

# Mechanisms of Adaptive Immunity to *Plasmodium* Liver-Stage Infection: The Known and Unknown

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## Abbreviations

CPS	Chemoprophylaxis sporozoites
CSP	Circumsporozoite protein
GAP	Genetically attenuated parasites
IVM	Intravital microscopy
RAS	Radiation-attenuated sporozoites
WSV	Whole sporozoite vaccination

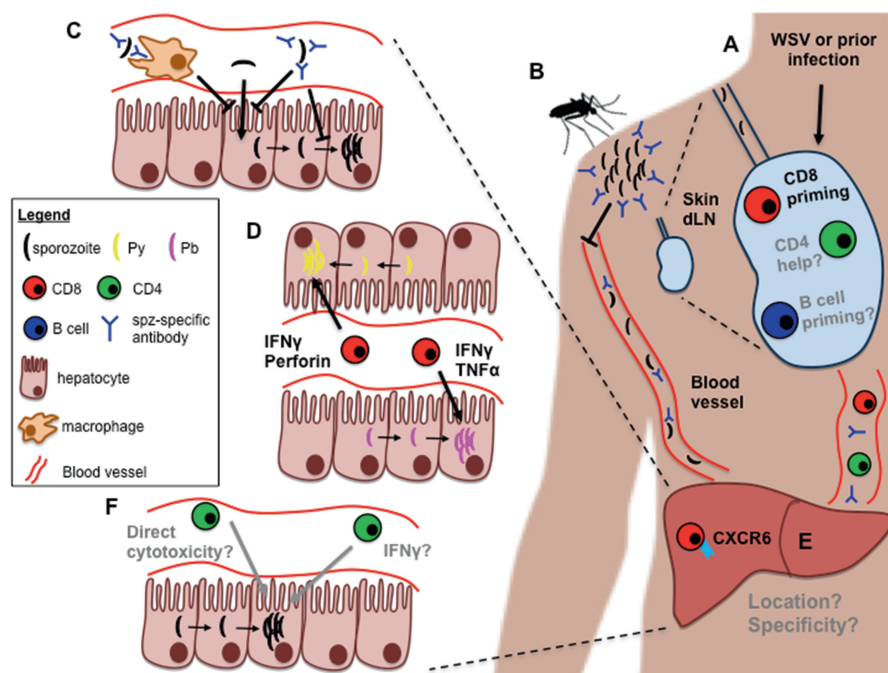
## 1 Introduction

Malaria infections remain a global health concern with a mortality rate of ~500,000 individuals per year [1]. Therefore, the need to develop a vaccine that provides life-long sterilizing immunity is of great importance. Sterilizing immunity to malaria infections involves preventing the liver-stage parasite from leaving the liver and initiating the symptomatic blood stage of infection. It should be noted this review focuses on memory responses and not the primary (effector) response following the initial exposure of a naïve individual to *Plasmodium* antigens, as this response, when assessed through physiological experiments, is incapable of controlling the parasite at the liver stage of infection. Whole sporozoite vaccination (WSV) approaches, such as radiation-attenuated sporozoites (RAS), viable sporozoites co-administered with the antimalarial drug chloroquine (chemoprophylaxis sporozoites, CPS), or liver-stage-arresting genetically attenuated parasites (GAP), have been most successful at providing sterilizing immunity relative to subunit, prime-boost vaccination strategies [2–5] through the actions of antiparasitic CD8 T cells, CD4 T cells, and antibodies (Fig. 1a). However, widespread application of WSV to human vaccination is complicated due to the high-dose requirement for RAS immunization, need for laboratory-reared mosquitoes, and logistical issues [6]. Application of WSV approaches to study the adaptive immune response against liver-stage infection may lead to the design of better subunit vaccines with easier deployment in the field.

