Tissue-specific cellular immune responses to malaria pre-erythrocytic stages

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Highlights

- Protective immunity against malaria can be achieved through immunisation with live attenuated *Plasmodium* sporozoites and targets the parasite pre-erythrocytic stages.
- Naturally exposed individuals remain at risk of malaria despite multiple sporozoite infections, and this could be explained by different mechanisms.
- Protective immunity relies primarily on effector $CD8^+$ T cells targeting the parasite in the liver.
- The generation of liver-resident parasite-specific memory $CD8^+$ T cells is emerging as a key determinant of protective immunity.

Abstract

Complete and long-lasting protective immunity against malaria can be achieved through vaccination with invasive live attenuated *Plasmodium* sporozoites, the motile stage inoculated in the host skin during a mosquito bite. Protective immunity relies primarily on effector $CD8^+$ T cells targeting the parasite in the liver. Understanding the tissue-specific features of the immune response is emerging as a vital requirement for understanding protective immunity. The small parasite inoculum, the scarcity of infected cells and the tolerogenic properties of the liver represent hurdles for the establishment of protective immunity in endemic areas. In this review, we discuss recent advances on liver-specific features of immunity including innate recognition of malaria pre-erythrocytic stages, $CD8^+$ T cell interactions with infected hepatocytes, antigen presentation for effective $CD8^+$ T cell responses and generation of liver-resident memory $CD8^+$ T cells. A better understanding of the factors involved in the induction and maintenance of effector $CD8^+$ T cell immunity against malaria pre-erythrocytic stages is crucial for the development of an effective vaccine targeting the initial phase of malaria infection.

Introduction

Malaria, caused by *Plasmodium* parasites, is one of the leading causes of mortality and morbidity in resource poor areas worldwide. Notwithstanding global control and elimination efforts, >400,000 people still die annually due to malaria (http://www.who.int/malaria/publications/world-malaria-report-2016/report/en). A highly efficacious malaria vaccine remains elusive. *Plasmodium* sporozoites are injected in the host skin by a female infected *Anopheles* mosquito. These sporozoites travel to the liver, invade hepatocytes and develop into exo-erythrocytic forms (EEF), which generate thousands of blood stage parasites. Targeting the malaria pre-erythrocytic stage is an ideal and attractive strategy for malaria vaccination. Inhibiting liver infection and development of malaria parasites can prevent both the disease-causing blood stages and the transmissible sexual stages.

Humans, rhesus monkeys and mice exposed to multiple doses of γ-radiation-attenuated sporozoites (RAS), the gold standard vaccine for malaria, can be fully protected against normal sporozoite challenge (reviewed in [1]). Alternative attenuation strategies, such as genetically attenuated parasites (GAP) or chemoprophylaxis with sporozoite infection (CPS), also induce sterile protection (reviewed in [2]). Whilst the use of attenuated parasites is a feasible approach for vaccination, they demand production of large quantities of infected mosquitoes that is not easily scalable to mass vaccination in poor settings. But, if we can discover the important features of a protective immune response, we can replicate these phenotypes by sub-unit vaccination. RTS,S/AS01, the most advanced malaria sub-unit vaccine candidate to date, is based on the circumsporozoite protein (CSP), the surface coat antigen of sporozoites. Yet, despite being designed to elicit different arms of the immune response, RTS,S/AS01 only provides partial protection in malaria-naïve and –experienced individuals [3•].

In rodent models and rhesus monkeys, protection conferred by RAS vaccination is largely dependent on effector $CD8^+$ T cells (reviewed in [4]). Depletion of $CD8^+$ T cells prior to challenge of immunised mice and rhesus monkeys consistently abrogated protection [5,6]. *P. falciparum* (*Pf)* RAS vaccination of humans induces high numbers of sporozoite-specific $CD8^+$ T cells producing IFN-γ [7]. Understanding the key features of host-parasite interactions and the induction of innate and adaptive immune responses, particularly parasite-specific $CD8^+$ T cells, is crucial for informing the development of an effective next generation malaria vaccine.

Immunisation with attenuated parasites versus natural infections: numbers matter

Despite repeated infections, individuals in endemic areas do not develop sterilising protection and those surviving episodes of childhood malaria remain vulnerable to intermittent infections [8]. Several possibilities, including the small number of parasites naturally transmitted by mosquitoes or the down-regulation of immunity by malaria blood infection, can explain the reasons behind the contrasting outcomes with those experimentally vaccinated with attenuated sporozoites (**Figure 1**).

