BREAKING THE CYCLE: ATTACKING THE MALARIA PARASITE IN THE LIVER

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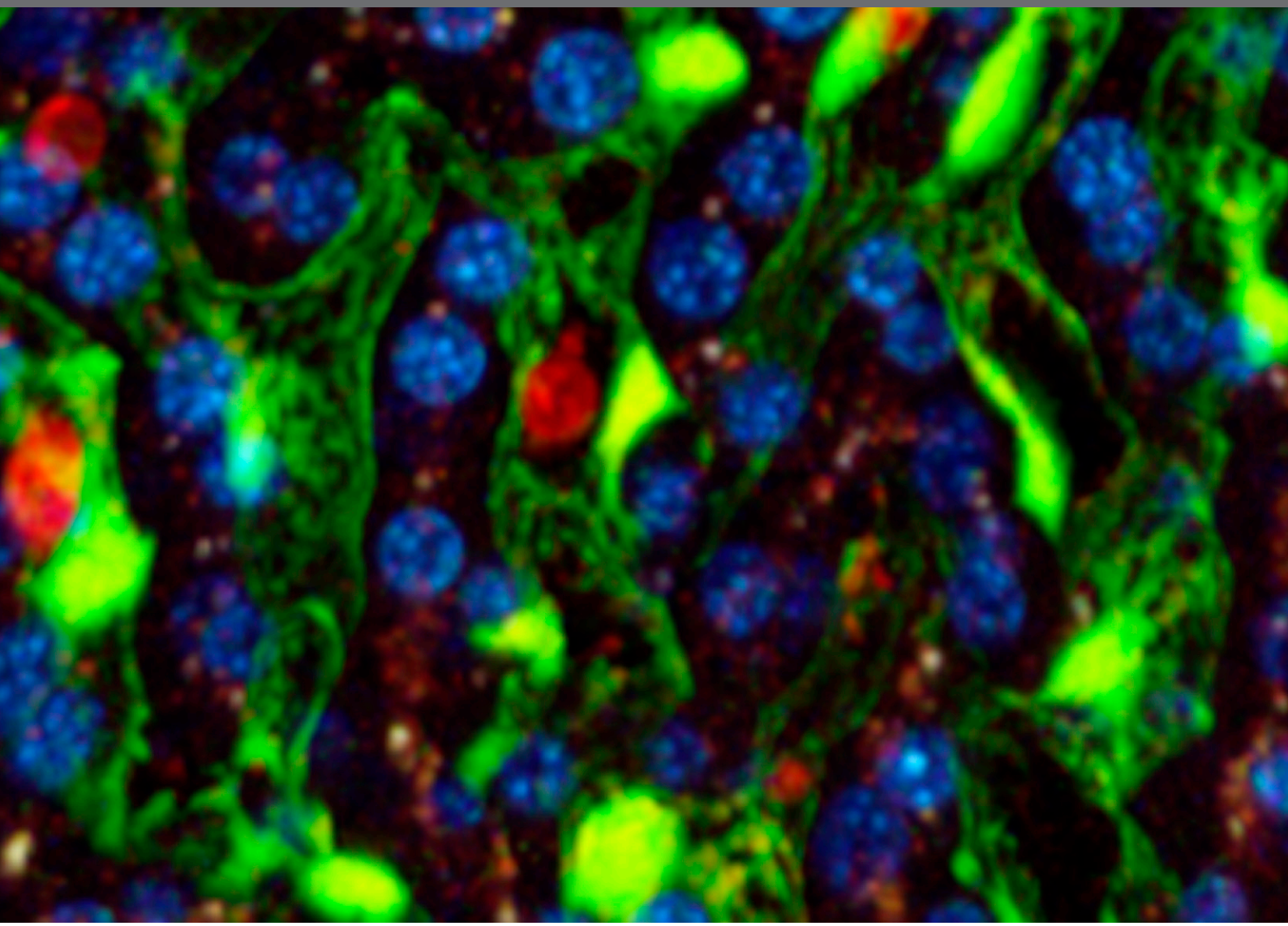

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BREAKING THE CYCLE: ATTACKING THE MALARIA PARASITE IN THE LIVER

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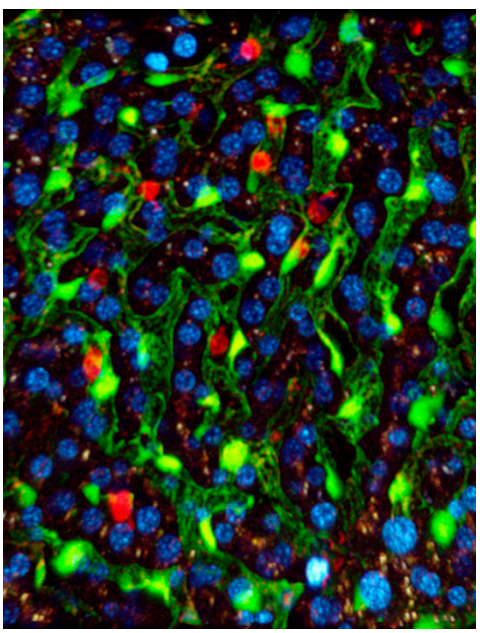

CD8 T cells (red) patrolling the liver sinusoids of a Tie2-GFP mouse with green fluorescent endothelia. The nuclei of the hepatocytes (dark) were labeled with Hoechst 33342 (blue). Maximum projection of an intravital microscopic Z-stack acquired with a Leica TCS SP2 AOBS confocal microscope, courtesy of M. Cabrera and U. Frevert. The image is closely related to the Frontiers review "Plasmodium cellular effector mechanisms and the hepatic microenvironment" by Ute Frevert and Urszula Krzych.

Despite significant progress in the global fight against malaria, this parasitic infection is still responsible for nearly 300 million clinical cases and more than half a million deaths each year, predominantly in African children less than 5 years of age. The infection starts when mosquitoes transmit small numbers of parasites into the skin. From here, the parasites travel with the bloodstream to the liver where they undergo an initial round of replication and maturation to the next developmental stage that infects red blood cells. A vaccine capable of blocking the clinically silent liver phase of the Plasmodium life cycle would prevent the subsequent symptomatic

phase of this tropical disease, including its frequently fatal manifestations such as severe anemia, acute lung injury, and cerebral malaria. Parasitologists, immunologists, and vaccinologists have come to appreciate the complexity of the adaptive immune response against the liver stages of this deadly parasite. Lymphocytes play a central role in the elimination of Plasmodium infected hepatocytes, both in humans and animal models, but our understanding of the exact cellular interactions and molecular effector mechanisms that lead to parasite killing within the complex hepatic microenvironment of an immune host is still rudimentary. Nevertheless, recent collaborative efforts have led to promising vaccine approaches based on liver stages that have conferred sterile immunity in humans – the University of Oxford's Ad prime / MVA boost vaccine, the Naval Medical Research Center's DNA prime / Ad boost vaccine, Sanaria Inc.'s radiation-attenuated whole sporozoite vaccine, and Radboud University Medical Centre's and Sanaria's derived chemoprophylaxis with sporozoites vaccines.

The aim of this Research Topic is to bring together researchers with expertise in malariology, immunology, hepatology, antigen discovery and vaccine development to provide a better understanding of the basic biology of Plasmodium in the liver and the host's innate and adaptive immune responses. Understanding the conditions required to generate complete protection in a vaccinated individual will bring us closer to our ultimate goal, namely to develop a safe, scalable, and affordable malaria vaccine capable of inducing sustained high-level protective immunity in the large proportion of the world's population constantly at risk of malaria.

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EDITORIAL
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Editorial: Breaking the cycle: attacking the malaria parasite in the liver

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Keywords: ***Plasmodium*, immunity, antigen-presenting cell, B cell, CD8 T cell, CD4 T cell, vaccine, adjuvant**

Plasmodium falciparum malaria remains one of the most serious health problems globally. Immunization with attenuated parasites elicits multiple cellular effector mechanisms capable of eliminating *Plasmodium* from the liver. However, malaria liver stage immunity is complex. The anatomic site of priming of naive *Plasmodium*-specific CD8 T cells, be it in the lymph nodes draining the site of *Plasmodium* antigen deposition by the mosquito or in the liver, may in fact determine the specificity of the effector CD8 T cells. The participation of particular antigen-presenting cells (Corradin and Levitskaya, 2014) and tissue signatures that influence the activation of intrahepatic CD8 T cells against malaria sporozoites (Morrot and Rodrigues, 2014) are still incompletely understood. Similarly, how effector CD8 T cells detect the few infected hepatocytes in the large liver and the mechanisms they use to kill the intracellular parasites are unknown. The unique immunological properties of the liver could explain why effector CD8 T cells are so inefficient in finding and eliminating the hepatic stages of *Plasmodium* (Bertolino and Bowen, 2015). Effector CD8 T cells require help from CD4 T cells, and antigen-presenting cells are thought to stimulate CD4 T cell licensing and enhance their capacity to optimally activate CD8 T cells (Crispe, 2014). Helper CD4 T cells also aid in the development of B cell-mediated immunity (Dups et al., 2014). High levels of interstitial antibodies can immobilize sporozoites in the skin and circulating antibodies can prevent the parasites from infecting the liver (Vanderberg, 2015). A better understanding of fine specificity and quantities of antibodies required for protection and the antigens recognized by neutralizing antibodies will facilitate the design of refined malaria vaccines that induce robust, long-lived, protective B cell responses (Dups et al., 2014).

Animal models have traditionally played an important role in the discovery of the basic parameters of CD8 T cell-mediated immunity, as ethical and practical limitations preclude study of the cellular and molecular mechanisms by which malaria vaccines induce protection in humans. Highlighting the complexity of *Plasmodium* liver stage immunity, comparison of murine malaria models led to the identification of protective memory CD8 T cell responses that differed quantitatively and qualitatively, depending on the *Plasmodium* species (Van Braeckel-Budimir and Harty, 2014).

The mechanisms effector CD8 T cells use to recognize and eliminate *Plasmodium* from the liver are also unknown. Adoptive transfer of circumsporozoite protein-specific CD8 T cells into transgenic mice that express matching MHC class I molecules either exclusively on hepatocytes or on dendritic cells suggests that recognition of hepatocytes is sufficient to confer protection (Huang et al., 2015). However, the formation of immunological synapses between T cells and hepatocytes has not been observed in the intact liver suggesting that classical granule-mediated cytotoxicity is dispensable for parasite killing. In fact, mounting evidence suggests that effector CD8 T cells elicited by attenuated sporozoite vaccines recognize a subpopulation of hepatic dendritic cells and use cytokines to eliminate the parasites at a distance, without direct contact with infected

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hepatocytes (Frevert and Krzych, 2015). This model is supported by the lymphogenic features of the liver (Frevert and Krzych, 2015). The contribution of Kupffer cells and other liver-resident and recruited antigen-presenting cells to the effector phase against *Plasmodium* likely varies with the immune status of the host (Bertolino and Bowen, 2015; Frevert and Krzych, 2015).

The observation that both antigen-specific and antigen-unrelated CD8 T cells cluster around infected hepatocytes led to the proposal that antigen-specific effector CD8 T cells recruit other T cells to the site of infection and that the resulting inflammatory microenvironment augments parasite killing by antigen-specific and antigen-unrelated bystander cells (Bayarsaikhan et al., 2015). The significance of antigen-dependent focal inflammation and its consequences for the elimination of the intracellular parasites is discussed (Fernandes et al., 2014).

Unlike *P. falciparum* and rodent species, *P. vivax* forms dormant liver stages that may relapse weeks, months, or years after the primary infection, leading to new bouts of illness. Non-human primate model systems have been developed to study the immunobiology of the relapse phenomenon and to screen for biomarkers for *P. vivax* and a related simian parasite (Joyner et al., 2015).

The liver is also a central player in the defense against *Plasmodium* blood stages. Activation of Toll-like receptors (TLRs), acute phase proteins, phagocytic activity, and cytokine-mediated pro- and anti-inflammatory responses are all part of the liver-inherent immune system (Wunderlich et al., 2014).

Efforts to develop a successful malaria vaccine have been the focus of substantial research activities for decades. Immunization with live-attenuated sporozoites can elicit sterile immunity. Whether attenuation is achieved by irradiation or genetic modification, CD8 T cells play an essential role in the resulting sterilizing protection in several experimental models. Inoculation of infectious sporozoites under chemoprophylaxis also confers long-lasting sterile protection against homologous parasite strains in humans, although the exact mechanism is unclear. Chloroquine, the first drug used for chemoprophylaxis, neither eliminates *Plasmodium* liver stages nor delays parasite development (Sahu et al., 2015). Anti-malarial compounds such as quinine, quinones, and resveratrol, which may be present in the diet of individuals living in some endemic areas, are thought to contribute to protection against blood stage infection (Dalai et al., 2015).

Unlike whole organism vaccines, most subunit vaccine candidates fail to induce substantial and lasting protection. Novel approaches are therefore under investigation to identify antigens responsible for protection against the different parasite stages (Chia et al., 2014). These include the mining of genomic, proteomic and transcriptomic datasets to rationally identify immunological signatures associated with more potent immunity than occurs after natural exposure (Proietti and Doolan, 2014). Another focus is on novel non-inflammatory nanoparticle-based adjuvants, which induce high CD8 T cell responses without expanding myeloid-derived suppressor cells or inflammation-reactive Tregs at the site of priming (Wilson et al., 2015).

We hope that this Frontiers eBook offers insight into the many efforts aiming at breaking the life cycle of *Plasmodium* in the liver. A more thorough understanding of the mechanisms leading to sterile protection is a prerequisite for developing a malaria vaccine that protects the 40% of the world's population at risk of infection. We thank all contributing authors for bringing a broad range of expertise to this Frontiers Topic.

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CD8 T-cell-mediated protection against liver-stage malaria: lessons from a mouse model

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Malaria is a major global health problem, with severe mortality in children living in sub-Saharan Africa, and there is currently no licensed, effective vaccine. However, vaccine-induced protection from *Plasmodium* infection, the causative agent of malaria, was established for humans in small clinical trials and for rodents in the 1960s. Soon after, a critical role for memory CD8 T cells in vaccine-induced protection against *Plasmodium* liver-stage infection was established in rodent models and is assumed to apply to humans. However, these seminal early studies have led to only modest advances over the ensuing years in our understanding the basic features of memory CD8 T cells required for protection against liver-stage *Plasmodium* infection, an issue which has likely impeded the development of effective vaccines for humans. Given the ethical and practical limitations in gaining mechanistic insight from human vaccine and challenge studies, animal models still have an important role in dissecting the basic parameters underlying memory CD8 T-cell immunity to *Plasmodium*. Here, we will highlight recent data from our own work in the mouse model of *Plasmodium* infection that identify quantitative and qualitative features of protective memory CD8 T-cell responses. Finally, these lessons will be discussed in the context of recent findings from clinical trials of vaccine-induced protection in controlled human challenge models.

