that a higher immunization dose with early arresting genetically attenuated parasite (EA-GAP) resulted in lower protection [36], indicating that with a higher dose fewer activated $CD8^+$ T-cells are in the liver. These data suggest that the higher dose results in more activated $CD8^+$ T-cells in the lungs, but this requires further investigation (Fig. 3A). Surprisingly, a single immunization with 50 mosquito bites yields 90% protection (**Chapter 7**), indicates that an effective vaccine, in a low dose combined with a single immunization, is sufficient for protection in naïve Dutch volunteers, despite expectations that boosting would be necessary.

**Timing of administration**

In a multiple-dose vaccination strategy, the timing of administration is crucial. Ideally, priming of $CD8^+$ T-cells should occur when the number of naïve $CD8^+$ T-cells is highest, which depends on the method used to expand the naïve $CD8^+$ T-cell pool (Fig. 3A). Followed by a parasite-specific immunization, which generally takes for naïve $CD8^+$ T-

cells 7-14 days to differentiate [8]. However, the exact timing of this peak in the human liver following LA-GAP immunization remains unknown. Moreover, vaccinations in seasonal malaria-endemic regions should consider the timing of immunization, as administering the vaccine before a malaria outbreak may prove to be the most effective strategy in these areas, since ongoing parasitaemia can suppress immune responses and reduce vaccine responsiveness [37].

**Route of administration**

The route of administration seems to be an important factor in improving protection against malaria. For instance, immunization of mice with cryopreserved RAS via intravenous (IV) injection resulted in 90-100% protection [38]. In contrast, immunization through ID, intramuscular (IM), or subcutaneous (SC) routes was significantly less effective in mice [39]. This discrepancy was thought to be due to the lower quantity of sporozoites (SPZ) that successfully reach the liver with these routes. However, other studies have demonstrated that despite having the same parasitic liver load after immunization (for which five times higher SPZ administration is needed for ID than for IV), ID administration still yields lower protection rates (53-68%) compared to IV administration (93-95%) [40]. ID administration appears to induce a larger production of the anti-inflammatory cytokine IL-10 by B- and T-cells, while resulting in fewer hepatic memory $CD8^+$ T-cells. LA-GAP vaccination in mice result in a lower protection (40%) after ID administration compared with IV (95%) and mosquito bite (100%) (Fig. 2) [41, 42]. The findings presented in this thesis emphasize several critical factors that influence vaccine-induced immunity, particularly the route of administration and the immune activation profiles in various tissues (**Chapters 2 and 6**). Our data reveal that $CD8^+$ and $CD4^+$ T-cells in the skin-draining lymph nodes, following LA-GAP SPZ administered via ID, exhibit a more regulatory immune activation profile compared with salivary gland extract (SGE). This regulatory activation may influence the quality of the immune response, warranting further investigation to enhance vaccine efficacy through ID administration. Even though it has not been investigated yet, we expect a comparable decrease in protection after LA-GAP ID administration compared with mosquito bite in humans.

| | Mosquito bite | IV | ID |
|---|---|---|---|
| Protection in mice | 100% (5-10 mosq, two doses) | 95% (10,000 SPZ, two doses) | 40% (50,000 SPZ, single dose) |
| Protection in human | 90% (50 mosq, single dose) | ? | ? |
| Complexity of administration | high | high | low |

**Figure 2**. Protection in mice and humans after LA-GAP immunization via mosquito bite, intravenous (IV) or intradermal (ID) administration. Followed by an overview of the complexity of the different administration methods.

