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

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Appendices

English summary

Each year, approximately 600,000 people die from malaria, a mosquito-borne parasitic infection, with 94% of cases occurring in sub-Saharan Africa. Among the various malaria species, *Plasmodium falciparum* (*Pf*) is responsible for the highest mortality rate. Unfortunately, interventions aimed at controlling mosquito populations, such as bed nets and insecticides, are not effective enough to eliminate the disease completely. As a result, researchers have been striving for years to develop an effective vaccine. In 2023, the World Health Organization (WHO) approved two malaria vaccines for the first time: RTS,S/AS01 and R21/Matrix-M. These vaccines target the circumsporozoite protein (CSP), which is present on the surface of malaria sporozoites (SPZ), the infectious form of the parasite. While this marks a significant milestone, these vaccines offer only 35–75% protection in endemic areas, require multiple booster immunizations, and exhibit decreasing effectiveness over time. Therefore, further research into more efficacious malaria vaccines is urgently needed.

Lifecycle

Malaria SPZ are injected into the skin by an *Anopheles* mosquito during a blood meal. Approximately 20% of the SPZ exit the skin and enter the bloodstream, where they travel through various organs before reaching the liver. In the liver, the SPZ infects hepatocytes and develops into a liver schizont filled with merozoites. This process takes 6 to 7 days, after which the liver schizont ruptures, releasing merozoites into the bloodstream to infect red blood cells. The merozoites then develop into the asexual blood stage, followed by the sexual blood stage. The sexual blood stage consists of male and female gametocytes, which, when ingested by a mosquito during a blood meal, develop into SPZ over the following 10-14 days, becoming capable of infecting new human hosts.

Attenuated malaria sporozoite vaccines

Over the years, researchers have attempted to develop vaccines targeting each stage of the parasite's life cycle. However, whole SPZ vaccines targeting the SPZ and liver stages have proven to be the most effective. The first protective whole SPZ vaccine was developed using radiation-attenuated SPZ (RAS). Since then, several whole SPZ vaccines have been created, including genetically attenuated SPZ. These SPZ have specific genes deleted, weakening the parasite. One example is the *Pf* GA1 parasite, a *Pf* SPZ engineered to stop at the early liver stage. Subsequently, the *Pf* GA2 parasite with a deletion of the *mei2* gene (*Pf*Δ*mei2)*, was designed to halt at the late liver stage, preventing merozoites from entering the bloodstream while allowing sufficient time for immune system recognition. *Pf* GA2 has demonstrated 89% protection, while *Pf* GA1

offers only 13% protection in human Dutch volunteers. These findings highlight the importance of the late liver stage in eliciting effective immunity against malaria. However, the specific immune cells responsible for protection during the liver stage in humans remain unidentified. To date, *in vitro* cultivation of *Pf* SPZ infection from liver stage to blood stage has not been successful with wild-type parasites. Consequently, it is not possible to determine at which point *Pf* GA2 parasites arrest development: whether they die, remain stalled in the late liver stage, or rupture as schizonts. Obtaining liver biopsies from malaria-infected volunteers is highly invasive, carries significant risk and offers a low probability of capturing *Pf*-infected hepatocytes due to the limited infectivity during this stage. These challenges hinder the investigation of immune mechanisms involved in liver-stage protection. To address this, a functionally equivalent model using the rodent-infective *Plasmodium berghei* (*Pb* GA2) has been developed to enable a detailed study of liver-stage immune responses.

Immune activation

Studies in mice have shown that $CD8^+$ T cells in the liver play a crucial role in eliminating the parasite, as their depletion abolishes RAS-induced protection. To recognize malaria-specific antigens, $CD8^+$ T cells must first be primed by antigen-presenting cells (APCs) like macrophages and dendritic cells. Once activated, they differentiate into epitope-specific $CD8^+$ T cells, some of which remain in tissues as resident memory T cells (Trm), ready to respond to infection. In the liver, Trm $CD8^+$ T cells are thought to mediate protection by specifically recognizing and eliminating malaria-infected hepatocytes. However, it remains unclear where and how naïve $CD8^+$ T cells are primed by APCs and which epitopes are presented by infected hepatocytes. Identifying these mechanisms could enable epitope-specific training of naïve $CD8^+$ T cells to enhance vaccine-induced protection. Additionally, an effective vaccine must confer long-term immunity. Understanding how $CD8^+$ T cells are trained could help extend their longevity and effectiveness.