Adaptive immunity against liver-stage infections is multifaceted, with contributions from the humoral and cellular arms. This review focuses on both arms of the adaptive immune response against preerythrocytic stage infection with a strong emphasis on the protective roles of antibodies and CD8 T cells. Lastly, we will highlight the caveats to our current understanding of what constitutes a protective memory anti-*Plasmodium* immune response and also describe major areas of research that would benefit from more attention.

## 2 Humoral Responses to Liver-Stage Infection

Antibodies have been shown to play a substantial role in providing sterilizing immunity to malaria infections. Importantly, antibodies against sporozoite antigens have been shown to inhibit infection via different mechanisms at distinct stages of



**Fig. 1** Adaptive immune responses to preerythrocytic antigens. Black lines and text indicate direct evidence to support mechanisms of protection. Gray lines and text indicate more data are needed to support the possible mechanisms of protection. (a) Upon WSV or infection, priming of sporozoite-specific CD8 T cells occurs in skin-draining lymph nodes (dLNs). It is unclear if the skin dLN is also a site for B cell priming or a location for CD4 T cells to provide help to CD8 T cells and B cells. After WSV or infection, antibodies and antigen-specific T cells are released into the blood where they can traffic throughout the body to provide multiple levels of protection, as described below. (b) After bites from infected mosquitoes, sporozoite-specific antibodies can reduce sporozoite deposition into the dermis and inhibit motility and therefore reduce entry to the underlying blood vessels. (c) Sporozoites travel through the blood to the liver where sporozoite-specific antibodies can increase opsonization and reduce sporozoite motility contributing to inhibition of hepatocyte invasion and subsequent cell traversal. (d) CD8 T cell-mediated killing of infected hepatocytes uses differential effector mechanisms depending on the rodent *Plasmodium* species. It is unclear if these cells are located in the liver sinusoids or parenchyma during this killing event. (e) Liver-localized CD8 T cell populations express CXCR6. The exact location (parenchyma or liver sinusoids) and specificity of these CD8 T cells following vaccination and challenge are unclear. (f) A role for cytotoxic CD4 T cells has been described in multiple models, but the mechanism as to how these cells contribute to sterilizing protection needs further investigation. It may be possible that infected hepatocytes upregulate class II MHC and become direct targets of cytotoxic CD4 T cells. Alternatively, CD4 T cells may inhibit infection via IFN $\gamma$  production

sporozoite infection. This section describes specific antibody and B cell response to sporozoite and liver-stage antigens and mechanisms of antibody-mediated inhibition of sporozoite infection.

## **2.1 *Mechanisms of Antibody-Mediated Inhibition of Preerythrocytic Infection***

The use of intravital microscopy (IVM) and fluorescent sporozoites has provided critical information to our understanding of how antibodies mediate inhibition of preerythrocytic infections depicted in Fig. 1b. Upon exposure to bites from infected mosquitoes, mice previously immunized with RAS showed significantly fewer sporozoites deposited into the dermis compared to nonimmunized control mice [7]. Immune complex formation between anti-circumsporozoite protein (CSP) antibodies from immunized mice and soluble CSP released by sporozoites into the saliva of infected mosquitoes likely contributed to partial obstruction of sporozoite release from the proboscis tip during a blood meal [7]. Additionally, mice passively immunized with anti-CSP antibodies also showed reduced dermal deposition of sporozoites after challenge with bites from infected mosquitoes. Importantly, none of the immunized mice developed parasitemia, as detected by blood smears, compared to the nonimmunized control mice, which developed parasitemia under similar infectious conditions [7]. Anti-sporozoite antibodies can also reduce sporozoite motility. Early in vitro studies showed that anti-CSP antibodies inhibited the motility of sporozoites and prevented invasion of cultured target cells [8]. More recent in vivo evidence supports the early in vitro data by demonstrating that in mice treated with anti-CSP antibodies, fewer sporozoites travel from the dermis to underlining blood vessels [9], a step which is necessary for trafficking to the liver for infection of hepatocytes.