Both ID and IV administration require mosquito dissection, which is labor-intensive and time-consuming. As an alternative, immunization via mosquito bite presents a rapid method for assessing whether the strain is effective enough to induce immune responses and provide protection (**Chapter 7**). However, this method poses several challenges: it is difficult to control the SPZ dose, local bite reactions to mosquito saliva can occur, mosquitoes exhibit preferences for human-specific odors and there is a general fear of using this unconventional route. Moreover, using mosquito bites for administration is impractical for large-scale vaccination, IV administration can be challenging [43], and intramuscular administration (IM) results in poor infectivity due to limited vascular access [44], making ID administration with cryopreserved SPZ the most optimal method for field use. However, with the decreased infectivity and lower immune activation after ID administration, there is an urgent need for adjuvants or delivery systems that can enhance responses toward effector memory $CD8^+$ T-cell activation following ID administration. A recent study in mice has shown that ID administration can substitute for IV administration when used with adjuvants. In this study, researchers combined a prime immunization with DNA encoding the *Plasmodium yoelii* CSP antigen, followed by a boost with adjuvanted 7DW8-5 RAS. 7DW8-5 is a synthetic glycolipid adjuvant that stimulates Th1 (IFNγ) responses, which induce $CD8^+$ T-cell responses. This combination, along with an ultra-low vaccination volume (2.5 µL) that promotes SPZ motility, resulted in sterile protection lasting at least four months [39]. These findings highlight the potential to improve LA-GAP protection following ID administration using adjuvants and an ultra-low injection volume. Furthermore, maintaining the viability of SPZ is crucial, as immunization with dead SPZ leads to reduced immune activation compared with live SPZ and makes hepatocyte invasion

impossible (**Chapter 2**). Therefore, effective methods for purifying and cryopreserving SPZ, as well as less time-consuming techniques for dissecting mosquitoes, are urgently required for the administration of LA-GAP vaccines in endemic areas.

## Proposed model to improve LA-GAP vaccine

Next to the route of administration, dose and timing, evidence suggests that immunization and challenge with a homologous strain are more effective than with heterologous strains [45]. For instance, studies indicate that heterologous protection in Malian adults was five times lower than homologous protection observed in naïve individuals [46]. Consequently, it is essential to enhance $CD8^+$ T-cell recruitment in the liver. One promising approach is the "prime-and-trap" strategy, designed to elicit robust cellular immunity by first priming $CD8^+$ T-cells and then strategically trapping them in the target organ, which is the liver in the context of malaria. Administration of LA-GAP SPZ serves as the trapping step, attracting the primed $CD8^+$ T-cells to the liver, where they subsequently will differentiate into liver-specific $CD8^+$ Trms. Numerous preclinical vaccination studies have used homologous and heterologous prime-boost strategies. However, to date, these studies have mainly relied on DNA vaccination [47]. We have demonstrated that LA-GAP immunization effectively primes and traps $CD8^+$ T-cells in the liver, making it an intriguing prospect to combine LA-GAP with a prime-and-trap strategy. Improvements to the LA-GAP vaccine can be pursued through one or more of the following steps (Fig. 3B):

1. Increase naïve $CD8^+$ T-cell population: Researchers have shown that a more diverse memory $CD8^+$ T-cell pool can be achieved when more naïve $CD8^+$ T-cells are available before antigen priming [48]. Naïve $CD8^+$ T-cells originate from hematopoietic stem cells (HSCs) in the bone marrow and migrate to the spleen [49]. Keratinocyte growth factor (KGF) has been shown to promote the output of naïve T-cells from the spleen [50]. For instance, KGF combined with a DNA plasmid tumor vaccination resulted in improved long-term survival, decreased tumor burden, and an increased number of tumor-specific $CD8^+$ T-cells [51], as well as IFNγ and TNF-producing $CD8^+$ T-cells in mice [52]. Additionally, recent unpublished data indicate that treatment with an IL-15 complex (IL-15C, which links IL-15 with the IL-15α subunit) increases $CD8^+$ and $CD4^+$ Trm-like cell numbers in the liver of mice. Further investigation is needed to determine if this enhancement leads to improved malaria protection. Therefore, combining KGF or IL-15C with LA-GAP vaccination merits further exploration of protective outcomes.

2. Epitope-specific $CD8^+$ T-cells: Understanding which antigens are presented during the liver-stage will help design a malaria-specific epitope-based vaccine that can improve

the diversity of the memory $CD8^+$ T-cell pool. The first epitope-based vaccine was developed in 1985 against *Vibrio cholerae* and *Escherichia coli*, paving the way for numerous subsequent effective vaccines [53]. This opens the possibility of investigating different epitopes and designing a heterologous epitope-based vaccine specific for the liver stage to enhance $CD8^+$ T-cell priming and recognition.