Keywords: CD8 T cells, memory, protection, *Plasmodium*, mice, humans

INTRODUCTION

Malaria represents an enormous global health problem. It is associated with around 200 million reported annual cases and more than 600,000 deaths, most of them recorded in sub-Saharan Africa (WHO, 2011). Current disease treatment is limited to antimalarial drugs targeting the symptomatic blood-stage infection. Given the enormous genetic plasticity of the parasite, the emergence of antimalarial drug resistance is inevitable and thus a major concern (Miller et al., 2013). Hence, the development of a protective malaria vaccine is alarmingly urgent.

Blocking *Plasmodium* infection at the level of the silent, liver stage of malaria represents an attractive strategy for disease prevention (Kappe et al., 2010). The earliest evidence of vaccine-induced, sterile, liver-stage immunity originates from mouse studies, in which it was demonstrated that vaccination with radiation attenuated sporozoites (RAS) prevented development of blood-stage *Plasmodium berghei* infection after sporozoite challenge (Nussenzweig et al., 1967, 1969). Importantly, RAS-induced sterile protection was confirmed in human subjects, in whom it was induced upon exposure to bites of more than 1000 sporozoite-bearing, irradiated mosquitoes (Clyde et al., 1973; Edelman et al., 1993; Hoffman et al., 2002). Persistent efforts to repeat the success of RAS in inducing sterilizing immunity using different vaccine formulations and regimens (e.g., which are easier to manufacture and can be administered through approved vaccination routes) have led only to a partial success (Moorthy et al., 2004; Dunachie et al., 2006; Bejon et al., 2007; Moorthy and Ballou, 2009; Agnandji et al., 2012). Therefore, RAS immunization still represents the gold standard for induction of sterile protection and, despite logistical challenges, has moved recently to Phase I clinical trial.

Besides being a potential vaccine candidate, RAS has been used as an invaluable tool for studying protective immune responses against liver-stage *Plasmodium* infections. Although the earliest work describes neutralizing antibodies as the main mechanism of RAS-induced protection (Potocnjak et al., 1980; Yoshida et al., 1980), more recently depletion studies and adoptive-transfer experiments have demonstrated that CD8 T cells have a leading role in protection against sporozoite challenge (Schofield et al., 1987; Weiss et al., 1988; Rodrigues et al., 1991). The critical role of CD8 T cells in protection against liver-stage malaria has been confirmed upon immunization with different formulations, such as recombinant vaccines [e.g., *Salmonella typhimurium*, vaccinia virus or adeno virus expressing *P. berghei* circumsporozoite (CS) protein] and genetically attenuated parasite (GAP; Sadoff et al., 1988; Lanar et al., 1996; Rodrigues et al., 1997; Jobe et al., 2007).

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 remain incompletely understood. The modest progress in this field is strongly influenced by the extreme genetic plasticity of the parasite, its complex life cycle and the paucity of defined antigenic targets for CD8 T cells. Filling these knowledge gaps is of an utmost importance, as this information would facilitate the development of successful pre-erythrocytic vaccine candidates.

Although highly desirable, studies of *Plasmodium* infection in humans are limited by multiple ethical and practical factors (inability to manipulate the immune response for mechanistic studies, difficulty to access relevant samples, etc.). Therefore, progress in understanding immunity against liver-stage malaria critically depends on the availability of suitable animal models. Infection of mice with *P. berghei* and *Plasmodium yoelii*, two rodent *Plasmodium* pathogens, closely resembles the early stages of human liver invasion, replication, and development within hepatocytes (Meis et al., 1983; Sturm et al., 2006; Baer et al., 2007). Additionally, clear differences in infectivity and pathogenicity displayed by these two *Plasmodium* species mimic the diversity of human *Plasmodium* infections (Sedegah et al., 2007). Therefore, the mouse model has proven to be invaluable for basic studies, such as host–parasite interactions and the underpinning of the immune mechanisms driving protection induced upon vaccination and sporozoite inoculation.

Here, we will present and highlight lessons about protective memory CD8 T-cell thresholds for liver-stage protection and effector mechanisms engaged by these cells, learned from vaccinations of mice with both subunit (*Plasmodium* CS-derived epitope) and whole-parasite (RAS and GAP) vaccine formulations. Finally, we will discuss the outcomes of recent vaccine clinical trials in light of our own findings and highlight the implications of the lessons learned for further development of liver-stage malaria vaccines.

INDUCTION OF CD8 T CELLS BY SUBUNIT VACCINES

SETTING THE NUMERIC THRESHOLD

Induction of sterilizing immunity against liver-stage parasite represents a challenge for at least two reasons. First, liver-residing or recruited CD8 T cells have to locate and eliminate all the infected hepatocytes/parasites to prevent progression of the infection from the liver to the blood stage. A single mosquito bite delivers a few hundred infectious sporozoites into the skin dermis. Given that only a fraction of sporozoites actually reaches the liver and infects hepatocytes, we estimate that only 1 out of 10^9 hepatocytes in humans or 1 out of 10^6 hepatocytes in mice are infected after mosquito infection. Similarly, low frequencies of infected hepatocytes are likely after intravenous (i.v.) challenge with 100–1000 virulent sporozoites, as used in many mouse studies. Thus, surveying CD8 T cells target extremely rare events, creating the proverbial "needle in a haystack" scenario. Second, the time interval between the start of the liver-stage infection and release of blood-stage merozoites is very short (7 days in *Plasmodium falciparum* and 2 days in *P. berghei/P. yoelii*), which means that CD8 T cells have a limited amount of time to perform their task (Sturm et al., 2006; Sturm and Heussler, 2007; Todryk and Hill, 2007). In the context of these spatial and temporal pressures, it is important to gain knowledge about the quantitative and qualitative features of protective memory CD8 T-cell response.

To address these questions, we used a mouse model to induce a stable, long-lasting memory CD8 T-cell response against a defined epitope (*P. berghei* $CS_{252-260}$). For this purpose, we exploited an accelerated prime-boost approach that is well established in our laboratory (Badovinac et al., 2005). In short, mice were vaccinated with mature dendritic cells (DCs), which had been incubated for 2 h with CS-derived peptide to allow surface peptide–MHC complexes to form (DC-CS), and a week later they received a booster vaccination with an attenuated *Listeria monocytogenes* expressing the same CS-derived peptide (LM-CS; Schmidt et al., 2008). This vaccination strategy (from here on abbreviated as DC-LM) has proven to be a robust tool for the generation and study of stable, long-lasting memory CD8 T-cell response against defined antigenic determinants without contribution by other components of the immune response (CD4 T cells, antibodies, NK cells; Schofield et al., 1987; Weiss et al., 1993; Doolan and Hoffman, 1999, 2000).

This vaccination approach allowed us to induce CS-specific CD8 T-cell immune responses with a magnitude of 1–7% of the total peripheral blood leukocytes (PBLs) (up to 20% of CD8 T cells). Strikingly, this response was stable and protective against repeated sporozoite challenges for at least 19 months (the life span of a laboratory mouse). Moreover, by titrating the booster vaccine dose, we were able to induce CD8 T-cell response with decreasing magnitude, which allowed us to determine the potential numeric threshold required for protection. Strikingly, we observed that more than 95% of animals with CD8+ T-cell frequencies exceeding a threshold of 1% of total PBLs were protected against sporozoite challenge, while more than 95% of animals with CD8 T-cell frequencies below this threshold developed blood-stage infection and were thus not sterilely protected (Schmidt et al., 2008). These findings demonstrate that sterile protection against sporozoite infection requires a remarkably strong CD8 T-cell response, representing a substantial fraction of the total CD8 T-cell pool and highly exceeding frequencies of antigen-specific CD8 T cells required for plausible protection against various viral and bacterial infections (Schmidt et al., 2008). On the other hand, given the previously mentioned spatial and temporal pressures on the sterilizing CD8 T-cell-mediated response, these results do not come as a complete surprise. It is not difficult to imagine that recognition and elimination of all the rare infected hepatocytes within 2 days require mobilization of extremely high CD8 T-cell numbers.

Thus, we describe a quantitative feature of protective memory CD8 T-cell response against liver-stage *Plasmodium*, and show that if met, this feature can potentially ensure life-long protection.

DISSECTING EFFECTOR FUNCTIONS UTILIZED BY PROTECTIVE MEMORY CD8 T CELLS

Various effector molecules, such as IFN-γ, TNF-α, perforin, FasL, and TRAIL, are utilized by memory CD8 T cells in protection against different infections (Raeder et al., 2000; Trapani and Smyth, 2002; Shrestha et al., 2006, 2008; Ishikawa et al., 2009). A few attempts were made to define the effector component of the CD8 T-cell responses against liver-stage *Plasmodium* infections. As these studies were based on RAS immunizations, which in addition to CD8 T-cell responses also induce also CD4 T cell and antibody responses, it is still not completely clear which pathways are engaged by memory CD8 T cells (Ferreira et al., 1986; Schofield et al., 1987; Tsuji et al., 1995; Renggli et al., 1997; Rodrigues et al., 2000). Furthermore, it is not clear whether CD8 T-cell responses against different *Plasmodium* species require the same effector pathways for sterile immunity. This information is of high relevance for the development of human

vaccines, which would ideally protect against multiple *Plasmodium* species.

To study effector functions in well-defined memory CD8 T-cell population, we used the DC-LM prime/boost approach. In contrast to vaccination with whole-parasite formulations, which in addition to CD8 T cells elicit also non-CD8 T-cell responses, this immunization approach allows focus only on effector pathways utilized by memory CD8 T cells (Schmidt et al., 2009). Memory CD8 T-cell responses against *P. beghei* and *P. yoelii* CS-derived peptides were induced in wild-type (wt) BALB/c mice together with mice deficient for various effector molecules (IFN-γ, perforin, FasL, and TRAIL). Additionally, TNF-α was depleted by neutralizing antibodies in vaccinated, wt mice to assess its role in CD8 T-cell-mediated sterile protection.

The most important finding of the study was that the pathways of memory CD8 T-cell-mediated protection against liver-stage infection were not completely overlapping for the two different *Plasmodium* species (Butler et al., 2010). Protection against *P. berghei* was diminished in the absence of IFN-γ and TNF-α but was not influenced by the absence of perforin. In contrast, the absence of perforin, but not TRAIL and FasL, completely eliminated protection against *P. yoelii*. In line with this finding, induction of generalized inflammation by treatment of animals with TLR9 agonist (CpG) 24 h-post sporozoite infection was sufficient to block the progression of *P. berghei*, but not *P. yoelii* infection to the blood stage. Susceptibility of *P. yoelii* sporozoites to CpG-induced inflammation was observed only during a very short window of 12 h-post infection. As *P. yoelii* displays higher infectivity in rodents compared to *P. berghei* (Sedegah et al., 2007), it is likely that more stringent control of parasite replication and development, involving direct killing of infected hepatocytes through the perforin pathway, are required for successful control of this infection at the liver stage.

Thus, effector mechanisms exploited by memory CD8 T cells in protection against liver-stage infection are *Plasmodium* species specific, a finding of high relevance for development of protective human vaccine targeting clinically relevant *P. falciparum* and *P. vivax*.

INFLUENCE OF *Plasmodium*–HOST INTERACTIONS ON MEMORY CD8 T-CELL-MEDIATED PROTECTION AGAINST LIVER-STAGE *Plasmodium* INFECTION

Although we demonstrated a clear threshold (>1% of PBL) for CD8 T-cell-mediated sterilizing immunity, our findings were limited to one mouse strain (BALB/c) and one *Plasmodium* species (*P. bergei*; Schmidt et al., 2008). The observation that some mouse strains, e.g., C57Bl/6 and B10.D2, are more difficult to protect against sporozoite infection upon RAS vaccination than BALB/c (Weiss et al., 1989; Doolan and Hoffman, 2000) made us wonder to what extent the protective threshold is influenced by host–parasite interactions defined by host-strain-specific background genes. Multiple studies have acknowledged the important role of non-MHC-linked host background genes on development of an immune response against infections (Hsieh et al., 1995; Diosi, 2002). Of equal importance, human malaria is caused by infection with multiple *Plasmodium* species, thus raising the question to what extent the protective threshold is influenced by different parasite species.

In our hands, following the DC-LM prime/boost with *P. yoelii* $CS_{280-288}$, mice developed high-magnitude memory CD8 T-cell responses that exceeded the defined threshold established with *P. berghei* model (>1% of total PBL). Interestingly, unlike our observations after *P. berghei* infection, these animals were highly susceptible even to a low-dose, *P. yoelii* challenge (Schmidt et al., 2011). Although we were able to substantially increase the size of memory CD8 T-cell population (>1.5% of total PBL), we were unable to find a defined, numerical threshold required for stable (defined as >80%) protection against liver-stage infection with *P. yoelii*. This observation was in line with the finding that protection against *P. yoelii* infection requires more stringent control of parasite replication, achieved by direct killing of infected hepatocytes by engagement of the perforin pathway (Butler et al., 2010). Thus, the protective memory CD8 T-cell threshold was highly influenced by the species of *Plasmodium* even in a single mouse strain.

In parallel with this, we observed that different strains of mice expressing the same MHC class I molecule ($H\text{-}2K^d$) that presents the CS-epitopes, but differing in background genes, display dramatically different levels of susceptibility to *P. berghei* sporozoite challenge (**Figure 1**). Importantly, in all these mouse strains, the DC-CS/LM-CS vaccination induced memory CD8 T-cell responses of similar magnitude and quality (e.g., production of IFN-γ; Schmidt et al., 2011). While more than 80% of immunized BALB/c and DBA/2 mice were protected against the infection, protection observed in mice with C57Bl/6 or closely related C57Bl/10 background genes (CB6F1 and B10.D2, respectively) was marginal or completely absent. Only after administration of a second booster vaccination dose, which induced extremely high-magnitude memory CD8 T-cell response (∼15% of the total PBL or ∼60% of the total CD8+ T cells), were B10.D2 and CB6F1 mice protected from sporozoite challenge. Furthermore, a study performed on reciprocal bone marrow chimeras between B10.D2 and BALB/c mice revealed that B10.D2 T-cell immune response reconstituted on a BALB/c background displays a superior protection in comparison to BALB/c T-cell response reconstituted on C57Bl/10 background. All the evidence results suggest that it is not the functional or quantitative property of memory CD8 T-cell response, but rather the host–pathogen interactions, determined by the host-background gene milieu and parasite species, that are key factors determining thresholds for memory CD8 T-cell-mediated protection. Of note, we have not identified a mouse background that is easier to protect against *Plasmodium* sporozoite infection.

This knowledge obtained in a mouse malaria model is of a particular relevance for understanding differences in protection observed in heterogeneous human populations and for development of successful malaria vaccines, which have to ensure protection against multiple, human *Plasmodium* species. Given the modest success of current human vaccines in inducing CD8 T-cell response, the extremely high numerical requirements for sterile protection against malaria may be an additional challenge for vaccine development.