Should sporozoites enter the bloodstream, antibodies may increase opsonization and phagocyte uptake, thus reducing the number of sporozoites capable of hepatocyte invasion (Fig. 1c). Anti-CSP antibodies can increase sporozoite opsonization [10], which has been shown to be a mechanism of protective immunity in response to vaccination in humans [11]. CSP expression and sporozoite motility are both required for productive infection of hepatocytes and cell traversal [12]. Importantly, CSP-specific antibodies have been shown to inhibit sporozoite infection of hepatocytes [13]. Therefore, sporozoites coated with antibodies show limited motility resulting in reduction of hepatocyte invasion and subsequent cell traversal (Fig. 1c). The capacity of anti-CSP antibodies to limit liver-stage infection is thought to be an important mechanism underlying the partial efficacy of the RTS,S vaccine, consisting of CSP fused to hepatitis B surface Ag, which was recently approved for use in humans by the EU [14].

By reducing the number of infected hepatocytes, antibody-mediated inhibition of liver-stage infections could substantially help a concurrent CD8 T cell response by limiting the number of infected hepatocytes that need to be targeted. The multiple mechanisms that anti-sporozoite antibodies use to inhibit infection explain why the antibody response targeting sporozoites is an essential component that contributes to sterilizing protective immunity in vaccinated individuals and will be an important feature in any new vaccines against malaria.

## 2.2 *Antibody and Specific B Cell Responses to Sporozoite Antigens*

Development of protective immunity after various immunization methods including RAS, CPS, and the subunit vaccine RTS,S has revealed a role for antibody-mediated protective immunity. The first monoclonal antibodies that mediated protective immunity were determined to be against CSP [15]. However, the role anti-CSP antibodies play in providing sterilizing protection in response to various vaccinations remains quite controversial [16–22]. Anti-CSP antibodies have been shown to be important for sterilizing immunity in response to RAS immunization RTS,S vaccination [17, 23–25]. However, anti-CSP antibodies not strongly correlate with protection after CPS [21]. This result with CPS vaccination is consistent with recent studies showing that blood-stage immunity contribute to protection in this model [26, 27]. Additional anti-sporozoite antibodies targeting antigens including TRAP and LSA-1 have also been associated with protection against infection [20], but the use of these antigens as targets in subunit vaccines did not result in protection [28, 29]. It is likely that antibodies to a variety of preerythrocytic antigens, and not just to a single specificity, contribute to sterilizing immunity in response to vaccination [19, 20].

To further understand the role that antibodies play in vaccine-induced sterilizing immunity, we first need a better idea of the spectrum of sporozoite and liver-stage antigenic targets and ways to evaluate the relevance of each target for protection. Having more tools including monoclonal antibodies and additional targets of protective epitopes in rodent models will be essential in determining the quantity and quality of antibodies required for a sterilizing immune response.

Although many studies have described a significant role of antibodies against liver-stage antigens in providing sterilizing immunity, the development and differentiation of liver-stage antigen-specific B cell populations have not been well described. Upon antigen encounter, naïve B cells can proliferate and differentiate into plasma cells or memory B cells [30]. However, the relative contribution of these distinct populations of B cells to liver-stage immunity has not been thoroughly investigated in rodent models of malaria, and the vast majority of information gathered on specific B cell responses to malaria infection has come from individuals who were naturally exposed or vaccinated. Of the studies conducted in humans in response to vaccination, an overwhelming majority of the work has been done studying blood-stage-specific B cell responses rather than liver-stage B cell responses [30]. One study that investigated the memory B cell response to a liver-stage antigen, CSP, in response to CPS immunization, determined that memory B cell responses increase with the number of immunizations but did not correlate with protection [21].

Because anti-sporozoite antibody responses are so important in contributing to sterilizing immunity, identifying how sporozoite-specific plasma and memory B cells are developed and maintained is of great importance. Blood-stage antigen-specific B cell populations have been identified using flow cytometry [31], but liver-stage antigen-specific B cell responses have not been described using this approach.