$CD8^+$ T-cell priming and recognition

Priming of $CD8^+$ T cells can occur in multiple lymphoid organs. For example, SPZ are injected in the skin by a mosquito, where they encounter dermal APC. These SPZ-stimulated dermal APC will travel to the skin-draining lymph nodes (skin-dLN) where they prime naïve $CD8^+$ T cells. The activated $CD8^+$ T cells will migrate to the liver. However, removal of the skin-dLN prior SPZ infection does not abolish liver-stage protection, indicating that $CD8^+$ T-cell priming relevant for liver-stage immunity takes place elsewhere. Therefore, we investigated the possibility of $CD8^+$ T-cell priming in other organs. Given that the lungs are highly vascularized and frequently serve as

immune surveillance hubs for circulating pathogens, we investigated whether they might also be involved in CD8$^+$ T-cell priming following GA2 SPZ immunization. It was previously unknown whether SPZ reached the lungs via circulation and how the immune system responded. In **Chapter 2,** we observed activated CD8$^+$ T cells in the lungs following *Pb* GA2 SPZ immunization in mice, indicating that SPZ travel through the lungs. Interestingly, we found an increased number of lung-specific Trm CD8$^+$ T cells, which had previously been described in the liver, after *Pb* GA2 SPZ immunization. Compared to liver activation (7 days post-immunization), lung activation occurred earlier (2 days post-immunization). These findings suggest that CD8$^+$ T-cell activation occurs in multiple organs. However, it remains unclear whether lung-activated CD8$^+$ T cells can directly eliminate SPZ or whether their presence benefits SPZ survival.
To investigate what memory CD8$^+$ T cells can recognize after they encounter SPZ-stimulated APCs, we cultured HLA-A*02-restricted human *Pf* SPZ-specific memory CD8$^+$ T cells *in vitro* in **Chapter 3** using a co-culture model with HLA-A*02 positive APCs. We demonstrate that repeated stimulation with *Pf* SPZ leads to the generation of a highly activated population of effector memory CD8$^+$ T cells (Tem). These cells exhibit robust functional markers, including CD137, IFNγ, and Perforin with distinct T cell receptor (TCR) clusters, upon re-stimulation with SPZ, suggesting antigen-specific recognition and cytolytic potential. After stimulation with different pre-erythrocytic-specific epitopes, we found activation of the SPZ memory CD8$^+$ T cells for complex-release (CRA) epitope GLLGNVSTV, indicating that this epitope is being processed and presented by *Pf* SPZ-stimulated APCs. This model can be used to identify additional epitopes recognized by SPZ-specific memory CD8$^+$ T cells, thereby refining vaccine target selection. However, further investigation is needed to determine whether these memory CD8$^+$ T cells can specifically recognize and eliminate *Pf*-infected hepatocytes.

Additionally, the CSP epitope YLNKIQNSL has been reported in literature to enhance CD8$^+$ T-cell activation in peripheral blood mononuclear cells (PBMCs) from individuals living in malaria-endemic regions and has also been associated with protective immunity in mice. Therefore, in **Chapter 4**, we investigated whether a memory HLA-A*02-restricted CD8$^+$ T-cell clone specific for the CSP epitope YLNKIQNSL can specifically recognize and eliminate *Pf*-infected human HLA-A*02 positive hepatocytes *in vitro*. Here we observed that these cells can recognize and kill around 45% of *Pf*-infected hepatocytes. This data provides unequivocal proof that hepatocytes can present *Pf* CSP epitope sequence YLNKIQNSL during *Pf* liver stage infection and consequently are targets for CD8$^+$ T-cell-mediated killing. This *in vitro* model can be used to identify

additional liver-stage epitopes, thereby increasing epitope diversity and improving vaccine efficacy.