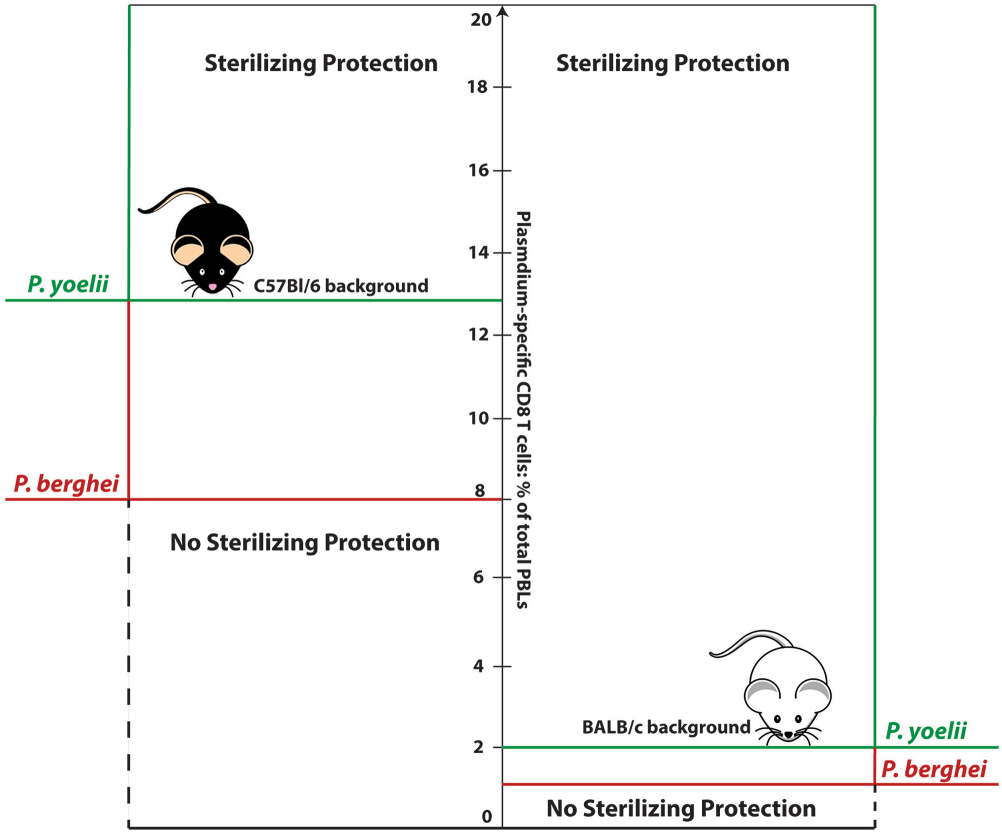

FIGURE 1 | Numerical thresholds required for memory CD8 T-cell-mediated sterilizing protection from sporozoite challenge: influence of host background genes and *Plasmodium* strain. Sterilizing protection of BALB/c mice against *P. berghei or P. yoelii* requires numbers of antigen- (CS-derived epitope) specific memory CD8 T-cell equivalent to ∼1% or 2% of total PBLs, respectively. Sterilizing protection of mice with C57Bl/6 background requires substantially higher CD8 T-cell numbers: ∼8% of total PBLs in the case of *P. berghei* infection, or ∼13% in the case of *P. yoelii* infection.

INFLUENCE OF UNRELATED INFECTIONS ON MAINTENANCE OF *Plasmodium*-SPECIFIC MEMORY CD8 T CELLS AND PROTECTION

In the parts of the world where malaria is endemic, infections with various, malaria-unrelated pathogens are also very common (Corbett et al., 2006; Magambo et al., 2006; Hotez and Kamath, 2009). Therefore, it is very likely that the burden of multiple infections has a substantial impact on the maintenance of both numbers and quality of *Plasmodium*-specific memory CD8 T cells, the major mediators of protection against liver-stage infection (Selin et al., 1996, 1999; Welsh and Selin, 2009). The phenomenon of attrition of antigen-specific memory CD8 T-cell populations has been described earlier in the context of infections with unrelated viruses (Selin et al., 1996, 1999). Specifically, these authors showed that antigen-specific memory CD8 T cells induced in mice by exposure to a viral infection underwent a decrease in frequency and numbers upon subsequent infection with unrelated viruses.

Investigating to which extent memory CD8 T cells are affected by unrelated infections and whether this potential effect can be reversed is of high relevance as it may provide basic information about development and reshaping of *Plasmodium*-specific immune response and useful guidelines for development of successful vaccine regimens.

In a separate study, we showed that exposure of mice to infections with unrelated pathogens (e.g., LCMV, LM, VacV, MHV-1) following DC-LM CS prime/boost vaccination induces dramatic decreases in frequency, but also in total numbers of antigen-specific memory CD8 T cells (Schmidt and Harty, 2011). Furthermore, more pronounced overall attrition was observed in the subpopulation of effector memory (Tem) compared to central memory (Tcm) T cells. This change in subset composition may have substantial functional consequences, as we previously suggested that Tem, but not Tcm are closely correlated with protection against sporozoite challenge (Schmidt et al., 2010). These drastic changes in numbers and composition of antigen-specific memory CD8 T-cell subsets resulted in seriously compromised protection, as only 10% of immunized mice exposed to malaria-unrelated infections were protected against sporozoite challenge. Importantly, the induced attrition did not hamper the capacity of memory CD8 T cells to expand upon antigen re-encounter. In line with this, a single-booster immunization after virus-induced attrition induced expansion of the memory population and restored the subpopulation structure (Tem vs. Tcm), and, most importantly, the protection against sporozoite challenge.

As multiple pathogens are endemic to the same geographic areas as malaria, it is very likely that vaccination-induced memory CD8 T-cell response will undergo a certain degree of attrition, which poses an additional concern for successful vaccination. The finding that despite the attrition, memory CD8 T cells remain responsive to booster vaccination, suggests that the use of prime and regular booster vaccination approach may be critical to preserve long-term immunity against malaria.

INDUCTION OF CD8 T CELLS BY WHOLE-PARASITE VACCINES

BROADENING OF THE ANTIGEN REPERTOIRE BY VACCINATION WITH RAS: IMPACT ON NUMERICAL THRESHOLDS

To date, only two immunization approaches have demonstrated a capacity to induce sterile protection against liver-stage malaria in both humans and animal models. Recently, an immunization approach based on multiple, low-dose inoculations of wt sporozoites through mosquito bites in individuals were also continually treated with the antimalarial drug chloroquine drug was evaluated. This approach was shown to successfully protect humans from a subsequent mosquito bit challenge (Roestenberg et al., 2011). The second approach is RAS vaccination, which still represents the gold standard of antimalarial protection (Clyde et al., 1973; Edelman et al., 1993; Hoffman et al., 2002). Nevertheless, critical parameters of protection, such as quantity and quality of the RAS-induced memory CD8 T-cell remain unknown.

After establishing that sterile immunity against sporozoite challenge in mice vaccinated with a single *Plasmodium* CS epitope requires mobilization of a large portion of total memory CD8+ T-cell pool (Schmidt et al., 2008), we decided to probe the hypothesis that broadening the antigenic targets would decrease the threshold of memory CD8 T cells required for protection. Multiple, low-magnitude, immune responses against broad range of antigenic determinants may have a superior protective capacity in comparison to monospecific response. For this purpose, the RAS vaccination regime was applied (Schmidt et al., 2010). To overcome the lack of well-defined MHC class I-restricted antigenic determinants, we took advantage of a surrogate activation marker approach recently described by our laboratory for measuring CD8 T-cell responses to bacterial or viral pathogens (Rai et al., 2009). Activation of effector or memory antigen-specific CD8 T cells by exposure to viral or bacterial infection induces down regulation of CD8α and up-regulation of CD11a. Importantly, these changes are stable in well-defined, antigen-specific CD8 T cells for the life of the laboratory mouse. This approach allowed us for the first time to follow and quantitatively and qualitatively characterize RAS-specific memory CD8 T-cell response and to assess the induced protection in multiple mouse strains.

To our surprise, broadening of the CD8 T-cell antigenic repertoire by vaccination with RAS did not measurably decrease the numerical threshold of memory cells required for protection (Schmidt et al., 2010). As shown by specific antibody depletion studies, the protection depended on CD8 T cells but not CD4 T cells (Schmidt et al., 2010). Similarly to our results with CD8 T cells specific for CS-derived epitopes, sterile protection was highly dependent on the mouse strain and *Plasmodium* species (**Figure 1**). Thus, BALB/c mice were relatively "easy" to protect, as a single dose (20,000) of RAS was sufficient to induce sterile protection against *P. berghei* challenge in > 80% of animals, and a single-booster immunization ensured protection against highly virulent *P. yoelii* challenge. On the other hand, protection observed in C57Bl/6 mice after a single RAS immunization was absent, despite similar memory CD8 T-cell numbers induced upon vaccination in both mouse strains. Solid, sterile protection of these mice against *P. berghei* sporozoite challenge required multiple booster doses and multiple-fold increase in the magnitude of the CD8 T-cell response. Antigen-specific CD8 T-cell populations representing even 40% of total CD8 T cells in B6 mice were not sufficient to protect against *P. yoelii* challenge at a memory time point.

Importantly, application of surrogate activation markers allowed us for the first time to follow and characterize RAS-specific memory CD8 T-cell responses and subsequent protection in populations of outbred mice, which may be a better model for genetically diverse human populations. In striking contrast to more or less uniform memory CD8 T-cell responses induced in inbred mice, responses measured in outbred groups varied substantially in magnitude and kinetics among individual animals. Despite wide distribution of immune responses, >80% of prime-boosted outbred mice were sterilely protected against homologous challenge with *P. berghei* and *P. yoelii* sporozoite infection. Importantly, this finding could potentially predict the distribution of immune responses upon whole-parasite immunization at the level of heterogeneous human populations.

MULTIPLE BENEFITS FROM TARGETING LATE-LIVER-STAGE ANTIGENS

Recently, a new sporozoite attenuation strategy has been developed, based on a direct manipulation (deletion) of target parasite genes (Mueller et al., 2005b). Such a targeted attenuation method may be superior in comparison to irradiation. While irradiation induces random lesions in DNA molecules and consequently gives rise to genetically diverse population of RAS, genetic manipulation allows for controlled production of well-defined and genetically similar parasites (genetically attenuated parasites – GAP; Chattopadhyay et al., 2009; Kappe et al., 2010). Additionally, while attenuation by irradiation arrests the parasite development at an early liver stage (Menard et al., 2013), targeting specific genes crucial for various metabolic processes allows for more or less custom design of GAP and production of both early- and late-liver-stage-arresting parasites (Mueller et al., 2005a,b; Aly et al., 2008). GAP have been tested in multiple pre-clinical studies and induced CD8 T-cell-mediated protection in murine malaria models (Labaied et al., 2007; Tarun et al., 2007).

Given the different degrees of intrahepatic development between early-liver-stage-arrested RAS and GAP and late-liver-stage-arrested GAP, we hypothesized that the latter expresses a broader spectrum of antigen determinants, and possibly induces superior protection. Consistent with this notion, vaccination with late-liver-stage-arrested GAP induced higher memory CD8 T-cell responses, which closely correlated with better protection induced in BALB/c, but also C57Bl/6 and Swiss Webster mice (Butler et al., 2011). Importantly, the antigenic specificity of the memory CD8 T-cell immune responses induced by early- and late-liver-stage-arresting vaccines was only partially overlapping.

From our study, it was clear that late-liver-stage-arresting GAP induce responses against broader spectrum of antigens compared to both RAS and early-liver-stage-arrested GAP. Thus, we concluded that superior protection induced upon vaccination with late-liver-stage-arrested GAP was based on the broadening of the antigenic repertoire.

These findings are particularly important in the light of recent finding of Tarun et al. (2008), who predicted that late-liver-stage and blood-stage parasites have substantial overlap in their antigen pools. This would mean that vaccination with late-liver-stage-arrested GAP may also induce cross-stage protection. Indeed, mice vaccinated with high numbers of late-liver-stage-arrested GAP, but not RAS, displayed high degree of protection against both liver- and blood-stage challenge (Butler et al., 2011).

Targeting a broad spectrum of antigens, particularly the ones shared by different developmental stages of the parasite, may increase the protective efficacy of a vaccine (**Table 1**). Not only do late-liver-stage-arrested GAP represent a promising vaccine candidate, but these parasites may also serve as an important model for determining novel CD8 T-cell antigens, which might be exploited for design of new generation of cross-protective subunit vaccines.

OUTCOME OF RECENT HUMAN TRIALS: NUMBERS DO MATTER

Although a substantial body of evidence has been collected pointing to the relevance of CD8 T-cell response in controlling liver-stage malaria, current vaccines have displayed rather modest capacity of inducing such responses in humans. The most advanced malaria vaccine is RTS,S, a subunit vaccine based on CS protein, which induces partial protection that correlates with antibody and CD4 T-cell immune responses, but no detectable CD8 T-cell response (Moorthy and Ballou, 2009). In the most recent field clinical trial in children this vaccine displayed ∼30% efficacy against clinical malaria in the target population of 6–12-month-old infants (Agnandji et al., 2012). Thus, there is a need for development of new vaccine candidates with the capacity to elicit CD8 T-cell response, and which can be used alone or in combination with RTS,S.

One of the promising CD8 T-cell-inducing approaches is based on heterologous prime-boost strategies using different viral vectors expressing the same CD8 T-cell-target antigens (Hill et al., 2010). Unfortunately, most of the vaccination regimens that were successful in mice failed when tested in humans (Ockenhouse et al., 1998; Moorthy et al., 2004; Bejon et al., 2006, 2007). Recently, a new promising prime-boost regimen based on chimpanzee adenovirus/MVA expressing *Plasmodium* thrombospondin adhesive protein (TRAP) fused to a multiple epitopes derived from several malaria antigens has been successfully tested in pre-clinical studies (Colloca et al., 2012), followed by two clinical trials in humans (O'Hara et al., 2012; Ewer et al., 2013). Results of the Phase IIa clinical trial revealed that 20% of vaccinated subjects had sterilizing immunity against standardized sporozoite infection, and 35% of vaccines displayed significant delay to a patency, which represent an additional measure of vaccine efficacy (Ewer et al., 2013). Although the degree of protection was modest, a very important finding of the study was the close correlation between the observed protection and the increased percentage of total CD8 T cells producing vaccine antigen-specific IFN-γ, over that induced in previous vaccine trials. Moreover, the efficiency of protection (sterile protection vs. delay to a patency vs. lack of protection) was dependent on the magnitude of IFN-γ-producing CD8 T cells, suggesting the existence of protective threshold, which is in line with our finding in a mouse model. The existence of protective CD8 T-cell thresholds may explain the failure of previous vaccination regimens tested in humans (Schmidt et al., 2008, 2010). On the other hand, it remains possible that protective memory CD8 T-cell thresholds in humans may be lower (<0.3% of total CD8 T cells) than those we observed in mice (>15% of total CD8 T cells). One of the possible explanations is the prolonged intra-hepatic phase of sporozoite infection in humans (6–8 days), compared to mice (2 days), providing human CD8 T cells with substantially more time for detection and elimination of infected cells, which might decrease the cell numbers required for successful protection.