Capacity to track liver-stage antigen-specific B cells through various vaccinations and natural infection will be important in determining how antibody production changes over time and thus alters protection. A particularly interesting question that should be addressed using such approaches involves how blood-stage infection may potentially affect the preexisting B cell response to liver-stage antigens or to subsequent sporozoite infections. Indeed, increased apoptosis of memory B cells and plasma cells has been described after blood-stage infection, but the specificity of those B cells has not been determined [32, 33].

One outstanding question regarding B cell activation and differentiation involves the priming of B cells in response to sporozoite antigens (Fig. 1a). Where and how is this occurring? Sporozoites are initially injected into the dermis of mammalian hosts, but not all will reach the bloodstream, and therefore some sporozoites remain in the dermis. Additionally, some sporozoites travel to skin-draining lymph nodes [34], which have been shown to be particularly important for CD8 T cell priming [35]. Is this also the case for sporozoite-specific B cells? This is an important question to address in regard to vaccination strategies. With new IVM technology, it may be possible to track sporozoites from initial deposition and determine where antigen-specific B cells are primed.

### **3 CD8 T Cells Are Potent Mediators of Protection Against *Plasmodium* Liver-Stage Infections**

*Plasmodium*-specific CD8 T cells targeting liver-stage-expressed antigens can provide sterilizing immunity in humans and rodents [36–41]. The importance of CD8 T cells as mediators of protection against *Plasmodium* has been appreciated as early as the 1980s from studies using rodents immunized with RAS [42, 43]. Because of the potent protection against *Plasmodium* induced by RAS vaccination, it has been considered the “gold standard” for vaccination. Consequently, WSV platforms are frequently used to study the generation of protective adaptive immunity against *Plasmodium* infection. In this section, we will highlight general features of what constitutes a protective CD8 T cell response, and also areas where more recently developed tools can increase our ability to study immunity mediated by *Plasmodium*-specific CD8 T cells.

#### **3.1 Quantitative and Qualitative Features of Protective Liver-Stage-Specific CD8 T Cells**

The degree of CD8 T cell-mediated control of *Plasmodium* infection is determined by the magnitude and the quality of the CD8 T cell response. Very large frequencies, >1% of all peripheral blood lymphocytes, targeting the liver-stage antigen CSP were required to provide sterilizing immunity in a rodent model of *P. berghei* infection [44]. The superior protection afforded by immunization with late

liver-stage-arresting GAP was dependent on a larger CD8 T cell response targeting a wider spectrum of liver-stage antigens, relative to RAS vaccination [45]. Thus, in rodent models, the magnitude of the *Plasmodium*-specific CD8 T cell response can be a predictive indicator of protection. Likewise, RAS immunization of humans demonstrated that individuals with larger CD8 T cell responses better protective outcomes [41]. However, in order to generate these responses, human subjects were exposed to the bites of large numbers of *Plasmodium falciparum*-infected mosquitoes through several exposures (~1000–3000 bites) [38] or through the delivery of 4–5 IV administrations of 135,000 *P. falciparum* sporozoites harvested from laboratory-reared irradiated mosquitoes [41]. Thus, only large RAS doses over multiple administrations were able to engender protective immunity of human volunteers. The need for large parasite-immunizing doses, multiple administrations, and laboratory-reared mosquitoes are just a few of the hurdles inherent to application of RAS vaccination in the field [6, 46]. Currently, subunit vaccination of humans, such as the recently licensed RTS,S vaccine [14], has not revealed robust sterilizing immunity [2–5, 47, 48]. In large part, this may be due to the failure of RTS,S to elicit detectable anti-CSP CD8 T cell response [49]. Therefore, it remains important to understand the superior immunity developed by WSV approaches, such as RAS, to lead to better design of subunit vaccines.