In mice, only ~20-50 *P. yoelii* (*Py*) or *P. berghei* (*Pb*) sporozoites are inoculated in the host skin during an infective bite and only a small fraction invades and develops inside hepatocytes (reviewed in [9]). $CD8^+$ T cell responses to CSP and sporozoites following *Py* and *Pf* RAS immunisation, respectively, are dependent on antigen dose so low inoculum equates to poor $CD8^+$ T cell responses [7,10]. In the *Py* model, $CD8^+$ T cell responses are not readily increased by repeated immunisation [11,12]. To achieve sterile protection in humans, more than 1,000 *Pf* infective bites (*Pf* RAS) are required [13]; this amount corresponds to almost ten years of exposure to *Pf* in a high malaria transmission area [14] but administered in a much shorter period. Sterile protection can also be achieved by the intravenous inoculation

of ~700,000 *Pf* RAS [7]. To protect humans under CPS, fewer *Pf* infective bites (~40) or cryopreserved sporozoites (~150,000) are needed [15,16•]. For CPS, the host is exposed to both pre-erythrocytic and blood stage (transient parasitemia) antigens, and sterile protection is observed only against a *Pf* sporozoite challenge [17]. Comparable to findings in humans, CPS induces efficient protection against *Pb* sporozoite infection, but not against blood stage challenge. This sterilising protection is abolished after depletion of $CD8^+$ T cells and is not affected by the lack of mature B cells [18,19]. In contrast, CPS vaccination not only induces high levels of antigen-experienced $CD8^+$ T cells but also targets blood stages of *Py* and *P. chabaudi* (*Pc*) [18,20–22]. In common, the two species used in these studies cause an acute parasitemia that can be naturally controlled by non-vaccinated hosts, indicating a lower stringency for the immune-control of the blood stage infection in comparison to the *Pb* and *Pf* lethal strains. Late arresting *Py* GAPs have been shown to provide superior protective immunity, suggesting a role of mid/late EEFs antigens in protection, and similar to RAS and CPS, sterile protection is dependent on the immunising dose of attenuated sporozoites [23]. These data suggest that exposure to a broad antigenic repertoire, including antigens shared between EEFs and blood stages, improves protection against the pre-erythrocytic stages. Additionally, the absence or the rapid clearance of infected RBCs by RAS/GAP or CPS vaccination, respectively, might impede the deleterious effect of blood infection on antigen presentation [24], the numbers and functionality of $CD8^+$ T cells [25] or the expansion of regulatory T cells during a prolonged blood-infection [26•]. Overall, the delivery of high doses of sporozoites seems to be a key requirement for the sterile protection elicited by immunization using live attenuated sporozoites. This high antigenic load is likely associated with overcoming the humoral and cellular effector thresholds necessary to sterilize the sporozoite infection [27,28].

Early responses to sporozoites and liver stages: innate immunity and hepatic responses

Information on how sporozoites interact with the innate immune system remains limited. *Pb* sporozoites induce the biphasic recruitment of CD11b^{+} Ly6C^{int} Ly6G^{hi} polymorphonuclear neutrophils, CD11b^{+} Ly6C^{-} Ly6G^{-} resident myeloid cells and CD11b^{+} Ly6C^{hi} Ly6G^{-} inflammatory monocytes in the skin inoculation site and the proximal draining lymph node (DLN), evoking a Th1 cytokine profile [29]. Migrating *Pb* sporozoites induce a signalling cascade *in vitro* in primary murine hepatocytes with MyD88-mediated NF-KB activation [30]. Although EEF development is clinically silent, accumulating evidence suggest that, as parasites replicate in the liver, functional innate immune responses are triggered that are dependent on both type I and II IFNs [31•,32•]. The type I IFN signaling pathway is activated in the livers of mice intravenously infected with either *Pb* or *Py,* a process that involves the cytosolic receptor melanoma differentiation-associated protein 5 (Mda5), suggesting sensing of parasite RNA, and requires the mitochondrial antiviral signalling protein (Mavs) and the transcription factors interferon-regulatory factors-3 (Irf3) and Irf7. Type I IFNs bind to Ifnar on hepatocytes and leukocytes, resulting in the subsequent recruitment of leukocytes to the liver at the end of the hepatic infection. Indeed, following the inoculation of *Py* GAP, type I IFN signalling is essential for the recruitment or expansion of CD49b^{+}CD3^{+} Natural Killer T (NKT) cells, one day after the peak of hepatic parasite release in the blood circulation. These NKT cells reduce liver infection during a subsequent and intertwined secondary *Py* sporozoite infection, presumably via the production of IFN-γ [32•]. However, type I IFN signalling does not impact the EEF growth after a primary *Pb* or *Py* sporozoite infection [31•,32•]. Furthermore, earlier reports using *Pb* and *Py* suggest that NKT cells have no role in protection against malaria pre-erythrocytic stages [33,34]. Notably, a type I IFN response of much lower magnitude was observed when lower doses of parasites were transmitted through mosquito bites [31•]. Hence, the significance of this response and its