Another recent human trial confirmed the importance of magnitude of CD8 T-cell response in protection against liver-stage malaria. Although it has been known for four decades that vaccination of human subjects with RAS via mosquito bites induces sterile protection against subsequent sporozoite challenge, only recently the first aseptic, radiation-attenuated, metabolically active, purified, cryopreserved, sporozoite vaccine was tested in a clinical trial, when it was injected into human subjects via two standard vaccination routes: subcutaneous (s.c.) and intradermal (i.d.; Epstein et al., 2011). Interestingly, the result of this first RAS clinical trial was the absence of protection in all the subjects, regardless of the dose received. With the hypothesis that the failure in protection was due to the suboptimal vaccination route rather than to lack of vaccine immunogenicity, Seder et al. (2013) performed another clinical trial using the

Table 1 | Comparison between early- and late-liver stage arresting whole-sporozoite vaccines.

Vaccine	Mode of inactivation	Stage of arrest	Percentage of sterilizing protection after a single dose	Protection against blood-stage challenge	Cross-species protection
RAS	Irradiation	Early liver	5	No	Partial
sap1^{-} GAP	Mutagenesis	Early liver	20	No	–
fabb^{-} GAP	Mutagenesis	Late liver	40	Yes	Yes

intravenous (i.v.) route for the vaccine delivery. The hypothesis was initially tested and confirmed in both non-human primate and a mouse model, as which revealed that i.v. immunization with RAS induces superior protection against sporozoite challenge when compared to s.c. immunization (Epstein et al., 2011). Using the previously described surrogate activation markers to enumerate antigen-specific CD8 T cells, authors showed that i.v. immunization induces immune response of substantially higher magnitude in liver and spleen than s.c. immunization. In line with these findings, and in striking contrast to s.c. vaccination, i.v. vaccination of human subjects induced sterilizing protection in 80% of subjects receiving the highest vaccine dose. Moreover, the protection correlated with numbers of CD8 T cells producing IFN-γ in a majority of protected subjects. These findings demonstrate superior protection induced by i.v. administration of RAS, and suggest induction of sufficient numbers of antigen-specific CD8 T cells as underlying mechanism of the observed protection.

Together these two studies suggest the translational value of our main finding in a mouse malaria model: the importance of memory CD8 T-cell numbers in sterilizing protection against liver-stage malaria and the potential existence of a definable threshold for sterilizing immunity. Besides being fundamental findings of high importance for further improvement of current and development of new malaria vaccine candidates and vaccination regimens, these findings also justify the use of a mouse as a suitable system for studying basic principles of immunity against pre-erythrocytic malaria stage.

CONCLUDING REMARKS

In summary, recent information generated in the mouse model of *Plasmodium* infection sets the cornerstone for further research of CD8 T-cell-mediated protection against liver-stage malaria. The most striking finding is that induction of extremely high numbers of memory CD8 T cells is a prerequisite for solid, sterile protection. Therefore, developing vaccines or vaccination regimens that will ensure induction of high-magnitude memory CD8 T-cell response against selected, highly protective target antigens is one means for successful immunization. However, it is still not known whether induction of additional immune components, such as CD4 T cells and antibodies, would positively influence the protection, for example, by decreasing the required numbers of CD8 T cells. Additionally, induction of CD8 T cells specific for antigens shared by (late) liver-stage and blood-stage parasite is a promising approach toward cross-stage, and possibly cross-species protection. Thus, one focus for future research should be discovery and characterization of such "shared" epitopes. Finally, the precise qualitative features leading to optimal memory CD8 T-cell protection against liver-stage *Plasmodium* infection remain to be determined.

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Tissue signatures influence the activation of intrahepatic CD8$^+$ T cells against malaria sporozoites

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Plasmodium sporozoites and liver stages express antigens that are targeted to the MHC-Class I antigen-processing pathway. After the introduction of *Plasmodium* sporozoites by *Anopheles* mosquitoes, bone marrow-derived dendritic cells in skin-draining lymph nodes are the first cells to cross-present parasite antigens and elicit specific CD8$^+$ T cells. One of these antigens is the immunodominant circumsporozoite protein (CSP). The CD8$^+$ T cell-mediated protective immune response against CSP is dependent on the interleukin loop involving IL-4 receptor expression on CD8$^+$ cells and IL-4 secretion by CD4$^+$ T cell helpers. In a few days, these CD8$^+$ T cells re-circulate to secondary lymphoid organs and the liver. In the liver, the hepatic sinusoids are enriched with cells, such as dendritic, sinusoidal endothelial and Kupffer cells, that are able to cross-present MHC class I antigens to intrahepatic CD8$^+$ T cells. Specific CD8$^+$ T cells actively find infected hepatocytes and target intra-cellular parasites through mechanisms that are both interferon-γ-dependent and -independent. Immunity is mediated by CD8$^+$ T effector or effector-memory cells and, when present in high numbers, these cells can provide sterilizing immunity. Human vaccination trials with recombinant formulations or attenuated sporozoites have yet to achieve the high numbers of specific effector T cells that are required for sterilizing immunity. In spite of the limited number of specific CD8$^+$ T cells, attenuated sporozoites provided multiple times by the endovenous route provided a high degree of protective immunity. These observations highlight that CD8$^+$ T cells may be useful for improving antibody-mediated protective immunity to pre-erythrocytic stages of malaria parasites.

Keywords: malaria liver stages, CD8$^+$ T cells, vaccine, migration and invasion, hepatocytes

INTRODUCTION

Malaria is a vector-born, parasitic illness caused by infection with *Plasmodium* spp. More than 3 billion individuals live in the endemic areas, which are mainly in tropical areas of the world. The disease causes approximately 600,000 annual deaths and most individuals are children living in Africa. In adults, pregnant women are vulnerable to malaria as pregnancy reduces their immunity. This problem has long been neglected but the maternal malaria causes an increased risk of spontaneous abortion, premature delivery and low birth weight of the fetus (Cotter et al., 2013; WHO Malaria Policy Advisory Committee and Secretariat, 2013; White et al., 2014). *Plasmodium* parasites have a complex life cycle, and the disease begins by the bite of an infected female Anopheline mosquito carrying the sporozoite form, which is the pre-erythrocytic stage of *Plasmodium* parasites. *Plasmodium* sporozoites are released from the secretory duct of the vector salivary gland where they develop and are injected into the bite site of the skin during blood meals. In the epidermis, the sporozoites actively cross the capillary vessels to enter the bloodstream from where they reach the liver (Khan and Waters, 2004; Miller et al., 2013).

After reaching the liver parenchyma, the sporozoites invade hepatocytes to divide and produce thousands of merozoites, which are the erythrocytic forms of *Plasmodium* parasites (Prudêncio et al., 2006). Parasite growth inside hepatocytes causes host cellular rupture, releasing the merozoites into the bloodstream, where they subsequently invade erythrocytes to initiate a cycle of intra-erythrocytic stage development. In this stage, the *Plasmodium* parasites continuously grow inside red blood cells, causing rupture and re-invasion of healthy erythrocytes and resulting in increased numbers of parasites in the bloodstream every 48 h (Prudêncio et al., 2006; Wright and Rayner, 2014).

Unlike the erythrocytic-stage of *Plasmodium* infection, which is responsible for the clinical symptoms and pathology of the

Abbreviations: PAMPs, Pathogen-associated molecular patterns; GPI, Glycosylphosphatidylinositol; TLR, Toll-like receptor; CSP, Circumsporozoite protein; RON4, Rhoptry resident protein; TRAP, Thrombospondin-related adhesion protein; ME-TRAP, Multiple epitope-thrombospondin-related adhesion protein; TLP, TRAP-Like Protein; TREP, TRAP-related protein; SPATR, Surface protein containing an altered thrombospondin repeat domain; AMA1, Apical membrane antigen 1; TAP, Vacuolar complex peptide transporters; LSA1, Liver-stage antigen 1; CelTOS, Cell-traversal protein for sporozoites; MVA, Modified vaccinia Ankara virus; AdHu5, Human type 5 replication-deficient adenovirus; TEM, Effector memory T cells; TCM, Central memory T cells; DC, Dendritic cell; LC, Langerhans cell; SC, Sinusoidal endothelial cells; KC, Kupffer cell.

disease, the liver stage of malaria is clinically silent but significantly relevant in the point of view of the host immune defense mechanisms (Frevert and Nardin, 2008). The hepatocyte cells are an obligatory destination for schizogony during the intrahepatic stage, which lasts for 2–7 days, depending on the mammalian host (5–10 days in humans), thus allowing the protective, cell-mediated immune responses to target the reservoirs of parasites in the liver. These hepatic reservoirs are a crucial target for immunological intervention achievements, as efficacious and effective pre-erythrocytic stage immunity would prevent the release of parasites from the hepatocyte and, consequently, the development of clinical disease and transmission of malaria (Doolan and Martinez-Alier, 2006; Duffy et al., 2012). The most clinically advanced malaria vaccine candidates for preventing disease are based on liver-stage *Plasmodium* antigens able to initiate protective immune responses and are expected to target endemic areas of greatest disease burden (Duffy et al., 2012; Birkett et al., 2013).

ANOPHELES VECTOR TRANSMISSION OF *PLASMODIUM* PARASITES IN THE SKIN SETS THE FIRST STAGE FOR INDUCTION OF CD8$^+$ T CELL-MEDIATED IMMUNITY AGAINST MALARIA SPOROZOITES

The sporozoite parasites are differentiated in the salivary glands of the mosquito vector and are inoculated in the vertebrate skin as the mosquito probes to locate the capillary vessels during the blood meal. During this phase, the sporozoites are injected into the skin tissue, a process that ends when the proboscis of the vector reaches the blood circulation and salivation no longer takes place (Vanderberg, 2014). Once in the skin, the sporozoites face a dangerous journey to the liver. They must find their way to hepatocytes, where they develop to the erythrocytic stage. Once the sporozoites enter the skin, the parasites are immediately targeted by innate host immune responses. Besides its own antigenic properties, the saliva proteins of the vector can influence several physiological responses in the skin, as the salivary contents have immunomodulatory properties (Demeure et al., 2005; Beghdadi et al., 2008; Hayashi et al., 2012; Mecheri, 2012).

The tissue trauma generated from the arthropod bite settles the cutaneous immune response behavior at the beginning of infection, as this injury generates endogenous danger signals (danger-associated molecular patterns) that, together with the exogenous PAMPs associated with *Plasmodium* parasites, play a crucial role in the initiation of the immune response (Naik et al., 2000; Gowda, 2007; Erdman et al., 2008; Greene et al., 2009; Fu et al., 2012; Gbédandé et al., 2013). While blood stage parasite PAMPs such as GPI or hemozoin/DNA have been identified (Gowda, 2007; Erdman et al., 2008), sporozoite PAMPs that could play a role in the skin remain to be defined. Thus, parasite-host factors during vector-mediated malaria transmission could be a determinant for activating the innate immune responses that would have an impact in the onset of parasite-specific adaptive immunity (Coban et al., 2007; Franklin et al., 2009; Gowda et al., 2012).

The arthropod vector also plays a role on this battlefield scenario because the *Anopheles* mosquito harbors an associated, symbiotic microbiome that is beneficial to host life related-traits, such as development, fecundity dietary, adaptation to the environment and immunity against pathogenic microorganisms, including *Plasmodium* parasites, in its digestive tract (Dinparast Djadid et al., 2011; Wang et al., 2011; Alvarez et al., 2012; Jiang et al., 2012; Ngwa et al., 2013). Glycosylphosphatidylinositol of *Plasmodium* parasites works as an exogenous PAMP to promote host cellular pro-inflammatory responses, mainly via TLR2 (Gowda, 2007). It is possible that, during the mosquito bite, the sporozoites are injected with an assortment of symbiotic microorganisms into the skin. Therefore, the mosquito microbiome itself could potentiate the innate signaling mechanisms involved in the skin immunity.

Recent studies using an experimental mouse model for malaria infection have substantially increased our comprehension of the host cellular responses that occur in cutaneous tissues during an *Anopheles* mosquito bite. An initial report has shown evidence that mosquito bites promote mast cell degranulation, leading to increased vascular permeability with plasma leakage and leukocyte infiltration into the injured tissue (Depinay et al., 2006). These host responses are initially followed by an accumulation of CD3$^+$ and B220$^+$ lymphocytes and CD11c$^+$ dendritic cells in the skin-draining lymphoid tissues, leading to hyperplasia of the organ at a time when no proliferation of these cells is observed, indicating a local sequestration of leukocytes from peripheral lymph nodes (Demeure et al., 2005).

These findings demonstrate that the subcutaneous transmission of the parasite by the vector potentially generates all of the requirements for priming the host adaptive immune responses in skin-associated tissues. However, no study has demonstrated how the antigens are processed in the tissue-nature source of the antigen-presenting cells that are retained in the skin-draining lymphoid organs after vector transmission of *Plasmodium* sporozoites. It is possible that the accumulated dendritic cell population in the skin-associated draining lymph nodes, which is critical for the priming of protective CD8$^+$ T cells against *Plasmodium* sporozoites, is derived from the cutaneous sites where the parasites are inoculated during a mosquito bite. All dendritic cell subsets of skin-draining lymph nodes are defined by the expression of the CD205, CD11b, CD11c, and CD8 markers, including a presumably blood-borne lymph node resident, CD8$^+$CD207$^+$ Langerhans cell population (Henri et al., 2010; Fehres et al., 2013). It remains unclear whether this cell population can transport antigens to lymph nodes and promote the initiation of T cell responses or, alternatively, if the parasite or its antigens can directly reach the skin-draining lymph nodes that are drained through the conduit network.

Recent studies using CD8$^+$ T cell transgenic mice specific for a MHC class I epitope present in the immunodominant surface protein of the sporozoite, the CSP, indicate that, in fact, the first signal of priming for the parasite-specific CD8$^+$ T cell response originates from the skin-draining lymphoid tissues associated with the site of vector transmission where the *Plasmodium* sporozoites are also found (Yamauchi et al., 2007). Within the first 48 h, these epitope-specific CD8$^+$ T cells secrete IFN-γ in lymph

nodes draining the site where the parasite was injected either by a vector bite or by dermal needle injection of the *Plasmodium* parasites (Chakravarty et al., 2007; Sinnis and Zavala, 2012; Ménard et al., 2013). However, in these experimental model studies, the parasites used in the infection were attenuated by irradiation, a matter that could change its infectivity in the skin because it has been shown that potent gliding motility is crucial for sporozoite exit from the cutaneous tissue (Vanderberg, 2014). These studies highlight the importance of the skin in the early stage of infection during the priming of the protective $CD8^+$ T cell responses that ultimately will limit the success of the parasite to reach the liver, where its multiplies and establishes its reservoir to continue to the blood-phase of infection.