While it is known that large magnitude CD8 T cell responses generated through vaccination are required to provide sterilizing immunity to rodents and likely to human subjects [44], less is known about the qualitative features (phenotype, functionality, specificity, localization, etc.) that are important for CD8 T cell-mediated clearance of parasite-infected hepatocytes. In regard to phenotype (Fig. 1d), CD8 T cells exhibiting an effector memory phenotype (CD62L<sup>lo</sup>) have correlated with better protection relative to central memory phenotype (CD62L<sup>hi</sup>) CD8 T cells in two independent studies [50, 51]. Importantly, these studies used distinct vaccination methods (RAS versus heterologous prime boost) to arrive at a similar conclusion. In regard to functionality, such as cytokine production and effector capacity, it has been repeatedly reported that expression of IFN $\gamma$  by CD8 T cells is required for a protective response [42, 52, 53]. Of interest, the cytolytic mechanism by which CD8 T cells mediate clearance of infection differs depending on the rodent *Plasmodium* infection [52]. For instance, when *P. berghei* CSP-specific CD8 T cells were induced using a prime-boost subunit vaccine approach in wild-type or various knockout mouse backgrounds, it was demonstrated that CSP-specific CD8 T cells depend on IFN $\gamma$  and TNF $\alpha$  to mediate protection from challenge (Fig. 1d) [52]. In contrast, in a similar prime-boost vaccination model inducing *P. yoelii* CSP-specific CD8 T cells, it was shown that CSP-specific CD8 T cells largely mediate protection using IFN $\gamma$  and perforin-mediated cytotoxicity following challenge. If these results extend into human *Plasmodium* species, it could have many implications. For example, it would be interesting to determine if a particular effector mechanism is more efficient at mediating parasite clearance and if this information can be applied to generating CD8 T cells via vaccination that preferentially utilize certain effector mechanisms. Collectively, details regarding the phenotype and functionality of *Plasmodium*-specific CD8 T cells have identified some important features that relate to protection, but much remains to be learned to enhance immunity to vaccination.



### 3.2 *Novel CD8 T Cell Epitopes Provide Additional Tools to Study Features of Protective Plasmodium-Specific CD8 T Cell Responses*

New CD8 T cell epitopes derived from liver-stage proteins expressed by rodent *Plasmodium* have been described enhancing the ability to study *Plasmodium*-specific CD8 T cell responses [54–57]. These novel epitopes will allow additional questions to be addressed such as how specificity of the CD8 T cell response may impact protective capacity. For instance, it is currently unclear whether all *Plasmodium*-specific CD8 T cell responses induced by WSV contribute to protective immunity—a question that has important implications for the identification of antigens for inclusion in subunit vaccines. To date, the majority of our understanding of the quantitative and qualitative features of protective CD8 T cell responses has tracked CD8 T cells directed against the immunodominant CSP epitopes: CS<sub>252–260</sub> in *P. berghei* and CS<sub>280–288</sub> in *P. yoelii* [44, 52, 58–64]. However, CD8 T cell-mediated protection following RAS is possible even in the absence of CSP-specific CD8 T cells [61], suggesting that currently undefined *Plasmodium*-specific CD8 T cells can mediate protection following challenge.

To date, subunit vaccination using prime-boost strategies to generate CSP- or TRAP-specific CD8 T cells has had limited success in protecting human subjects [2–5]. Thus, it remains necessary to continue to define and study *Plasmodium* CD8 T cell epitopes to identify candidate antigens for inclusion in subunit vaccines. Fortunately, with the description of novel rodent *Plasmodium* CD8 T cell epitopes, studies can expand beyond tracking the immunodominant CSP response. Since the magnitude of endogenous CD8 T cells primed following WSV is generally small ([54, 56] and unpublished data), additional tools such as TCR transgenic [65] or retrogenic mice [66] may be necessary to enhance the small numbers of endogenous precursor CD8 T cells, which limit the resolution in which these responses can be tracked. Alternatively, tools to generate *Plasmodium*-specific CD8 T cells via heterologous prime-boost methods could help overcome CD8 T cell numerical limitations typically inherent to the WSV approach. Prime-boost vaccination strategies can help determine the protective capacity of these new CD8 T cell epitopes through examining individual CD8 T cell specificities in the absence of any other anti-*Plasmodial* response, which can ultimately help in the design of a successful subunit vaccine.