relevance in humans still remains uncertain, as well as its impact on the acquisition of immunity by vaccination using live attenuated sporozoites. During EEF development, the parasite exploits diverse cellular pathways and several host and immune factors are modulated, including Bcl-2, p53, IL-6, heme oxygenase and the autophagy machinery (reviewed in [35,36] and [37]). The relationships amongst the modulation of host cell factors, the innate immune system and the development of protective $CD8^+$ T cells against pre-erythrocytic stages remain to be established.

Antigen capture and presentation leading to CD8+ T cell priming

How parasite antigens are processed and presented for primary activation of antigen-specific $CD8^+$ T cells is not well understood, but likely depends on the nature and spatio-temporal exposure of parasite-derived antigens. Sporozoites migrate through various cell types during their journey from the skin to the liver. During cell traversal, sporozoites shed antigens in the host cell cytosol, which can be processed and directly presented [38] or captured for cross-presentation by dendritic cells (DCs). After dermal inoculation, a fraction of *Pb* sporozoites actively migrate to the DLN [39], and can prime protective *Py*CSP-specific $CD8^+$ T cells [40]. Lymph-node resident $CD8\alpha^+$ DCs capture antigens from migratory malaria sporozoites and induce *Pb*CSP-specific $CD8^+$ T cell responses [41]. Intravenous inoculation of live attenuated sporozoites is a more efficient vaccination approach as compared to intradermal inoculation, in both human and rodents models [7,42]. Various factors likely concur to protective immunity induced by immunization with live attenuated sporozoites administered intravenously (**Figure 2**). A recent study revealed that the lower protective efficacy of *Py* GAP administered via the intradermal route is not linked to low hepatic parasite numbers, but correlates with a shift towards regulatory immune responses [43•]. In particular, more interleukin-10-producing B and T cells but fewer hepatic memory $CD8^+$ T cells and $CD8\alpha^+$

168 DCs were found in the liver and skin DLNs after intradermal injection, as compared to
169 intravenous inoculation. Intravenous injection of *Pb* RAS leads to a CD8α$^+$ DC-dependent
170 splenic priming of CD8$^+$ T cells specific for an antigen expressed in pre-erythrocytic and
171 blood stages [44]. CD8α$^+$ DCs accumulate in the liver after *Pb* RAS immunisation [45,46],
172 however a role for hepatic DCs in both priming of CD8$^+$ T cells and protection remains poorly
173 characterised. In the liver, sporozoites traverse Kupffer cells (KC) and liver sinusoidal
174 endothelial cells (LSEC) prior to infecting hepatocytes [47]. In other systems, both KC and
175 LSEC can function as APCs and could present parasite antigens to CD8$^+$ T cells, resulting in
176 either tolerance or enhanced immune responses in inflammatory conditions [48,49].
177 How antigens expressed exclusively during EEF development are presented to the
178 immune system is unclear. After invasion of hepatocytes, *Plasmodium* parasites replicate
179 within the PV membrane (PVM), which constitutes a barrier preventing access of antigens to
180 the host cell cytosol and the MHC class I presentation pathway. RAS and GAP invade
181 hepatocytes where they undergo arrested development into EEFs. Attenuation leads to
182 parasite death and possible breakdown of the PVM, which could also enhance antigen
183 presentation and priming of protective CD8$^+$ T cells. Although antigen presentation by
184 hepatocytes tends to have a tolerising effect (reviewed in [50]), hepatocytes were shown to
185 prime CD8$^+$ T cells specific for CSP [51]. Furthermore, a recent study revealed that
186 presentation of antigens expressed in hepatocytes leads to differentiation of systemically
187 primed CD8$^+$ T cells into liver-resident memory cells that are critical for protection [52••].
188 During the effector phase of an efficient immune response, protective CD8$^+$ T cells
189 recognize parasite-derived peptides displayed with MHC class I molecules on the surface of
190 infected hepatocytes, leading to parasite elimination [40,53,54]. In *Py*-infected hepatocytes,
191 antigen processing and presentation follows an endosomal-independent, TAP-dependent
192 pathway [53,55] requiring an intracellular source of parasite antigens. From thousands of