3. Enhance $CD8^+$ T-cell expansion: To improve existing LA-GAP vaccines, the use of adjuvants is particularly noteworthy, as they can enhance the immune response. There are two categories of adjuvants: immunostimulants (danger signal molecules that activate and mature APCs, e.g. via Toll-like receptors (TLR)) and delivery systems (carrier materials that facilitate antigen presentation) [54]. TLR adjuvants have shown effectiveness in malaria vaccines by enhancing immunity (e.g. IL-12, which promotes adaptive immune responses), antigen presentation, and biasing toward protective responses (a balanced Th1/Th2 response) [55]. RTS,S/AS01 is a combined adjuvant vaccine, where RTS,S acts as the delivery system (CSP on a virus-like particle) and AS01 serves as a TLR4 agonist immunostimulant adjuvant [56]. However, since this subunit vaccine relies on antibody responses and does not achieve sterile protection, alternative approaches capable of inducing stronger cellular immunity are urgently needed. For the enhancement of $CD8^+$ T-cell responses, cytokines like IL-15, IL-12, and IL-2, produced after DC stimulation, seem to be essential [57]. TLR3, TLR4, TLR7/8, and/or TLR9 agonists can induce these responses [58, 59]. Thus, combining LA-GAP vaccination with a TLR agonist could be highly promising in the fight against malaria in endemic regions. Moreover, liver-homing ligands or chemokines (CXCL10, CCL5) can recruit $CD8^+$ T-cells in the liver which makes it interesting to investigate the combination with LA-GAP [60]. Overall, these investigations are crucial for advancing the effectiveness of LA-GAP vaccines and improving malaria control strategies.

4. Long-term protection and T-cell exhaustion: With the high prevalence of malaria-infected mosquitoes, the longevity of $CD8^+$ T-cell responses may be crucial for the complete elimination of malaria. Our research suggests that a single administration of LA-GAP provides effective protection (**Chapter 7**), yet the duration of LA-GAP protection in humans remains unknown. Immunization of three to five times (200 mosquito bites) with EA-GAP PfGAP3KO (which involves deletions in pre-erythrocytic stage proteins P52, P36, and SAP1) results in 50% protection one month later and only 16.7% protection six months later in humans [61]. A study involving Dutch naïve volunteers who received whole SPZ immunization under CPS (administered three times monthly, 12-15 mosquitoes each time), resulted in 100% protection when challenged 28 days post-chloroquine prophylaxis [62]. However, a re-challenge 28 months later revealed a sustained protection of 66%, marking the longest duration of immunity observed to

date [63]. In animal models, mice receiving three weekly immunizations (30,000 SPZ via IV administration) with RAS, LA-GAP, and CPS displayed a decrease in protection of around 25% (for RAS), 0% (for LA-GAP), and 25% (for CPS) ten weeks following the third immunization, with waning effects noted for all three after nine months [36]. Overall, these observations indicate that protection diminishes over time in both mice and humans.

Interestingly, repetitive boosting with homologous listeria monocytogenes antigens does not seem to affect the lifelong survival of $CD8^+$ T-cells in mice, although it does impact the phenotype and function of memory $CD8^+$ T-cells, which is influenced by the time elapsed since the last antigen encounter [64]. During repeated natural malaria infections, there appears to be a regulatory effect from the immune system [65]. The parasite's ability to persist without causing severe disease suggests a balanced host-parasite relationship. This implies that multiple booster strategies may not enhance protection and could lead to immune cell regulation and/or exhaustion.
Additionally, cancer cells have been shown to dampen the immune response through immune checkpoint PD-1 upregulation [66]. In the context of malaria, it has been reported that PD-1 can mediate up to a 95% reduction in parasite-specific $CD8^+$ T-cells [67]. Other immune checkpoints, such as CTLA-4, LAG-3, and TIM-3, have also been identified on $CD8^+$ T-cells following malaria infection in mice [68, 69]. These immune checkpoints can contribute to T-cell exhaustion, resulting in a loss of function of parasite-specific $CD8^+$ T-cells. Exploring the potential of combining anti-immune checkpoint therapies with treatments like IL-2, IL-7, and IL-15 may promote cell memory without causing excessive immune activation and could benefit long-term protection. However, a recent study indicated that increased expression of multiple checkpoint molecules is associated with enhanced function rather than exhaustion, suggesting that treatment with anti-immune checkpoint would actually lower protection [70]. Notably, this study did not consider immune responses over time, leaving uncertainties about whether these markers influence long-term functionality. Thus, this strategy remains high-risk, with potential outcomes including decreased $CD8^+$ T-cell protection or hyperactivation leading to autoimmunity, warranting thorough investigation concerning timing and dosage. Furthermore, other immune cells, particularly $CD4^+$ T-cells, are believed to play an essential role in achieving long-term $CD8^+$ T-cell protection [71, 72]. In the liver, we observed a significant increase in $CD11c^+$ $CD8^+$ T-cells following immunization (**Chapters 2 and 6**). Notably, antigen-specific hepatic $CD11c^+$ $CD8^+$ T-cells are described as short-lived memory cells [73] that can suppress $CD4^+$ T-cell activation [74]. This suppression poses challenges for $CD8^+$ T-cells