These findings demonstrate that $CD8^+$ T cells can be primed in the lungs and that memory $CD8^+$ T cells can recognize and kill *Pf*-infected hepatocytes through CSP epitope YLNKIQNSL. To fully leverage these effector functions, vaccine-induced $CD8^+$ T-cell responses must be robust, epitope-specific, and effectively distributed across relevant tissues. Furthermore, individuals in malaria-endemic regions often show reduced protection compared to those in non-endemic settings. This may be caused by differences in genetics, pathogen exposure, and potential mismatches between vaccine strains and wild-type parasites. Lab-adapted genetically attenuated SPZ, like GA2, may differ from naturally circulating strains, leading to suboptimal $CD8^+$ T-cell priming and impaired recognition of *Pf*-infected hepatocytes. Therefore, a better understanding of the optimal vaccine formulation, such as SPZ biology, route of administration and dose can profoundly shape the magnitude and quality of immune responses.

Inducing GA2 SPZ vaccine efficacy

Biological variation in SPZ, such as their age at the time of immunization, can significantly influence vaccine performance. In literature, SPZ collected between 10- and 21-days post–mosquito blood meal have been used interchangeably, despite limited understanding of how SPZ biology changes over time or how such changes affect their immunogenicity and protective efficacy. Therefore, we examined in **Chapter 5** the differences in motility, immunogenicity and infectivity between day 14 and day 20 *Pf* SPZ post-blood meal. Here we found that younger *Pf* SPZ (14 days post-blood meal) displayed greater motility, macrophage activation and hepatocyte infectivity, but elicited weaker $CD8^+$ T-cell responses than older *Pf* SPZ (20 days post-blood meal). Future studies should determine whether higher hepatocyte infectivity or stronger $CD8^+$ T-cell activation offers greater protection, insights that will be critical for improving SPZ vaccine design.

Additionally, mouse models have shown that the route of vaccine administration significantly impacts vaccine efficacy. For example, intradermal (ID) SPZ immunization, which is easier to administer than intravenous (IV) immunization, results in a lower parasitic liver load and diminished protection after repeated SPZ immunizations followed by antiparasitic treatment. Identifying the most effective route could improve vaccine efficiency, reduce the necessary dosage, and lower costs. We explored in **Chapter 6** why ID immunization results in lower protection. Our findings revealed a lower number of $CD8^+$ Trm cells and reduced activation of $CD8^+$ T cells and DN T cells in

the spleen, lungs and liver after ID *Pb* GA2 immunization compared with IV *Pb* GA2 immunization. In the skin, skin-dLN and the liver we identified a large population of myeloid cells with a predominantly regulatory phenotype following ID *Pb* GA2 SPZ immunization. These cells exhibited decreased CD86 expression in the skin and increased PD-L1 expression in the skin-dLN. These results suggest that myeloid cells in the skin and skin-dLN orchestrate a regulatory immune response resulting in a lower number of $CD8^+$ Trm cells and diminished T cell activation in the spleen, lungs and liver after ID *Pb* GA2 SPZ immunization as compared to IV *Pb* GA2 immunization. Recent studies have shown that reducing the injection volume can enhance vaccine efficacy via the ID route. Together, these insights provide an important foundation for optimizing ID immunization strategies to achieve protective immunity comparable to IV delivery.

Finally, recent studies have shown that multiple booster immunizations may paradoxically diminish protection, highlighting the need to determine the minimum number of doses required for effective immunity. In **Chapter 7,** we used a human clinical study to demonstrate that a single *Pf* GA2 SPZ immunization administered via mosquito bites provides 90% protection. Elevated cytokine-expressing $CD4^+$ T cells were associated with this protection. This unprecedented single-dose efficacy highlights the GA2 vaccine potential, warranting further research to optimize administration routes and dosages for maximal protection in endemic regions.

Conclusion

Altogether, the studies presented in this thesis demonstrate that $CD8^+$ T cells can be primed in the lungs, that memory $CD8^+$ T cells are capable of recognizing specific *Pf* epitopes, and that they can effectively recognize and kill *Pf*-infected hepatocytes. In addition, we have shown that the efficacy of GA2 SPZ vaccines can be enhanced by optimizing both the age of the SPZ and the route of administration. Finally, we demonstrated that a single dose of GA2 SPZ can confer up to 90% protection in humans. These findings provide important insights into the immune mechanisms activated by GA2 immunization and offer strategies for improving vaccine design, contributing to the long-term goal of eradicating malaria completely.