proteins expressed by sporozoites and liver-stages, so far, only a few antigens, conserved among plasmodial species, are known to elicit a $CD8^+$ T cell-dependent protection against a sporozoite infection. Among them are the two most abundant surface proteins of sporozoites, CSP [56] and the thrombospondin-related anonymous protein (TRAP/SSP2) [57]; a protein involved in the wounding and traversal of host cells by ookinetes and sporozoites (CelTOS) [58]; an asparagine-rich protein that regulates the initial development of liver stages (SLARP/ SAP1) and a putative serine hydroximethytransferase (SHMT) [59]. *Pf* liver-stage antigen 1, liver-stage associated protein 2 and UIS3 antigens also elicit $CD8^+$ T cell-dependent protection in a challenge model where heterologous *Pb* sporozoites over-express these antigens via the strong *uis4* promoter [60,61]. The expression of the OVA MHC-I epitope fused to the HSP70 or the green fluorescent protein in the parasite cytosol via the constitutive *hsp70* promoter can also lead to the elimination of infected hepatocytes by OVA-specific $CD8^+$ T cells [53], showing that cytosolic, membrane, secreted and PVM antigens can be potentially presented on the surface of infected hepatocytes.

Effector functions of protective $CD8^+$ T cells

Different effector molecules can be utilised by both effector and memory $CD8^+$ T cells to protect against infections, including IFN-γ, tumour necrosis factor (TNF), perforin, granzyme, FasL and TNF-related apoptosis-inducing ligand (TRAIL). The mechanisms by which $CD8^+$ T cells inhibit the development of pre-erythrocytic stages remain poorly understood. Studies using antigenically distinct *Pb* strains showed that bystander killing of parasites does not occur during the $CD8^+$ T cell response to malaria parasites [62], indicating that elimination of infected parasites is likely mediated by direct recognition of infected hepatocytes by antigen-specific $CD8^+$ T cells. Systemic depletion of IFN-γ which is produced not only by $CD8^+$ T cells but also by $CD4^+$ T, NK T and NK cells, consistently abolishes

sterile protection in rodent models immunised with *Pb* or *Py* RAS [5]; IFN-γ activates the L-arginine-dependent inducible nitric oxide synthase (iNOS) pathway, which leads to the production of nitric oxide (NO) that is toxic to the developing EEFs (reviewed in [1]). Lytic factors appear to be dispensable in the effector function of $CD8^+$ T cells against pre-erythrocytic stages. Mice deficient for perforin, granzyme B or FasL and immunised with either *Pb* or *Py* RAS are completely protected against sporozoite challenge [5,63]. However, the roles for other immune mechanisms were not properly studied in these gene-deficient animals. Several experiments have also been performed using peptide-stimulated activated $CD8^+$ T cells or vaccine-induced $CD8^+$ T cells. Activated $CD8^+$ T cells specific for a cytoplasmic antigen in EEFs and generated by peptide-stimulation were shown to eliminate developing parasites in the liver in the absence of IFN-γ [53]. Effector *Py*CSP-specific $CD8^+$ T cells that are deficient of perforin, granzyme B or FasL, and generated following vaccination with recombinant vaccinia virus, were capable of targeting the developing EEFs [64]. Finally, an immunisation strategy involving priming with DCs and boosting with recombinant *Listeria monocytogenes* to generate memory *Pb*CSP- or *Py*CSP-specific memory cells showed the importance of both IFN-γ and TNF in protection against *Pb* and *Py*, whilst perforin was only involved in protection against *Py,* providing evidence of species-specific effector mechanisms for parasite killing [65].