THE HEPATIC ENVIRONMENT AS A SPECIALIZED NICHE CONSTITUTED WITH PROFESSIONAL ANTIGEN-PROCESSING CELLS FOR MHC CLASS I ANTIGENS

Studies have revealed that the time from the infective bite to the arrival of the parasite in the liver is approximately one to a few hours (Vanderberg, 2014). During this phase, the parasites must be prepared for the grand finale moment that culminates in the invasion of hepatocyte. Microarray analysis of the sporozoite transcriptome indicated a differential gene expression profile in the salivary gland forms compared to the sporozoites co-cultured with hepatocyte cells. In these studies, 21 genes were confirmed to be up-regulated in sporozoites co-cultured with hepatocytes. These genes were clustered into two patterns of expression, one transiently up-regulated and the other with sustained, increased expression (Rosinski-Chupin et al., 2007; Mikolajczak et al., 2008). These different expression profiles indicate genes that express sporozoite surface proteins that are involved in hepatocyte invasion and transiently expressed and other sustained-expression genes that are involved in the development of parasites inside hepatocytes (Mikolajczak et al., 2008).

Recently, it has been determined that, in fact, there is a shift in the sporozoite forms from the arthropod vector to the mammalian host that is responsible for the activation of the steady-state form of the parasite to its adaptation to the hepatic stages (Coppi et al., 2011). Migrating sporozoites switch to an invasive phenotype in the vertebrate host to reach and colonize the liver. This is well demonstrated in the context of the circumsporozoite protein, which is required for the interaction of the parasite with hepatocyte cells. In salivary glands, the CSP expressed by the sporozoites has a masked domain at its carboxy terminus. During its adaptation in the vertebrate host, the CSP is proteolytically processed in the liver tissue, thus exposing its C-terminal domain, which binds to heparan sulfate proteoglycans on the hepatocyte membrane as the parasite contacts these target cells during hepatic invasion (Coppi et al., 2011). This process is responsible for changing the status of the sporozoite into a hepatic invasive form, which contrasts from the skin form in that the cell transversal activity is the most important mechanism responsible for parasite invasion.

In the liver parenchyma, the parasite needs to glide through tissues and transverse hepatocytes (Ishino et al., 2004). However, this mechanism is not required for final hepatocyte invasion of the parasite before entering the intra-hepatic developmental stage (Ishino et al., 2004, 2005; Kariu et al., 2006; Amino et al., 2008). The invasion mechanism for this step has been shown to be dependent on the recognition of receptors for the region II-plus of the CSP on the basolateral domain of the plasma membrane of hepatocytes (Cerami et al., 1992) and the expression of genes such as the RON4 and the trombospondin-related adhesive proteins TRAP, TREP, TLP, and SPATR, which have roles in sporozoite motility and binding to hepatocyte cells (Sultan et al., 1997; Moreira et al., 2008; Combe et al., 2009). Inside hepatocytes, the sporozoites reside adjacent to the nucleus, and it has been suggested that this topological location may be useful for the parasite's control of the host cells, which occurs by translocation of the CSP into the host nucleus to modulate hepatocyte gene transcription (Singh et al., 2007). During the liver stage, the *Plasmodium* is highly metabolically active and expresses several genes to support its growth and development to blood-stage merozoites (Duffy et al., 2012). It has been shown that the gene products of liver-stage sporozoites are accessible to the host MHC class I-dependent antigen-processing machinery that is required for $CD8^+$ T cell recognition and are thus considered potential vaccine targets against the disease (Birkett et al., 2013). Among these genes, the immunogenic properties of *Plasmodium* liver-stage antigen-1 have been investigated, and this antigen has been currently evaluated in vaccine protocols aimed at inducing protection from malaria liver-stage parasites (Hill et al., 1992; Pichyangkul et al., 2008; Rodríguez et al., 2009; Cummings et al., 2010).

The choice of liver-stage development genes as vaccine targets seems to be of relevance because the antigens can be expressed early or late during parasite development in the liver, thus varying the efficacy of the immunity to the infected hepatocytes. However, the paucity of liver-stage, parasite-specific epitopes that are recognized by $CD8^+$ T cells has constrained vaccine development approaches. Several preclinical evaluations strongly support the importance of CSP-based vaccine approaches to confer protection mediated by $CD8^+$ T cells, but this remains to be determined in humans (Zavala et al., 1985; Khusmith et al., 1991; Kumar et al., 2006). Recently, studies using genome-wide epitope profiling have identified new potential antigen targets in the TRAP (Hafalla et al., 2013). These epitope-mapping studies should improve the design of vaccine protocols based on recombinant viral vectors and DNA vaccines encoding the entire TRAP antigen sequence, which have been shown to induce a protective-mediated immune response against malaria liver-stage infection (Ewer et al., 2013; Ferraro et al., 2013; Hafalla et al., 2013).

Importantly, it has been shown that, besides the early expressed antigens by liver-stage sporozoite forms, such as the TRAP and CS genes, late antigens expressed during the intrahepatic development of blood-stage merozoites inside hepatocytes are also targets of the host-protective $CD8^+$ T cell responses. Virus-vector vaccines expressing the AMA1 from *Plasmodium* parasites induce sterile protection associated with cell-mediated immunity (Chuang et al., 2013; Schussek et al., 2013; Schwenk et al., 2013). AMA1 is a highly conserved antigen in all apicomplexa parasites and is expressed in the merozoite stage of the *Plasmodium* life cycle (Remarque et al., 2008). During the intrahepatic stage of *Plasmodium* parasites, this antigen is expressed in

the late-stage of development of sporozoite forms to the merozoite blood stage. In mice, vaccine-induced AMA1-specific CD8+ T cells are associated with sterile protection against experimental malaria infection. In humans vaccinated with a combined genetic vaccine expressing AMA-1 and CSP of *P. falciparum*, 4 of the 15 volunteers (27%) were sterile protected against infection caused by infected mosquito bites (Chuang et al., 2013). Based on these findings, it is worthy to speculate that a better approach for vaccine design should combine the use of antigen targets expressed during the early stages of parasite infection in hepatocytes, e.g., the CSP and TRAP antigens, with the late-expressed AMA1 antigen from *Plasmodium* parasites to potentiate the effectiveness of a vaccine against malaria liver-stage infection.

The exposure of infected hepatocytes to sporozoites developing antigens in the course of infection has been proposed as important for the acquisition of anti-malaria adaptive immune responses, but the basis of hepatocyte antigen processing and presentation are not well understood. Recent studies have investigated the effects of parasite replication on the MHC class I pathway in hepatocytes. A HC-04 cell line isolated from primary human hepatocytes supports the full development of *Plasmodium falciparum* and *Plasmodium berghei* sporozoites to exoerythrocytic merozoite forms in the same time frame as observed *in vivo* (Ma et al., 2013). In these studies, Levitskaya's group has demonstrated that infected human hepatocytes supporting the replication and development of *Plasmodium* parasites to merozoites did not change the mRNA expression of the molecular components of the MHC class I pathway, such as the non-proteolytic (α1 and α2) and proteolytic (β1, β2, and β5) subunits of the proteosome, the elements of the TAP1 and TAP2 that are involved in the non-classical MHC class I cross-presentation pathway of vacuolar antigens, or ER chaperones (calnexin, calreticulin and Erp57) responsible for the complex assembly and stability of MHC class I molecules (Ma et al., 2013). These studies also demonstrated that infected hepatocytes were able to up-regulate the surface expression of MHC class I molecules in response to pro-inflammatory IFN-γ and TNF-α cytokines.

Interestingly, the findings indicated that human hepatocytes infected with *Plasmodium* parasites express the MHC class I processing and presentation machinery of the cross-presenting pathway and are able to stimulate and induce the activation of antigen-specific MHC class I-restricted effector CD8+ T cells (Balam et al., 2012). This phenomenon is of relevance because it has been shown that the *in vivo* activation of CD8+ T cells specific for CS depends on the cross-presentation of parasite antigens to the class I vacuolar pathway via TAP1 (Cockburn et al., 2011). However, it is important to note that, as the CS protein is an early antigen expressed by the sporozoite liver stages, it is possible that the antigen-processing restriction for the antigens expressed in the late-phase, intra-hepatic developmental stages of *Plasmodium* parasites may have different restrictions and pathways.

The cross-presentation MHC class I pathway in the liver seems to be extended to different subsets of hepatic cells, and this function was observed in liver sinusoidal endothelial cells as well as Kupffer cells at low antigen concentrations (Ebrahimkhani et al., 2011). Interestingly, the antigen cross-presentation by liver cells induced efficient CD8+ T cell expansion comparable to classical dendritic cells obtained from the spleen (Plebanski et al., 2005; Ebrahimkhani et al., 2011). However, T cells that proliferated following activation by contact with antigen-presenting liver cells expressed lower levels of T cell activation markers and intracellular IFN-γ, suggesting that dendritic cells may have a role in compensating the full activation program of CD8+ T cells that are initially activated in the hepatic parenchyma (Ebrahimkhani et al., 2011), a scenario that could support the full priming of CD8+ T cells in the liver. Alternatively, it is possible that the primary CD8+ T cells activated in the draining lymphoid organs during skin infection may support further activation and differentiation by liver cells once the parasite targets the hepatic parenchyma during malaria infection (**Figure 1**).

CD8+ T CELLS AGAINST MALARIA LIVER STAGES ARE DEFINED AS DIFFERENT SUBSETS AND MEDIATE PROTECTION IN THE COURSE OF A NATURAL OR VACCINATION-INDUCED IMMUNE RESPONSE

As in most infections caused by intracellular pathogens, CD8+ T cells are essential for developing acquired immunity against malaria liver-stage infection (Shrikant et al., 2010; Gebhardt and Mackay, 2012; Remakus and Sigal, 2013). Several independent investigations have demonstrated a critical role for CD8+ T cells in adaptive immunity against the pre-erytrocytic stage of *Plasmodium* infection using different approaches, such as *in vivo* depletion, reconstitution and adoptive transfer studies (Doolan and Martinez-Alier, 2006). Former studies in experimental rodent models indicated that immunization with irradiated *P. berghei* and *P. yoelli* sporozoites is able to induce protective immune responses mediated by CD8+ T cells, and *in vivo* depletion of this T cell subset abolished the acquired protective immunity (Schofield et al., 1987; Weiss et al., 1988; Seguin et al., 1994; Doolan and Hoffman, 1999, 2000). Although CD8+ T cells are considered to be the primary effector cells in the protective immune responses induced by irradiated sporozoites, CD4+ T cells specific to parasite-epitope antigens associated with MHC class II complexes expressed by infected hepatocytes are also thought to exert a role in host protection (Weiss et al., 1993).

Depending on the mouse strain used in the experimental rodent model of malaria infection, there is a requirement for CD4+ T cells in the effector responses induced by immunization with irradiation-attenuated sporozoites. It has been shown that C57BL/6, B6.129, and B10 mice immunized with irradiated sporozoites and previously depleted of CD4+ T cells were not protected against sporozoite challenge (Doolan and Hoffman, 2000; Doolan and Martinez-Alier, 2006). These results indicate a critical role for CD4+ T cells in the protective, immune-mediated responses against malaria liver-stage sporozoites. However, this CD4+ T cell requirement was not observed when BALB/c, B10.BR, and A/J mice were infected (Doolan and Hoffman, 2000). The CD4+ T cell requirement indicates a helper function-dependence for the induction or maintenance of effector CD8+ T cell responses, similar to how it occurs in other models. This dependence was further investigated using CD8+ T cell receptor (TCR) transgenic mice against the SYIPSAEKI epitope from the *Plasmodium yoelli* CSP antigen (Carvalho et al., 2002).

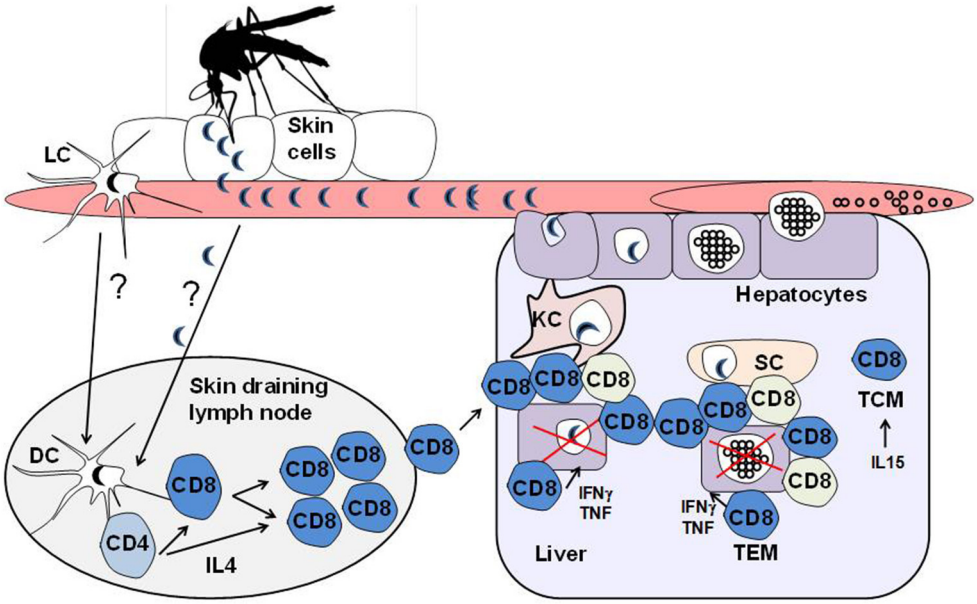

FIGURE 1 | Model depicting the activation of CD8+ T cells against *Plasmodium* liver-stage sporozoites. Malaria is an infectious disease that begins with the bite of an infected female Anopheline mosquito carrying the sporozoite forms, which is the pre-erythrocytic liver stage of *Plasmodium* parasites. *Plasmodium* sporozoites are released from the secretory duct, where they develop, and are injected into the bite site of the skin during blood meals. The motile sporozoites can then actively disseminate through the skin, bloodstream and draining lymphoid tissues. It is thought that the dendritic cell population in the skin-associated draining lymph nodes, possibly derived from the cutaneous sites where the parasites are inoculated during the vector bite, is critical for the priming of protective CD8+ T cells against the *Plasmodium* sporozoites, which are shown to be dependent on IL4-secreting CD4+ T helper cells. Although the epidermis and draining lymphoid tissues are thought to initiate the priming of anti-parasite CD8+ T cells, these effector cells can undergo further developmental steps in the liver following stimulation with parasite antigens from sporozoites as they develop into the erythrocytic merozoite forms of Plasmodium parasites in the hepatic parenchyma. It has been shown that the hepatic sinusoids are enriched with cells that are able to cross-present MHC class I antigens and therefore act as a barrier that is specialized to process and present antigens to intrahepatic CD8+ T cells. The liver antigen-presenting cells include dendritic cells, sinusoidal endothelial cells as well as Kupffer cells. The vast repertoire of antigen-presenting cells found in the journey of the parasite from the skin to the liver could be a determinant for generating distinct CD8+ T cell subsets during infection. In fact, it has been shown that effective protection against malaria liver stages is associated with distinct, intra-hepatic immune responses that are characterized by the induction of CD8+ T cell subsets with differences in the gene expression profiles associated with the cell trafficking responses that could play roles in the local immunity by tissue resident memory/effector cells. In the figure, the different colored CD8+ T cells represent the heterogeneous effector populations found in the liver following sporozoite infection.