In addition to characterizing the protective capacity of single CD8 T cell epitopes, general features of protective CD8 T cell epitopes can be identified to help in predictive epitope screens of the *Plasmodium* proteome. The *Plasmodium* parasite encodes over 5000 open reading frames [67, 68], which consequently present a challenge in screening for new CD8 T cell epitopes for inclusion for a subunit vaccine. As CD8 T cell epitopes are described and studied, general features (antigen localization, abundance, expression patterns, etc.) of protective CD8 T cell epitopes be revealed to help in future screens for additional protective epitopes.



### 3.3 *Localization of Protective CD8 T Cell Responses*

Several in vitro and in vivo studies have demonstrated CD8 T cell-mediated killing of parasite-infected hepatocytes, but increased technological advances in cellular imaging, particularly IVM, have the potential to reveal information regarding the location of CD8 T cell responses following vaccination or challenge. Multiple studies have provided evidence that protective *Plasmodium*-specific CD8 T cells target and kill liver-stage-infected hepatocytes [69–71]. More recently, IVM was applied to study the mechanism in which CD8 T cells target *Plasmodium*-infected hepatocytes. Using GFP-expressing sporozoites to infect mice, a loss of GFP fluorescence was observed in hepatocytes surrounded by clusters of CSP-specific in vitro-generated effector CD8 T cells, suggesting the direct killing of *Plasmodium*-infected hepatocytes via a mechanism requiring multiple antigen-specific CD8 T cells [72]. These observations help provide an explanation regarding the large CD8 T cell numerical requirements for protection since several CD8 T cells were associated with killing of a single infected hepatocyte [44]. However, it is still unclear where protective *Plasmodium*-specific CD8 T cells are localized following vaccination and the movements of these cells upon challenge. There is an increasing interest in the role of tissue-resident memory CD8 T cell populations generated following infection or vaccination in multiple models (reviewed elsewhere [73, 74]). However, to date it remains unclear how the resident memory populations identified in the skin, brain, and mucosal tissues compare phenotypically and functionally to populations of *Plasmodium*-specific CD8 T cells localized in the liver of vaccinated mice. It will be important to thoroughly describe *Plasmodium*-specific CD8 T cells within the liver, particularly in regard to whether these cells are resident within the parenchymal tissue, or alternatively, whether they are associated with the endothelial barrier of the liver sinusoids. Recent IVM data suggests that hepatitis B virus-specific in vitro-generated effector CD8 T cells in the liver are localized in the vasculature and do not require migration into liver parenchymal tissue to kill infected hepatocytes [75]. While this is a different infection setting, it is possible that *Plasmodium*-specific CD8 T cells in the liver exhibit a similar location in the vasculature during killing of parasite-infected hepatocytes (Fig. 1e). This result has important implications as most studies of liver-resident memory CD8 T cells in *Plasmodium* have utilized perfusion techniques to eliminate circulating cells, but it is possible perfusion techniques may dissociate important “resident” CD8 T cell populations, closely associated with the vasculature, from analysis.

To date, liver-resident memory populations generated by WSV have been described as CD8 T cells that remain in the liver following perfusion [76, 77], but their contribution to protective immunity is unclear. CXCR6 expression has also been described as an important molecule involved in CD8 T cell liver homing and residence [78]. Expression of chemokine receptor CXCR6 may be a marker to identify these liver-resident *Plasmodium*-specific CD8 T cell populations (Fig. 1e) [76, 77]. For example, CXCR6 expression on CD8 T cells was shown to be required for long-term maintenance of *Plasmodium*-specific CD8 T cell populations in the liver [76].