to differentiate into long-lived memory CD8⁺ T-cells. These findings suggest that the CD11c⁺ CD8⁺ T-cells in the lungs, akin to those in the liver, may also be short-lived memory cells. Further investigation into whether blocking CD11c⁺ on CD8 T cells could result in long-term protection would be worthwhile.

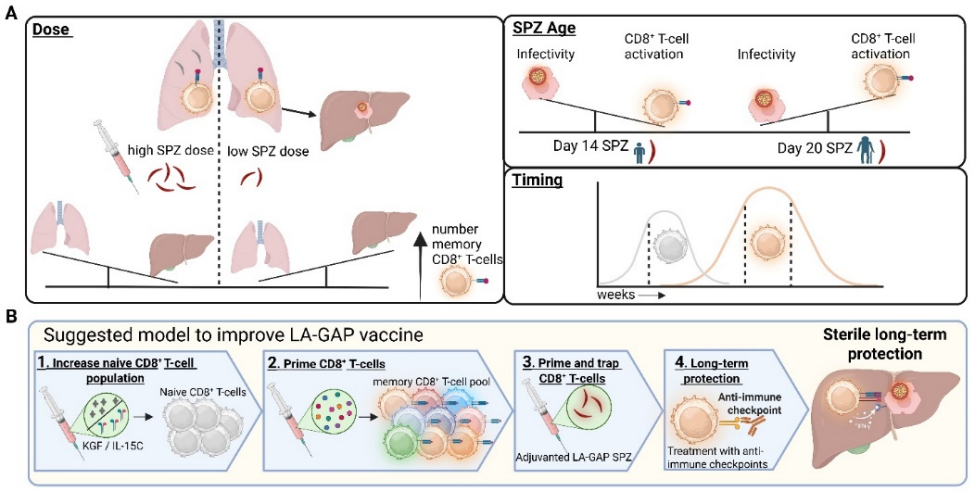


**Figure 3. A** Dose and SPZ age influence protection and CD8⁺ T-cell responses. Higher doses may enhance lung and liver-stage immunity (upper left), while older SPZ show reduced infectivity and T-cell activation (upper right), highlighting the importance of timing (lower right). **B** A suggested model to improve LA-GAP vaccine. 1. Treatment with KGF or IL-15C to enhance the naïve CD8⁺ T-cell population. 2. Epitope-specific priming of naïve CD8⁺ T-cells. 3. Prime and trap CD8⁺ T-cells in the liver with LA-GAP ID immunization in combination with adjuvants. 4. Treatment with anti-immune checkpoint to lower T-cell exhaustion and improve long-term protection.



## Differences between mouse and human

Over the years, our understanding of the parasite's lifecycle, development, immunology, drug discovery, and vaccine development has primarily relied on mouse models [75]. In this thesis, we utilized a mouse model to investigate immune cell priming in the lungs and the differences between IV and intradermal ID administration (**Chapters 2 and 6**). Using a mouse model allowed us to explore questions that would be impossible to investigate in humans. However, several vaccine studies in mice demonstrated protection but did not achieve the same level of efficacy in humans [76]. This discrepancy highlights the differences between mice and humans. Mouse models typically use genetically cloned, pathogen-free mice, which lack the HLA-type diversity found in humans. Additionally, the skin, the first organ for SPZ entry, is much thinner in mice compared to humans [77]. Thus, it is essential to investigate whether the