In the TCR transgenic system, transferring of CSP-specific, transgenic CD8+ T cells into normal mice subsequently immunized with irradiation-attenuated sporozoites can induce a protective immune response against sporozoite challenge (Sano et al., 2001). This protective response was not observed when CD8+ TCR transgenic cells were transferred into normal mice that had been previously depleted of CD4+ T cells or IL-4 gene knockout recipient mice (Carvalho et al., 2002). In the absence of CD4+ T cells, effector antigen-specific CD8+ T cells begin a normal activation program with proliferative expansion in the first stages of the T cell differentiation program. However, after this initial phase, CD8+ TCR transgenic cells activated in the absence of CD4+ T helper cells fail to develop further, which compromises the effector pool of antigen-specific CD8+ T cells, as represented by a reduced number of cells and lower frequencies of IFN-γ secreting, antigen-specific TCR T cells. Using adoptive transfer and reconstitution approaches with gene-knockout cells to dissect the CD4+ T cell helper function, studies on the CSP-specific CD8+ TCR transgenic system indicated a requirement for the interleukin loop involving IL-4 receptor expression on CD8+ cells and that IL-4 secreted by CD4+ helper T cells was necessary for the full development of antigen-specific CD8+ T cells in the protective cellular-adaptive response against *Plasmodium* liver-stage parasites (Carvalho et al., 2002; Morrot et al., 2005).

Many studies of malaria as well as listeriosis and hepatitis models have raised attention to the complexity of the adaptive immune response in the hepatic microenvironment (Shrikant et al., 2010; Condotta et al., 2012; Gebhardt and Mackay, 2012; Claassen et al., 2013; Remakus and Sigal, 2013). Although these studies point to a central role for CD8+ effector/memory T cells in the elimination of infectious pathogens in the liver, the cellular and molecular interactions that underscore the mechanisms leading to pathogen killing are still poorly understood. In malaria, *in vivo* studies have investigated the dynamics of effector CD8+ T cells in the hepatic microenvironment. The immunization of naïve BALB/c mice with radiation-attenuated sporozoites significantly increased the presence of CD8+ T cells patrolling the sinusoids (Guebre-Xabier et al., 1999; Cabrera et al., 2013). Importantly, studies using the TCR CSP-specific transgenic system have approached intravital dynamic

imaging of *Plasmodium* elimination and revealed that parasite elimination frequently involves the recruitment of antigen-specific CD8$^+$ T cells to the liver parenchyma and the spatial distribution of these cells in a cluster surrounding the infected hepatocytes. These clusters of parasite-specific CD8$^+$ T cells reveal an efficacious, cell-mediated protective immunity mechanism against antigen targets expressed by sporozoite forms during malaria liver-stage infection (Cockburn et al., 2013).

Multiple effector mechanisms are likely to mediate *Plasmodium* liver-stage elimination by CD8$^+$ T cells (Frevert and Nardin, 2008). The protective effector mechanisms exerted by CD8$^+$ T cells in the pre-erythrocytic malaria stages in infected or vaccinated hosts varies depending on the infected animal model or vaccination regimen (Doolan and Martinez-Alier, 2006; Frevert and Nardin, 2008; Duffy et al., 2012). Although the literature has advanced our knowledge of protective host CD8$^+$ T cell immunity in rodent models, little is known about the cellular events that occur in the intrahepatic malaria stage of naturally infected humans. CD8$^+$ T cells can induce infected host cell killing by redundant mechanisms in which IFN-γ and TNF-α are identified as important players of the non-cytolytic pathways of infected hepatocytes during liver-stage infection of *Plasmodium* parasites (Butler et al., 2010). Another classical mechanism is mediated by the granule exocytosis pathway, which is a common characteristic of CD8$^+$ T cells and NK cells. Cytotoxic granules are a product of the secretory lysosomes that contain the pore-forming proteins perforin and granulysin, which are granzymes that mediate apoptosis in target cells (Frevert and Nardin, 2008). A preclinical trial of an optimized DNA vaccine approach that targets multiple sporozoite and liver-stage antigens, including CSP, LSA1, TRAP, and CelTOS, demonstrate the acquisition of antigen-specific CD8$^+$ granzyme B$^+$ T cells in non-human primates (Ferraro et al., 2013). The elimination of *Plasmodium* liver-stage parasites is likely to be mediated by direct recognition of infected hepatocytes by antigen-specific CD8$^+$ T cells, as this event occurs without a bystander effect that could promote parasite killing over the distance from the synapse that is induced between these cells in the liver parenchyma (Cockburn et al., 2014).

The multiple effector mechanisms deployed by CD8$^+$ T cells against the pre-erythrocytic-stage of the *Plasmodium* parasite is suggestive of a possible different commitment of the intrahepatic CD8$^+$ T cell lineage in malaria liver-stage infection. These cells exhibit a transcriptional profile with a distinguishable expression of immune function genes, cell cycle control and cell trafficking (Tse et al., 2013). In fact, it has been shown that effective protection against malaria liver stage is associated with distinct, intra-hepatic immune responses that are characterized by the induction of different CD8$^+$ T cell subsets. Studies have demonstrated that the protective T cells that are induced by attenuated malaria parasites induce changes in the CD8$^+$ T cell population, which is characterized by upregulation of CD11c on effector CD3$^+$CD8$^+$ T cells in the liver, spleen and peripheral blood. The majority of these cells are CD11c^{hi}CD44^{hi}CD62L$^-$ and secrete pro-inflammatory cytokines and cytotoxic markers such as IFN-γ, TNF-α, interleukin-2, perforin and CD107a. CD11c expression is lost as the CD8$^+$ T cells progress to memory phase (Cooney et al., 2013).

Other studies have demonstrated that vaccine-induced immunity using attenuated *Plasmodium* sporozoites is accompanied by the presence of intrahepatic effector memory (EM) CD8$^+$ T cells characterized by a CD44^{hi}CD45RB^{lo}CD62L^{lo}CD122^{lo} phenotype and central memory (CM) CD8$^+$ T cells with a distinguished phenotype, CD44^{hi}CD45RB^{hi}CD62L^{hi}CD122^{hi}, that are maintained by IL-15-mediated homeostatic proliferation (Krzych and Schwenk, 2005). The EM CD8$^+$ T cells promptly secrete IFN-γ upon sporozoite challenge, and these intra-hepatic memory CD8$^+$ T cells can be boosted by re-exposition to sporozoite antigens (Krzych and Schwenk, 2005). This feature could be of relevance, considering that it has recently been shown that the liver environment can keep a persisting depot of liver-stage antigens from irradiated sporozoites over 8 weeks after immunization, which is required for optimal development of protective immune responses mediated by CD8$^+$ T cells (Cockburn et al., 2010).

The presence of different T cell memory subsets may implicate a self-competition of CD8$^+$ T cells for the antigen, which, in turn, would limit the expansion/magnitude of the EM and CM pools of memory CD8$^+$ T cells (Hafalla et al., 2002, 2003; Cockburn et al., 2010). This intricate, self-regulatory mechanism exerted by activated CD8$^+$ T cells may have implications for the development of malaria liver-stage vaccines, considering it has been demonstrated that a large threshold for memory CD8$^+$ T cell frequencies is required for long-term protection. In these studies, the authors have developed a model of epitope-specific immunization regimes to induce a large memory CD8$^+$ T cell response capable of protecting mice from sporozoite challenges (Schmidt et al., 2008). Although the issue concerning the persistence of parasite antigens for the maintenance of memory T cells still remains elusive as some studies have demonstrated that primaquine treatment of immunized mice with irradiated sporozoites does not affect the protective responses (Krzych et al., 2014). However, since the primary primaquine action is target to the pre-erytrocytic liver stage parasite development, its effect does not interfere with the tissue depot of sporozoite antigens responsable for the activation/maintenance of host specific T cells (Krzych et al., 2014). Although the supporting mechanisms responsible for the persistence of memory T cells remains still controversial, a robust and sustainable intrahepatic CD8$^+$ T cell response is the target of the vaccine designs aimed at avoiding the intrahepatic development of malaria blood-stage merozoite forms, which are ultimately responsible for the clinical signs of the disease. It is possible that varying the route and/or nature of the protective antigen regimen in the prime-boost strategy implied in the vaccine protocols would overcome this restriction. This is of particular relevance and should be taken into consideration because sustainable protection against malaria is characterized by acquisition of strong, IFN-γ-secreting, intrahepatic CD8$^+$ memory T cells (Nganou-Makamdop et al., 2012).

DEVELOPMENT OF A RECOMBINANT VACCINE AGAINST THE PRE-ERYTHROCYTIC STAGE OF MALARIA

The only malaria vaccine that has reached Phase III clinical trials consists of a recombinant version of the *P. falciparum* CSP

that is administered in an adjuvant system. The efficacy of this vaccine formulation is approximately 30–50% and greatly correlates with the antibody titers to *P. falciparum* sporozoites and, to a minor extent, to CSP-specific CD4$^+$ T cells capable of secreting two or more cytokines (Birkett et al., 2013). Because this vaccine formulation already elicits antibodies and CD4$^+$ T cell-mediated immunity, to improve the vaccination efficacy, simultaneous stimulation of effector mechanisms mediated by CD8$^+$ T cells can be useful. Currently, the heterologous prime-boost regimen has achieved the best results in terms of a strong, protective immune response mediated by CD8$^+$ T cells specific for the pre-erythrocytic stages of malaria parasites (Hill, 2011; Soares et al., 2012; Teixeira et al., 2014).

Unfortunately, the results obtained after heterologous prime boost vaccination in a mouse model have not been duplicated in non-human primates or in humans (Birkett et al., 2013). Most of the studies rely on the analysis of the protection efficacy from the different vaccine regimens (Hill et al., 2010; Hill, 2011; Birkett et al., 2013). As the protection against malaria liver stage directly correlates with the acquisition of strong IFN-γ secreting intrahepatic CD8$^+$ T cells (Nganou-Makamdop et al., 2012), the levels of these responses elicited in the different vaccines would be an important parameter to guide the efficiency of a vaccine design. However, due to the absence of a direct method to identify and phenotype malaria vaccine-induced intrahepatic CD8$^+$ T cells in humans and non-human primates during pre-clinical and clinical tests, it should be important to determine whether the levels of antigen-specific CD8$^+$ T cells found in the liver correlates with the peripheral CD8$^+$ T cell responses from PBMC blood found in murine models, using a subunit vaccine for proof-of-concept.

The results of clinical trials using the heterologous prime-boost vaccination regimen with the *pfcsp* and *pfama-1* genes were recently published (Hill et al., 2010). This protocol consisted of priming with recombinant plasmid DNA, followed by a booster immunization with AdHu5, both expressing the *pfcsp* and *pfama-1* genes from the 3D7 strain of *P. falciparum*. Upon experimental challenge by exposure to the bites of mosquitos infected with the homologous parasite strain, only 27% of the individuals were sterilely protected (Bruder et al., 2012).

In other studies, individuals were initially immunized with recombinant adenoviral vector type 63 from chimpanzees that contained a synthetic gene encoding the polypeptide denominated ME-TRAP (Sheehy et al., 2012). The individuals received boost vaccinations with a recombinant MVA also containing this same gene (Bejon et al., 2007). In additional heterologous prime boost Phase II studies, 3 of 14 individuals were sterilely protected from infection after exposure to *P. falciparum*-infected mosquitoes (Bejon et al., 2007).

DEVELOPMENT OF AN ATTENUATED PARASITE VACCINE AGAINST THE PRE-ERYTHROCYTIC STAGE OF MALARIA

Although it has been long established that attenuated sporozoites provide a high degree and relatively long-lived protective immunity in most hosts, including men, vaccination regimens using whole, attenuated sporozoites were, until recently, considered a difficult path for the development of a product for mass vaccination. The recent development of cGMP *P. falciparum* sporozoite cultivation methods has now made this path a tangible possibility (Epstein et al., 2011). This strategy offers several advantages. Attenuated sporozoites express and present the whole spectrum of immunogens associated with the sporozoite- and liver-stages of malaria to the host (Butler et al., 2011; Epstein et al., 2011; Duffy et al., 2012; Spring et al., 2013). As the attenuated parasite is still able to infect target tissues, such as skin and liver parenchyma, in its natural route of infection, it induces the development of specific antibodies, CD4$^+$, CD8$^+$, and $\gamma\delta$ T cells. This wide range of immunogens and mechanisms of defense has a better chance of eliminating all pre-erythrocytic stages, which is required for sterile protection. In agreement with this idea, a recent study showed that protocols using multiple intravenous injections of radiation-attenuated sporozoites could provide sterile, protective immunity to 100% of vaccinated individuals (Seder et al., 2013). This observation may lead to a rational strategy for the development of a vaccine against malaria infection. Although important challenges should be overcome to face a large scale generation of *Plasmodium* sporozoites. To this goal, the development of aseptic, purified and cryopreserved *Plasmodium falciparum* sporozoites has received important priority (Seder et al., 2013). Improvements to stabilization of cryopreserved sporozoites are critically important to maintain high infectivity for the development of a vaccine using radiation-attenuated sporozoites. This is particularly critical to reduce vaccine wastage by prolonging the time after thawing the cryopreserved *Plasmodium* parasites during the vaccination.