Live cell imaging was utilised to dissect the fine mechanisms of $CD8^+$ T cell recognition of infected hepatocytes. Polyclonal $CD8^+$ T cells from mice immunised with *Py* GAP were shown to establish immunological synapses *in vitro* and utilise perforin to induce massive apoptosis of infected hepatocytes, with no detectable production of IFN-γ and TNF [66]. Intravital imaging revealed that *Py*CSP-specific effector $CD8^+$ T cells (generated by peptide-stimulation or a recombinant viral vaccination), as well as non-specific $CD8^+$ T cells, form clusters around infected hepatocytes, a process requiring G protein-coupled receptors

[53,67]. Targeting by *Py*CSP-specific effector $CD8^+$ T cells showed heterogeneity in the death phenotypes of the parasite, implying that multiple and redundant mechanisms are involved [67]. Taken together, these findings uphold the view that elimination of infected hepatocytes occurs in a multifaceted process.

The role of liver-resident memory $CD8^+$ T cells in protective immunity

To induce sterilising immunity against EEFs, $CD8^+$ resident or recruited to the liver must locate and eliminate all parasites to prevent progression to the blood stage infection [62], in a limited amount of time (2 days in mouse, 7-10 days in humans). $CD8^+$ T cells must find rare events: estimated at 1 out of 10^9 hepatocytes in humans and 1 out of 10^6 hepatocytes in mice [4]. Consequently, extremely high numbers of circulating vaccine-induced effector $CD8^+$ T cells are required to scan, locate and kill infected hepatocytes in the short amount of time the parasites are in the liver [28,68].

Accumulating evidence indicates a vital role for liver-resident $CD8^+$ T cells in protective immunity to pre-erythrocytic stages. Long-term protection after immunisation with *Pb* RAS and CPS correlates with sustained IFN-γ responses of hepatic $CD8^+$ memory T cells [69]. Recent studies in non-human primates have also correlated liver $CD8^+$ T cell numbers with protective efficacy after intravenous RAS vaccination [70•]. Memory *Py*CSP-specific $CD8^+$ T cells express high levels of CXCR6 [71]. Poor CXCR6 expression in these cells results in a reduction of both liver-associated memory and protective immunity [72], suggesting a role of resident $CD8^+$ T cells in protection. Intravital imaging documented the presence of motile $CD8^+$ T cells within the liver sinusoids of *Py* RAS vaccinated mice, suggesting that memory T cells survey for liver infection by patrolling the sinusoids [73]. More recently, a study based on transgenic $CD8^+$ T cells specific for a *Pb* antigen expressed in pre-erythrocytic and blood stages, identified a population of memory $CD8^+$ T cells in the

liver that express a distinct phenotype ($CD69^+$ $KLRG1^{lo}$) from splenic memory cells that are $CD69^-$ $KLRG1^{hi}$ [52••]. Detailed phenotypic analysis revealed that liver Trm cells also lacked CD103 expression and differentially expressed a number of surface markers, with higher levels of CXCR3, CXCR6, CD101, BTLA, FR4, Ly6ae, CD25, CD31, CD93, IL-4R, CD127, gp130, CD200R, and CD43, but lower levels of CX3CR1 and NKG2D as compared to circulating effector memory T cells [52••]. Induced following Pb RAS immunisation, these tissue-resident memory T (Trm) cells have the core gene signature of Trm cells from gut, skin and lung. Parabiosis experiments in mice showed that these cells do not recirculate, confirming their liver-resident status [52••]. Intravital imaging revealed that liver-resident cells depend on LFA-ICAM-1 interactions [74]. An immunisation strategy, which involves systemic DC-targeted priming followed by the expression of the antigen on hepatocytes to trap circulating primed $CD8^+$ T cells in the liver, enabled conversion to Trm cells [52••]. In this study, DC-targeted priming was achieved by conjugating the peptide antigen to a monoclonal antibody that targets the surface receptor Clec9A, which is expressed by $CD8\alpha^+$ DCs, and a recombinant adeno-associated virus that targets hepatocytes was used to express the antigen in the liver. This prime-trap vaccination strategy was shown to protect against normal *Pb* sporozoite challenge [52••].