Further studies need to define these liver-resident CD8 T cells, their protective capacity, and how they differ from circulating CD8 T cell populations in addition to their differences from the more strict definitions of resident T cell populations in other tissues and model systems [73]. Comparisons of these populations will be helpful in determining the characteristics of liver-resident CD8 T cells exhibit, their importance in vaccine-induced protection, and how we create or modify liver-localized CD8 T cell responses against *Plasmodium* to foster better liver-stage immunity.

## 4 Role of CD4 T Cells to Liver-Stage Infection

The role CD8 T cells play in sterilizing immunity to liver-stage infection in rodent models of malaria is well characterized, but the role of CD4 T cells is mouse strain dependent. CD4 T cell depletion studies revealed that the two major mouse strains used in rodent malaria models, C57BL/6 and BALB/c, showed differential requirements for CD4 T cells in protection against *P. yoelii* sporozoite challenge [39]. While depletion of CD4 T cells had no impact on protection of RAS-immunized BALB/c mice, CD4 T cell-depleted C57BL/6 mice previously immunized with RAS were not protected [39]. How CD4 T cells mediate protection in these mouse strains needs further investigation, but based on the role CD4 T cells play in other infection models, these cells may play a cytotoxic role or provide help to B cells and CD8 T cells, both of which have been shown to contribute to sterilizing immunity. This section focuses on the roles that CD4 T cells may be playing in response to liver-stage infection and highlights remaining questions that still need to be addressed.

### 4.1 Role of CD4 T Cell Help in Protective Immunity to Liver-Stage Infection

CD4 T cells have the capacity to differentiate into various subsets including Th1, Th2, Tfh, Treg, and ThCTLs [79]. However, CD4 T cell differentiation in response to malaria infections has not been widely investigated in part due to lack of identifiable epitopes that induce detectable responses. Nevertheless, it is likely that Tfh plays a strong role in antibody response to liver-stage antigens. Given the fact that antibody responses to sporozoites can mediate sterilizing protection after vaccination, Tfh are likely playing a role in helping B cells in terms of memory B cell formation and antibody production. CD4 T cell help for B cells is an area that requires further investigation, and information gathered may be applied to enhance specific components of a protective vaccine.

A few studies have identified a strong role for CD4 T cell help of CD8 T cells in malaria infections. CD4 T cells were found to be important for the expansion and survival of CD8 T cell effector and memory responses after sporozoite infection, indicating that the size of the CD8 T cell memory pool after WSV or RAS

immunization was highly dependent upon helper T cells [80]. Interestingly, the memory CD8 T cells that did form in the absence of CD4 T cells exhibited normal effector functions but were unable to protect against sporozoite challenge. The size of the memory CD8 T cell response is especially important in mediating sterilizing protection as discussed in aforementioned Sect. 3.1 [81, 82]. Thus, the absence of CD4 T cells produced functional but significantly fewer antigen-specific CD8 T cells, resulting in an inability to protect against liver-stage infection. Specifically, IL-4 production by CD4 T cells was shown to contribute to the expansion of malaria-specific CD8 T cells [83]. IL-4 production from CD4 T cells may also promote antibody production from B cells during malaria infections, but this has yet to be described.

To date, only a few studies have addressed the role CD4 T cells play in providing B cell and CD8 T cell help in response to liver-stage antigens. Given the fact that both antibodies and CD8 T cells play such an important role in providing protective sterilizing immunity against liver-stage infections, more work on how CD4 T cells contribute to helping both B cells and CD8 T cells is needed.