SUMMARY AND PERSPECTIVES

The skin tissue acts as natural barrier against invading pathogens. The recent advances in skin immunobiology and studies pointing to the importance of the skin for the induction of the first signs of an anti-*Plasmodium*-adaptive responses induced by CD8$^+$ (Sinnis and Zavala, 2012)T cells against whole sporozoite forms in natural infections have substantiated a new concept in malaria biology and will consolidate the efforts for designing an efficacious vaccine against the pre-erythrocytic stages (Ménard et al., 2013). Recent advances in rodent models indicate a parasite skin-developmental pathway leading to generation of merozoites in the skin hair follicles that are infective to erythrocytes (Gueirard et al., 2010). This feature is able to promote a shortcut in the malaria cell cycle and should reshape all the current goals for malaria vaccine development strategies which target the induction of immunity to prevent the clinical signs of disease and/or interrupt transmission to support the eradication of this infirmity. Vaccines aimed at targeting the pre-erythrocytic stages of *Plasmodium* parasite infection can reach the spectrum of the vaccine goals against malaria, as any intervention at the pre-erythrocytic stage of the parasite cycle would have an implication in the further development of the parasite to the blood-associated disease symptoms and the sexual-stage parasites that are required for vector transmission.

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Liver-inherent immune system: its role in blood-stage malaria

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The liver is well known as that organ which is obligately required for the intrahepatocyte development of the pre-erythrocytic stages of the malaria-causative agent *Plasmodium*. However, largely neglected is the fact that the liver is also a central player of the host defense against the morbidity- and mortality-causing blood stages of the malaria parasites. Indeed, the liver is equipped with a unique immune system that acts locally, however, with systemic impact. Its main "antipodal" functions are to recognize and to generate effective immunoreactivity against pathogens on the one hand, and to generate tolerance to avoid immunoreactivity with "self" and harmless substances as dietary compounds on the other hand. This review provides an introductory survey of the liver-inherent immune system: its pathogen recognition receptors including Toll-like receptors (TLRs) and its major cell constituents with their different facilities to fight and eliminate pathogens. Then, evidence is presented that the liver is also an essential organ to overcome blood-stage malaria. Finally, we discuss effector responses of the liver-inherent immune system directed against blood-stage malaria: activation of TLRs, acute phase response, phagocytic activity, cytokine-mediated pro- and anti-inflammatory responses, generation of "protective" autoimmunity by extrathymic T cells and B-1 cells, and T cell-mediated repair of liver injuries mainly produced by malaria-induced overreactions of the liver-inherent immune system.

Keywords: liver, hepatic immune system, tolerance, innate immunity, blood-stage malaria, *Plasmodium*, erythrocytes, "protective" autoimmunity

LIVER-INHERENT IMMUNE SYSTEM

The liver is well known for its pivotal role in detoxification of drugs, toxins, and other harmful substances, in protein, lipid, carbohydrate, and vitamin metabolism, in steroidogenesis and bile secretion as well as for its supporting functions of other organs. Less known is the fact that the liver also plays a key immunoregulatory role (Selmi et al., 2007; Crispe, 2009; Nemeth et al., 2009; Parker and Picut, 2012). Indeed, the fetal liver is the primary hematopoietic organ of mammals, and, postnatally, the liver has retained the capability to exert important functions of both innate and adaptive immunity (Parker and Picut, 2012).

The liver-inherent immune system acts locally in the liver, but with systemic implications. Its two major functions are: (i) to sense pathogens, as, e.g., RNA- and DNA-viruses, bacteria, fungi, parasitic protozoans, and to activate efficacious inflammatory and immune responses against these pathogens; (ii) to recognize concomitantly non-pathogenic antigens as in harmless food compounds and to activate delicate mechanisms of immune hyporesponsiveness to generate tolerance (Selmi et al., 2007; Crispe, 2009; Nemeth et al., 2009; Parker and Picut, 2012). Both "antipodal" functions benefit from the dual blood supply of the liver (Nemeth et al., 2009). The hepatic artery delivers approximately 20% oxygenated blood from the circulation, while the portal vein provides approximately 80% venous blood from the gastrointestinal tract. The total blood of the human body is estimated to pass the liver approximately 25 times per hour. During liver passage, the blood percolates through a honeycomb of sinusoids with an approximate diameter of 5–7 μm, by which the velocity of the blood flow is slowed down to 25–250 μm/min (Nemeth et al., 2009). This facilitates the liver cells to recognize blood-delivered pathogens.

Pathogen recognition receptors (PRRs) represent a fundamental part of the liver-inherent immune system, that sense the evolutionary highly conserved pathogen-associated molecular patterns (PAMPs; Broering et al., 2011; Savva and Roger, 2013; Kesar and Odin, 2014). There exists a large reservoir of hepatic PRRs encompassing scavenger receptors (SRs) recognizing their targets by specific glycosylation patterns, modified protein motifs, and lipid moieties (Sorensen et al., 2012), cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptors recognizing bacterial peptidoglycans, the retinoic acid inducible protein-I (RIG-I) recognizing structural features of viral ssRNA, and, in particular, Toll-like receptors (TLRs; Thompson and Locarnini, 2007). The binding of TLRs by their respective ligands activates

innate and ensuing adaptive immune responses. There have been identified 10 functional human TLRs and 12 functional mouse TLRs, with TLRs 1–9 conserved in both humans and mice (Kawai and Akira, 2010).

Toll-like receptors are expressed by the majority of liver cells, though to varying levels (Chen and Sun, 2011). The TLRs 1, 2, 4, 5, and 6 are expressed on cell surfaces, whereas the TLRs 3, 7, 8, and 9 are intracellularly localized in endolysosomal membranes (Kawai and Akira, 2006). The TLRs, except TLR3, signal via the adaptor protein myeloid differentiation factor-88 (MyD88) ultimately activating nuclear factor kappa B (NFκB)-initiated and other pathways.

Effectful responses of the liver-inherent immune system against pathogens are generated by the orchestrated interplay of differently specialized liver-resident cell populations whose main "immune" functions are shortly outlined in the following.

Liver sinusoid endothelial cells (LSECs) form the fenestrated sinusoids-lining endothelium, which separates the sinusoid lumen, without any basement membrane, from the underlying space of Disse and the parenchyma tissue. LSECs represent about 45% of the non-parenchymal cells of the liver (Selmi et al., 2007; Jenne and Kubes, 2013). They express the SRs-A, SR-B, and SR-C as well as different TLRs. In response to ligands for TLRs 1, 2, 4, 6, and 9, LSECs produce tumor necrosis factor α (TNFα), and TLR3 ligands induce interferon-γ (IFNγ), TNFα, and interleukin (IL)-6 (Wu et al., 2010). Moreover, LSECs express major histocompatibility complex (MHC) class I and II molecules, co-stimulatory molecules CD40, CD80, and CD86 and adhesion molecules, as, e.g., intercellular adhesion molecule (ICAM) required for interaction with lymphocytes. Currently, LSECs are envisioned as to represent an intricate platform for antigen presentation (Selmi et al., 2007; Jenne and Kubes, 2013; Nakamoto and Kanai, 2014).

Hepatocytes (HCs) of the liver parenchyma encompass about 70–80% of all liver cells (Nemeth et al., 2009; Jenne and Kubes, 2013). HCs can express TLRs 1–9, though only TLR2 and TLR4 have been reported to be ligand-responsive to date (Liu et al., 2002). HCs induce different innate immune responses (Nemeth et al., 2009; Jenne and Kubes, 2013), but are also able to initiate adaptive immune mechanisms. Indeed, HCs express MHC I molecules, and inflammatory conditions even induce expression of MHC II antigens (Franco et al., 1988), enabling HCs to present antigen to naive T cells (Selmi et al., 2007; Nemeth et al., 2009; Jenne and Kubes, 2013).

Different soluble components produced by HCs majorly contribute to the acute phase response (APR), the first line of innate systemic defense against pathogens. Acute inflammation induces HCs to transiently increase their production of "positive" acute phase proteins (APPs), whereas that of other "negative" APPs are decreased (Steel and Whitehead, 1994; Parker and Picut, 2012). The major positive APPs include serum amyloid A (SAA) and C-reactive protein (CRP) in humans as well as SAA and the CRP-homolog serum amyloid P (SAP) in mice, whose serum concentrations during an APR can increase up to 1000-fold over normal levels (Steel and Whitehead, 1994). Apart of these major APPs, there are numerous other proteins produced in the liver during infection, as, e.g., lipopolysaccharide binding protein, haptoglobin, α2-macroglobulin, different proteinase inhibitors, α1-antitrypsin, and α1-antichymotrypsin (Steel and Whitehead, 1994). Among the negative APPs are the plasminogen activator inhibitor-1 (PAI-1) and the liver isoform of alkaline phosphatase (AP; Steel and Whitehead, 1994). The latter has been used classically as a serum biomarker for hepatic disease states such as hepatitis, steatosis, cirrhosis, drug-induced liver injury, and hepatocellular carcinoma (Pike et al., 2013).

Other soluble substances produced by HCs are complement components, e.g., C3, that opsonizes pathogens for elimination, and C9, that forms the membrane attack complex for final microbe lysis (Parker and Picut, 2012). Moreover, HCs produce a number of circulating growth factors and inflammatory cytokines, amongst which is also IL-6, that in turn induces massive production of APPs by HCs (Parker and Picut, 2012). This pleiotropic cytokine exerts both pro- and anti-inflammatory properties and takes actions through two different signaling mechanisms (Heinrich et al., 2003; Drucker et al., 2010; Scheller et al., 2011). IL-6 "classic signaling" is restricted only to HCs and immune cells, which are activated through their specificity-defining membrane IL-6 receptor α (IL-6Rα) and the recruitment of two chains of the signal-transducing membrane receptor GP130, thus activating the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. However, IL-6 can also communicate with all other cells in the body by a process termed "IL-6 trans-signaling." Hereby, IL-6 binds to the naturally occurring soluble IL-6Rα, which is derived by shedding of the ectodomain of membrane IL-6Rα and by alternative mRNA splicing in humans, but not mice. The IL-6/IL-6Rα complex can bind to GP130 expressed ubiquituously in surface membranes of all cells. However, the IL-6/sIL-6Rα complex can be inactivated by binding soluble GP130 (sGP130), thus blocking IL-6 trans-signaling (Knupfer and Preiss, 2008).

Kupffer cells (KCs) are located in the sinusoid lumen, adhere to LSECs and represent about 30% of the non-parenchymal liver cells (Parker and Picut, 2012). These liver-resident macrophages constitute about 80–90% of the whole reticuloendothelial system in the human body (Bilzer et al., 2006). The KCs presumably derive from both local hematopoiesis in the liver as well as from bone marrow-derived monocytes that enter the liver and eventually differentiate to liver-resident macrophages (Nemeth et al., 2009; Jenne and Kubes, 2013). KCs are very versatile with a lifespan reaching from 1 week until 1 year. They express different surface receptors allowing the detection, binding, and phagoyctosis of pathogens. For instance, KCs express ligand-responsive TLRs 2–4 and TLR9 (Nakamoto and Kanai, 2014), SRs, the complement receptors (CR)1 and CR3 as well as Fc receptors. Even if KCs are not able to internalize and destroy pathogens, they have captured on their surface as, e.g., *Listeria monocytogenes*, they recruit neutrophils from the circulation, which then take over destruction of these pathogens (Gregory et al., 1996). Moreover, KCs are involved in waste disposal, which also includes phagocytosis of activated and apoptotic "self" cells, as, for example, neutrophils, platelets, and T cells. Upon stimulation, KCs produce a series of cytokines among which are TNFα, IL-1β, IL-6, IL-12, and IL-18 (Kopydlowski et al., 1999). Moreover, KCs are capable of antigen presentation. They express MHC I, MHC II and co-stimulatory molecules required for activation of T cells. In particular, KCs

can activate natural killer T (NKT) cells patrolling the sinusoids (Jenne and Kubes, 2013).

Hepatic stellate cells (HSCs) or Ito cells reside in the space of Disse, represent about 5–8% of total liver cells, and their dendritic-like protrusions wrap around the sinusoids (Jenne and Kubes, 2013). Apart from vitamin A storing and fibrinogenetic cells, HSCs are able to present lipid antigens to NKT cells, enhance proliferation of NKT cells and may present antigen to $CD8^+$ T cells (Nemeth et al., 2009; Jenne and Kubes, 2013). Upon activation, human HSCs express TLR4, along with CD14, and the mouse HSCs TLRs 2 and 4 express vascular cell adhesion molecule 1 (VCAM-1), transforming growth factor β1 (TGF-β1) and monocyte chemoattractant protein-1 (MCP-1; Selmi et al., 2007; Jenne and Kubes, 2013).

Dendritic cells (DCs) are concentrated around the portal triad and around the central veins of the liver. There exist at least five DC-types of with different "immature" phenotypes: lymphoid DCs ($CD8a^+$ $B220^-$ $CD11b^+$), myeloid DCs ($CD8^-$ $B220^-$ $CD11b^+$), plasmacytoid DCs ($CD8a^-$ $B220^+$), mixed lymphoid/myeloid DCs ($B220^-$ $CD11b^-$), and natural killer (NK) DCs ($B220^-$ $CD11c_{int}$ $CD69^+$ $2B4^+$ $DX5^+$; Selmi et al., 2007; Nemeth et al., 2009; Jenne and Kubes, 2013). Hepatic DCs differ from their counterparts in any classical lymphoid organ not only by their immature phenotype, but also by less immunogenicity, higher productions of cytokines, and higher endocytotic activity (Hsu et al., 2007). They produce TNFα and IL-6 in response to diverse ligand-activated TLRs. The ability of DCs to activate T cells can be impaired by LSECs, which may be important for induction and maintenance of immune tolerance. The major function ascribed to hepatic DCs is to control the balance between oral and portal autoimmune diseases (Selmi et al., 2007; Nemeth et al., 2009).

Liver-resident lymphocytes, i.e., NK cells, NKT cells, T cells, and B cells, account for about 25% of the non-parenchymal cells of the liver. They differ from their corresponding cell populations in other tissues in several aspects (Selmi et al., 2007; Crispe, 2009; Jenne and Kubes, 2013).

Natural killer cells are large granular lymphocytes (LGLs), represent about 35–50% of all liver-resident lymphocytes and comprise a very heterogenous pattern (Nemeth et al., 2009). NK cells in the liver are also more frequent than in any other tissues. For instance, these innate lymphocytes are enriched more than threefold in comparison to those in blood (Crispe, 2009). They possess diverse PRRs which do not only recognize PAMPs, but also damage-associated molecular patterns (DAMPs). NK cells express ligand-responsive TLRs 1–4 and TLRs 6–9 (Sawaki et al., 2007). Upon activation and in very close contact with target cells, which may be opsonized by antibodies, NK cells release perforin and various granzymes from their intracellular vesicle stores by exocytosis, which kills foreign microbes (Lodoen and Lanier, 2006) and also malignant cells (Herbermann et al., 1975; Notas et al., 2009). In addition, activated NK cells secrete numerous cytokines, as, e.g., IFNγ, and are therefore considered as to shape subsequent immune responses including modulation of MHC expression by HCs and HSCs (Crispe, 2009).