Conclusion

Despite the strong evidence for the role of $CD8^+$ T cells in sterile protection against malaria, critical qualitative and quantitative characteristics of the protective response and effector mechanisms engaged by $CD8^+$ T cells have only started to emerge recently. Whilst multiple immune mechanisms appear to contribute to protection against pre-erythrocytic stages, the generation of liver-resident parasite-specific memory $CD8^+$ T cells is emerging as a key determinant of protective immunity. The design of strategies inducing this type of

response and the identification of protective target antigens will be instrumental for the development of an efficacious malaria vaccine.

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Figure legends

Figure 1. Potential factors contributing to the lack of protective immunity during natural *Plasmodium* infection. Under natural transmission conditions, only a few sporozoites are injected by an infected mosquito into the host skin. The motile sporozoites enter the blood stream by traversing a dermal capillary, are transported to the liver and traverse across liver sinusoidal endothelial cells (LSEC) or Kupffer cells (KC) to reach hepatocytes. Sporozoites invade hepatocytes inside a vacuole, where they replicate into thousands of merozoites, which once released into the bloodstream invade erythrocytes and initiate the blood stage infection. A combination of factors concurs to the lack of protective immunity in naturally exposed individuals. Infected mosquitoes inject very low numbers of sporozoites (1). Dermal inoculation is associated with immune regulatory mechanisms (2). The liver environment is prone to immune tolerance (3). The membrane of the parasitophorous vacuole limits diffusion of parasite liver stage antigens and exposure to the immune system (4). The blood stage infection that follows complete parasite development in the liver has immunosuppressive effects on liver stage immunity (5).

Figure 2. Potential factors contributing to protective immunity against liver stages after immunisation with live attenuated sporozoites administered intravenously. Immunisation with live attenuated sporozoites administered intravenously is the most efficient approach to confer full protection against normal sporozoite challenge. Under these conditions, high numbers of sporozoites can be inoculated (1), allowing systemic delivery of antigens (2). Alteration of the parasitophorous vacuole integrity in arrested liver stage parasites likely favours exposure of liver stage antigens to the immune system (3). Sporozoite and liver stage antigens can be captured and presented by $CD8\alpha^+$ DCs in the spleen and/or the liver draining lymph nodes (DLN) for priming of naïve $CD8^+$ T cells (4). Exposure of parasite antigens in