## **4.2 Role of Cytotoxic CD4 T Cells in Protective Immunity**

Several studies have identified a role for cytotoxic CD4 T cells in protection against liver-stage infections. Some of the first studies to describe cytotoxic CD4 T cells in sterilizing protection were performed in murine models using CD4 T cell clones specific for CSP and non-CSP epitopes [84, 85]. More recent studies identified a role for cytotoxic CD4 T cells following RAS immunization in  $\beta$ 2M knockout mice, which lack endogenous CD8 T cells [86]. Liver-stage-specific cytotoxic CD4 T cells have also been described in humans after various immunization methods [87–89]. However, despite the numerous reports describing cytotoxic CD4 T cells, the mechanisms of cytolytic action remain quite controversial and require further investigation (Fig. 1f). This is in part due to the variability in direct cytolytic capacity of described CD4 T cell populations and the means in which such cytotoxic T cell populations were identified [84–88]. Only some specific CD4 T cell clones showed direct cytolytic capability, whereas others did not. Moreover, CD4 T cells that did show cytolytic potential were in response to peptide-pulsed autologous cells or parasitized RBC-stimulated cells [85, 87, 88]. Other studies showed CD4 T cells increased degranulation markers (CD107a/b) after parasitized RBC stimulation, suggesting cytolytic activity [88]. However, direct cytolytic activity of CD4 T cells against infected hepatocytes was not measured. This may call into question if CD4 T cells can directly lyse infected cells via similar mechanisms as CD8 T cells (i.e., perforin/granzymes). Combined, these studies highlight the many unknowns about the actual role of cytotoxic CD4 T cells in response against liver-stage *Plasmodium* infections, especially in regard to the recognition of infected hepatocytes. Upregulation of class II MHC on hepatocytes has been described in some disease states [90], but it has yet to be determined during *Plasmodium* infection.

Many questions still need to be addressed to determine the actual role cytolytic CD4 T cells play in sterilizing immunity against liver-stage infections. How do CD4 T cells mediate cytotoxicity if not by direct recognition of infected hepatocytes? Neutralization of IFN $\gamma$  in  $\beta$ 2M knockout hosts reduced the protective capacity, indicating IFN $\gamma$  may play a partial role in protection against liver stage [86]. CD4 T cells may, however, be mediating cytotoxicity indirectly via IFN $\gamma$ -induced NO production [91]. Do cytotoxic CD4 T cells also play a role in the presence of CD8 T cells, which have been shown to be a major contributor to protective immunity in most animal models, whereas CD4 T cells have only been shown to contribute to protection in a few mouse models [39]? The role cytotoxic CD4 T cells play in sterilizing immunity to liver-stage infection may not be as straightforward as described in some studies, and more work could help address some of these underlying questions.

## 5 Conclusion

Adaptive immune responses against liver-stage infections are critical for mediating protection against malarial disease. Highlighted in this review and depicted in Fig. 1 are mechanisms of the adaptive immune response known to contribute to protective immunity (black lines/text). Moreover, we have identified certain holes in our current understanding of the adaptive immune response to liver-stage antigens that will be important areas of future studies that will enable the development of more effective vaccines (gray lines/text). A large portion of the current literature has applied WSV approaches in rodent models of malaria to arrive at an understanding of the critical importance of antibodies and CD8 T cells that recognize sporozoite and liver-stage antigens. Although general features of these protective antibody and CD8 T cell responses are understood, there remains a need to continue to define these responses since the majority of the antigens targeted by CD8 T cells, and likely antibodies, have not been characterized. Further, the helpers of these responses, namely, CD4 T cells, require attention as these responses may be critical in manipulating CD8 T cells and antibodies qualitatively following subunit vaccination into responses capable of mediating lifelong sterilizing immunity. Whether CD4 T cells can contribute in the direct killing of hepatocytes will be important to determine as it may provide critical assistance to antibodies and CD8 T cells in clearing the infected liver before symptomatic blood stage. Further, the impact on CD8 T cell-mediated protection due to specificity and location is important area for further research as enhance the quality of the CD8 T cell response may help overcome issues in vaccination approaches that are incapable of eliciting quantitatively large responses. Taken together, despite the advances in understanding of the role of antibodies and CD8 T cells in the adaptive immune response against liver stage, what qualitative features impact protection and how can we manipulate vaccine-induced responses to provide better protective immunity is an important area of research that may dictate the design of a successful human subunit vaccine.

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