regulatory immune responses observed in the skin-draining lymph nodes, where dermal immune cells travel to, in mice (**Chapter 6**) also occur after SPZ ID immunization in humans. Moreover, the liver plays a critical role in $CD8^+$ T-cell priming. The differences in the development of the liver stage between *Pf* (6-7 days, human) and *Pb* (2-3 days, mice) complicate studies focussing on immune priming and recognition. During infection, APCs can, after encountering the parasite, release cytokines such as IL-12 and IL-15. These cytokines can attract $CD8^+$ T-cells to the site of infection [78]. The shorter liver stage during *Pb* infection may result in different levels of priming and protection in mice compared to humans. Furthermore, only 61% of the epitopes between *Pf* and *Pb* are comparable [79], leaving room for mismatches in priming and recognition. Therefore, while a mouse model can provide valuable insights, it has limitations, particularly concerning the delicate liver-stage correlation to *Pf* in humans.

With controlled human malaria infection, we can account for all immune factors and investigate protection following LA-GAP immunization (**Chapter 7**). However, controlled human malaria infection only allows for the examination of peripheral immune cells, making it difficult to study liver-resident $CD8^+$ T-cells, which are present at a frequency 100 times higher in the liver than in the peripheral blood of non-human primates [80]. To address this challenge, we have attempted to establish a human *in vitro* model to investigate the priming of $CD8^+$ T-cells (**Chapter 3**) and the recognition of *Pf*-infected hepatocytes (**Chapter 4**). Despite the limitations of this model, such as the absence of other immune cells, cytokines, and nutrients, it provides insights into what can be presented and recognized during the liver stage of malaria.

## Future perspectives

This thesis demonstrates that a single LA-GAP immunization delivered via mosquito bite is effective enough to achieve 90% protection (**Chapter 7**). The next steps in improving LA-GAP involve enhancing its immunogenicity and simplifying its administration. To achieve this, we need to identify which epitopes can be recognized by $CD8^+$ T-cells.
We developed a model to prove the cytotoxicity of CSP epitope sequence YLNKIQNSL-specific $CD8^+$ T-cells, and the presentation of CSP epitope sequence YLNKIQNSL by *Pf*-infected hepatocytes. This model can be used to examine the presentation of various epitopes (**Chapter 4).** Cultivating different epitope-specific memory $CD8^+$ T-cells will be necessary, although the limited knowledge of all epitopes present poses a limitation. To overcome this, we cultured *Pf*-SPZ memory $CD8^+$ T-cells through multiple co-culture rounds with *Pf* SPZ-stimulated APCs (**Chapter 3**). These cells can recognize all epitopes presented by *Pf* SPZ-stimulated APCs, and with TCR sequencing, we have identified the

TCRs present on these *Pf*-SPZ memory CD8$^+$ T-cells. To ascertain which specific epitope is recognized by which TCR, it would be beneficial to culture separate clones from this SPZ-specific CD8$^+$ T-cell pool and measure the activation of these cells after stimulation with different epitopes. This approach will clarify which epitopes can be presented by APCs and recognized by CD8$^+$ T-cells following SPZ infection or immunization. However, this method primarily demonstrates the recognition of SPZ-specific epitopes and does not encompass any recognition of liver-stage-specific epitopes. Therefore, similar studies should be conducted using liver-stage-specific memory CD8$^+$ T-cells. However, the low infectivity of *Pf* during the liver stage poses a challenge for culturing *Pf* liver-stage-specific CD8$^+$ T-cells, a technical problem which needs to be overcome. Once possible, this approach will help determine: 1) whether *Pf*-infected hepatocytes can function as APCs and prime naïve CD8$^+$ T-cells, and 2) which epitopes are presented by *Pf*-infected hepatocytes after cloning the different memory cells. Additionally, data comparing EA-GAP and LA-GAP immunization indicate that the development of the parasite during the liver stage plays a crucial role in protection. Therefore, it is essential to first develop an *in vitro* liver-stage model that can sustain the parasite throughout the entire liver stage.

## Concluding remarks

This thesis highlights the crucial role of CD8$^+$ T-cell priming in establishing protective immunity against liver-stage malaria. It also emphasizes how factors such as the age of SPZ and the routes of vaccine delivery can influence this immunity. The demonstrated efficacy of a single LA-GAP immunization, which has shown high levels of protection in human volunteers, represents a significant milestone in malaria vaccine development. These findings lay the groundwork for the rational design of next-generation malaria vaccines and open avenues for new strategies that target both effector and regulatory pathways, aiming for lasting and comprehensive protection.

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