600 the liver leads to the differentiation of activated $CD8^+$ T cells into tissue-resident memory T
601 (Trm) cells that patrol the liver sinusoids (5). Aborted liver stage development prevents the
602 appearance of an immunosuppressive blood stage infection (6). Upon reinfection or
603 challenge, effector $CD8^+$ T cells form clusters around infected hepatocytes and can eliminate
604 parasites through direct killing of the infected cell and/or through the release of cytokines that
605 inhibit parasite development (7).
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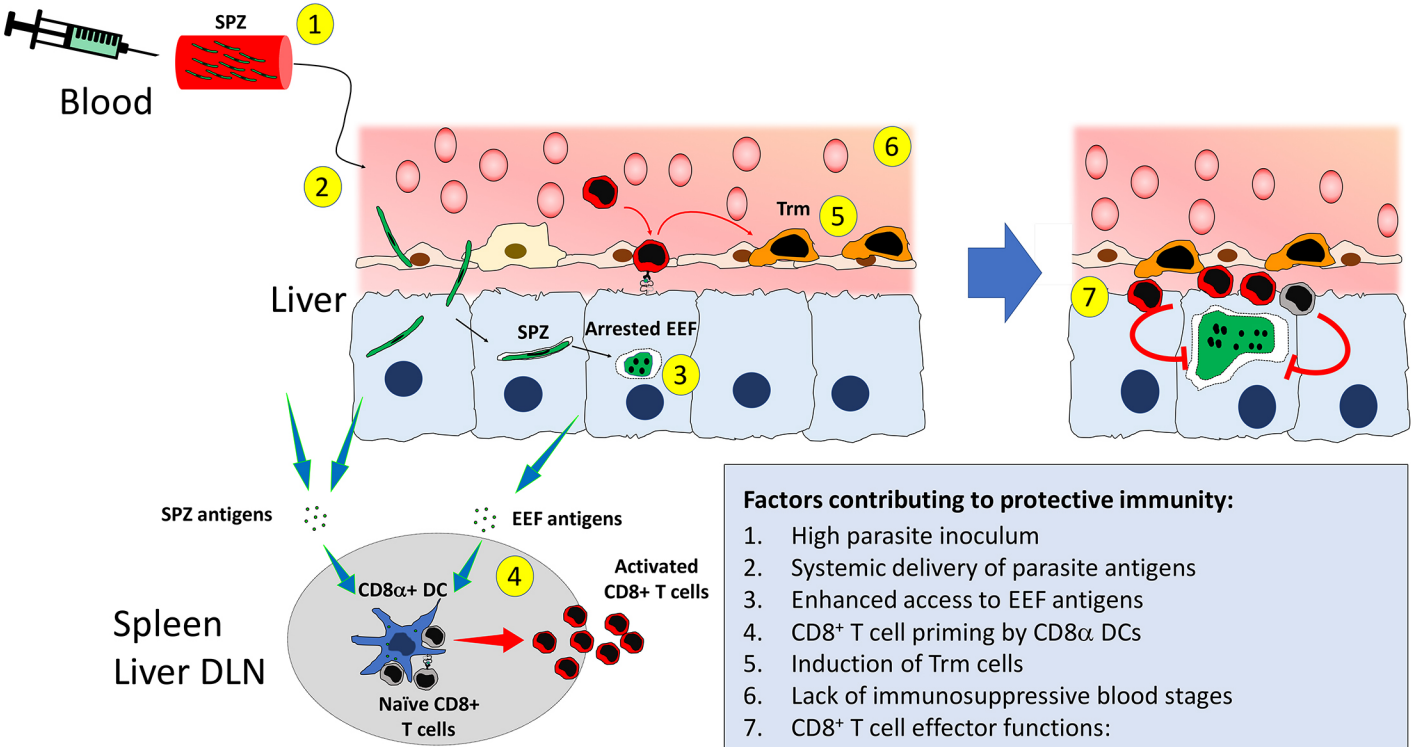
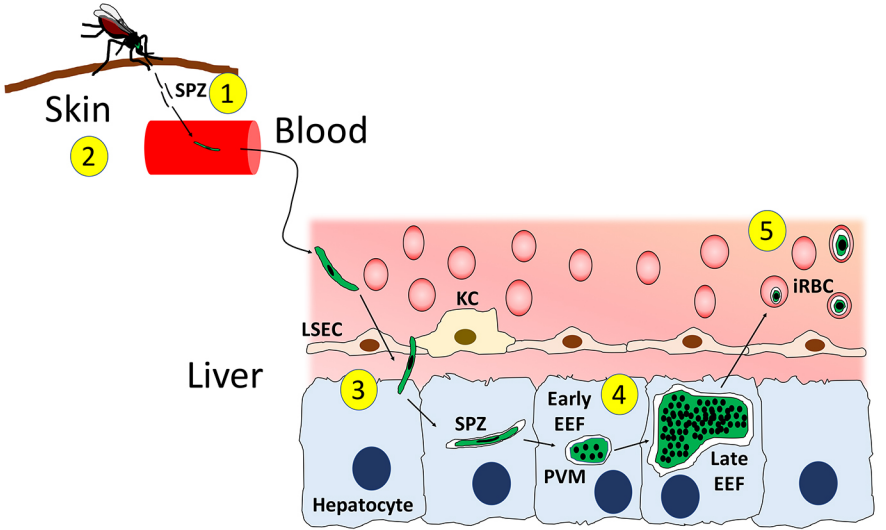


Factors contributing to protective immunity:

1. High parasite inoculum
2. Systemic delivery of parasite antigens
3. Enhanced access to EEF antigens
4. $CD8^+$ T cell priming by CD8α DCs
5. Induction of Trm cells
6. Lack of immunosuppressive blood stages
7. $CD8^+$ T cell effector functions:
   - direct killing of infected cells
   - parasite inhibition through cytokine secretion



Lack of protective immunity:

1. Low parasite inoculum
2. Regulatory mechanisms induced in the skin
3. Immune tolerance in the liver
4. Limited access to EEF antigens
5. Regulatory mechanisms induced by blood stage infection