Natural killer T cells are an abundant population amongst liver-resident lymphocytes (Crispe, 2009; Nemeth et al., 2009; Jenne and Kubes, 2013). Besides characteristic NK markers, NKT cells also express a limited repertoire of T cell receptors (TCRs). They are mainly $CD4^+$, are restricted by CD1d molecules, and display a LGL-morphology. There exist two populations, i.e., $NK1.1^+$ TCR^{int} (NKT cells) and $NK1.1^-$ TCR^{int} (Abo et al., 2012). Invariant NKT (iNKT) cells express the invariant TCRα (Vα14-Jα18) in mice and Vα24-Jα18 in humans and respond to various lipids in context with the non-classical MHC I molecule CD1d (Bendelac et al., 2007). The CD1-dependent iNKT make up to 30% of total hepatic lymphocytes, whereas their content in blood is only 0.5%. Another group of NKT cells are the CD1d-independent $NK1.1^+$ T cells. Hepatic NKT cells are characterized by peculiar features: (i) They apparently are the only cell populations among the liver-resident lymphocytes, which actively patrol the hepatic vasculature for detecting pathogens. (ii) They can also detect insults in further distant tissues and then act as modulators of the global immune status. (iii) They might play a role in liver autoimmune diseases upon tolerance breakdown (Nemeth et al., 2009; Abo et al., 2012).

T cells contribute about 25% to the liver-resident lymphocytes (Selmi et al., 2007; Crispe, 2009; Nemeth et al., 2009). Most of them are extrathymic T cells developing outside the thymus and express only intermediate amounts of TCR (Seki et al., 1991; Abo et al., 2012). $CD8^+$ T cells are more abundant than $CD4^+$ T cells in the liver (Selmi et al., 2007). Moreover, double negative $CD4^-$ $CD8^-$ T cells occur in the liver and many of them express $_{\gamma,\delta}$TCR instead of the conventional $_{\alpha,\beta}$TCR (Nemeth et al., 2009). Most hepatic T cells have an activated phenotype; for instance, CD25 and CD69 are expressed on $CD8^+$ T cells. The T cells in the liver differ in general from those in blood, lymph nodes, and spleen by a lower ratio of CD4/CD8, higher percentage of double positive $CD3^+$ $CD4^+$ $CD8^+$ and double negative $CD3^+$ $CD4^-$ $CD8^-$, as well as higher abundance of $_{\gamma,\delta}$TCR cells. The latter represent about 15–25% of hepatic T cells, but only 3% of blood T cells (Nemeth et al., 2009). T cells can be activated through their TLRs 2, 3, and 9 (Gelmann et al., 2004; Komai-Koma et al., 2004). Importantly, the liver also contains several subsets of regulatory T (T_{reg}) cells, and specific T_{reg} cells are assumed to play an important role in induction and maintenance of tolerance (Selmi et al., 2007; Crispe, 2009).

B cells represent less than 10% of the liver-resident lymphocytes in the human liver. In mice, the fetal and adult liver contains $B220^{low}$ B-1 cells, being either $CD5^+$ or $CD5^-$ (Tachikawa et al., 2008; Baumgarth, 2011; Abo et al., 2012), with CD5 as a negative regulator of B cell receptor signaling (Lankester et al., 1994). Incidentally, B-1 cell development is negatively regulated by the nuclear pregnane X receptor in the liver (Casey and Blumberg, 2012). The majority of intrahepatic B cells resembles conventional $B220^{high}$ B-2 lymphocytes. B-2 cells are IgM^{low} IgD^{high} $CD23^+$, while B-1 cells are IgM^{high} IgD^{low} $CD3^-$ (Tachikawa et al., 2008). B-1 and B-2 cells produce low affinity autoreactive IgM antibodies, also termed natural antibodies (Baumgarth et al., 2000). Most natural antibodies are polyreactive with numerous "self" and "non-self" antigens and are involved in efficient control of pathogens from circulation (Baumgarth et al., 2000), in the clearance of senescent erythrocytes (Lutz et al., 1990), in removal

of apoptotic bodies (Duan and Morel, 2006), and in autoimmune diseases (Ochsenbein and Zinkernagel, 2000).

THE LIVER: EFFECTOR ORGAN AGAINST BLOOD-STAGE MALARIA

The malaria-causative agents are parasitic protozoans of the genus *Plasmodium*. These are transmitted by *Anopheles* and reach via the bloodstream the liver, in which they invade HCs and undergo asexual multiplication (Frevert and Nacer, 2013). This pre-erythrocytic development of the parasites in the liver proceeds clinically asymptomatic, it is not preventable by the liver-inherent immune system, though it may possibly benefit, at least transiently, from the tolerogenic properties of the liver (Frevert, 2004). Then, merozoites are released from HCs, which penetrate erythrocytes and the ensuing asexual multiplication in erythrocytes is associated with morbidity and mortality of malaria.

Blood-stage malaria afflicts about 225 million people and kills about 781,000 people, mainly African children, worldwide per annum (Garcia, 2010; WHO, 2010). The majority of the malaria-afflicted people can obviously cope with the infections. Repeated blood-stage infections in malaria endemic areas obviously lead, though slowly, to acquisition of natural immunity against the blood-stage malaria. This natural immunity is not solid: it reduces disease symptoms, but it does not prevent re-infections with elevated blood parasitemias during malaria season. It is specific for species and strain of *Plasmodium*, and it is short-lived (Cohen and Lambert, 1982). It appears as if the slow acquisition of natural immunity may be associated with increasing tolerance generated by the liver against malarial blood-stages, though experimental evidence for this view is lacking.

The spleen is currently considered as to be the central effector site of host immunity against blood-stage malaria (Chotivanich et al., 2002; Engwerda et al., 2005; Del Portillo et al., 2012). The spleen is equipped with mechanisms to eliminate senescent and aberrant red blood cells (RBCs) including parasitized RBCs (pRBCs). However, previous studies with the experimental malaria *P. yoelii* have already revealed that mice, after vaccination and splenectomy, are still able to clear blood-stage infections, albeit at delayed recovery (Playfair et al., 1979; Dockrell et al., 1980). Also, splenic uptake of injected labeled pRBCs during infections with *P. yoelii* decreased with progressing course of infection. Concomitantly, however, the liver has increased its uptake of pRBCs.

Similarly, it has been found in *P. chabaudi*-infected mice that, during the crisis phase of infection, which is characterized by a dramatic decline of peripheral parasitemia from about 35–55% at peak parasitemia to about 1–2% within 3–4 days, the spleen, though dramatically enlarged, becomes "closed" for entry of particulate material. Indeed, the spleen excludes uptake of injected labeled pRBCs or inert polystyrol beads, but not that of fluorescently labeled bovine serum albumin (Krücken et al., 2005, 2009). Concomitantly, however, the liver has increased its trapping capacity of particles (Krücken et al., 2005, 2009). Moreover, the importance of the liver against blood-stage malaria is particularly demonstrated in lymphotoxin β receptor (LTβR)-deficient mice which have severe defects of the spleen and have also lost all other secondary lymphoid organs (Fütterer et al., 1998). Nevertheless, these LTβR-deficient mice are even more resistant to blood-stage malaria of *P. chabaudi* than the corresponding control mice (Wunderlich et al., 2005). These findings support the view that the liver with its immanent immune system is also a major—if not essential—player of the host defense against blood-stage malaria, though this aspect has been largely neglected by research to date.

LIVER EFFECTORS TOWARD BLOOD-STAGE MALARIA

Evidence is increasing that the liver-resident immune system is obviously able to respond to blood-stage malaria with the development of efficient anti-malaria effector mechanisms.

Toll-like receptors are activated in the liver by blood-stage malaria. Indeed, experimental malaria with *P. chabaudi* induces significant increases in mRNA expression of *Tlr 1*, *2*, *4*, *6*, *7*, and *8*, varying between 4- and 21-fold in the liver of C57BL/6 mice (Al-Quraishy et al., 2014). By contrast, the expression of *Tlr3*, *Tlr5*, and *Tlr9* does not respond to malaria infection at all. It is conspicuous that the mRNA levels of both the surface-localized TLR1, TLR2, and TLR6, as well as the intracellular TLR7 and TLR8 are increased.

TLR1 and TLR6 are currently regarded as auxiliary receptors, which both form heterodimers with TLR2 in the plasma membrane (Kawai and Akira, 2010; Oliveira-Nascimento et al., 2012). These heterodimers of both TLR2/1 and TLR2/6 recognize a broad spectrum of different PAMPs, in particular multiple diacyl lipopeptides of various infectious agents such as viruses, bacteria, fungi, and parasites (Oliveira-Nascimento et al., 2012; De Almeda et al., 2013; Misch et al., 2013; Zhang et al., 2013). PAMPs recognition then activates a cascade of downstream reactions eventually leading to innate and adaptive immune responses directed against pathogens and, also, to protection from those adverse processes induced by host responses to infections (Kawai and Akira, 2010; Oliveira-Nascimento et al., 2012). In human malaria with *P. falciparum*, the TLR2/1 and TLR2/6 complexes recognize specific components of the glycosylphosphatidylinositols (GPIs), that are regarded as major factors contributing to malaria pathogenesis (Krishnegowda et al., 2005). Thus, TLR2/1 recognizes intact 3-acyl-GPIs and TLR2/6 the sn-2-lyso-GPI components (Krishnegowda et al., 2005). Remarkably, expression of *Tlr6* and *Tlr1*, but not that of *Tlr2*, *Tlr7*, and *Tlr8*, are under epigenetic control, since blood-stage infections of *P. chabaudi* induce alterations of the DNA methylation status of the promoters of *Tlr1* and *Tlr6* genes (Al-Quraishy et al., 2013). This malaria-induced epigenetic fine-tuning of *Tlr6* and *Tlr1* in the liver may be an important initial step of the host response against *Plasmodium* blood stages. Since DNA methylation is stable (Bird, 2002), it may be further suspected that epigenetic fine tuning of *Tlr6* and *Tlr1* may contribute to memory against homolog re-infections.

In contrast to *Tlr1* and *Tlr6*, the DNA methylation status of the *Tlr8* gene promoter is not affected by *P. chabaudi* infections at all. The function of TLR8, intracellularly localized in endosomal membranes, are not yet well understood (Cervantes et al., 2012; Kesar and Odin, 2014). TLR8 has been shown to sense single-stranded RNA of viral origin and bacterial RNA as well as to induce NFκB-dependent cytokines and type I IFNs. However, increasing information suggests that TLR8, possibly in cross-talk

with TLR7 (Wang et al., 2006), is involved in the generation of autoimmunity (Krieg and Vollmer, 2007; DeMaria et al., 2010). Remarkably, dysregulations of *Tlr8* and *Tlr6* occur, when *P. chabaudi* infections are induced by testosterone to take a lethal outcome in female mice (Al-Quraishy et al., 2014).

ACUTE PHASE RESPONSE

An APR is experimentally inducible by blood-stage malaria, without any preceding pre-erythrocytic development of parasites in the liver, in humans and model systems. When in human volunteers an APR is induced by injecting *P. falciparum*-infected RBCs, serum levels of α1-acid glycoprotein (AGP) peak within 1 week (Klainer et al., 1968). AGP inhibits *in vitro* growth of *P. falciparum* by more than 80% at a concentration of 2.5–3.0 mg/ml, which resembles those levels reached during a typical APR in humans (Friedman, 1983). Other APPs such as α1-antitrypsin, transferrin or α2-macroglobulin have been reported to be not as effective against *P. falciparum in vitro* (Friedman, 1983). Patients suffering from malaria reveal elevated serum CRP which binds to the patients' RBCs. These in turn loose their normal discoid-shape, increase membrane fluidity and hydrophobicity and decrease their effective complement-regulatory proteins (Ansar et al., 2009). In mice, blood-stage infections of both self-healing *P. chabaudi* and *P. vinckei* as well as lethal *P. berghei* induce an APR characterized by increased serum levels of the major APP SAP (Balmer et al., 2000). In accordance, the mRNA levels of *Saa 1–3* in the liver increased by 65- to 80-fold at peak parasitemia of *P. chabaudi* infections (Krücken et al., 2005). By contrast, a reduced APR in association with a significant extension of primary parasitemia is induced in IL-6-deficient mice in response to blood-stage infections. This suggests that the APR induced by blood-stage infections contributes to an antiparasitic and/or immunomodulatory immune response (Balmer et al., 2000).

It is remarkable, however, that negative APPs, as, e.g., haptoglobin, PAI-1, and AP with potentially efficient anti-malaria activity, are decreased during the APR against blood-stage malaria. For instance, haptoglobin is malaria-protective, since it is toxic to *P. falciparum*-infected RBCs. Its main function is to bind hemoglobin released from erythrocytes and to support its elimination, thereby also preventing oxidative stress in the blood (Imrie et al., 2004). Hypohaptoglobinemia has been introduced as a biochemical and epidemiological marker of *P. falciparum* malaria (Mohapatra et al., 1999). In experimental *P. chabaudi* malaria, the *Pai-1* gene is downregulated in the liver. Since *Pai-1*-deficient mice cannot control parasitemia as good as wild type mice, PAI-1 has been suggested to be required for parasitemia control (Krücken et al., 2005). AP is also reduced at peak parasitemia during self-healing *P. chabaudi* infections (Wunderlich et al., 2005). AP exerts anti-inflammatory effects, as it is capable of dephosphorylating potentially deleterious molecules and of reducing inflammation and coagulopathy systemically (Pike et al., 2013). Obviously, the blood stages of the malaria parasites benefit from the down-regulation of negative APPs during an APR.

INFLAMMATORY RESPONSE

Course and outcome of both human and experimental blood-stage malaria critically depend on a finely tuned balance between both pro-inflammatory cytokines, as, e.g., IL-1β, TNFα, IFNγ, IL-6, and IL-12, and anti-inflammatory cytokines such as IL-4 and IL-10 (Bakir et al., 2011; Perkins et al., 2011).

In different experimental models, the liver has been shown to produce different cytokines in response to primary blood-stage malaria. However, there are differences between lethal and self-healing infections. In lethal *P. chabaudi* malaria, there occurs biphasic, two-wavy increase in serum IL-1β, TNFα, and IL-6 with the first wave peaking on day 1 *p.i.* and the second wave peaking at maximal parasitemia on day 8 *p.i.* (Wunderlich et al., 2012). In parallel, the liver biphasically produces mRNAs coding for IL-1β, TNFα, and IL-6 (Krücken et al., 2009). By contrast, the hepatic production of IFNγ mRNA reveals a lower and more uniform increase during precrisis. This pattern of mRNA production is totally reverted after protective vaccination of mice. Then, the liver produces a pronounced biphasic increase of IFNγ mRNA at higher levels, while the mRNA levels of IL-1β, TNFα, and IL-6 are lower and the biphasic production pattern has largely disappeared (Krücken et al., 2009). This indicates that protection against blood-stage malaria is associated with low production of IL-1β, TNFα, IL-6 and a high biphasic production IFNγ by the liver. Incidentally, a two-wavy production pattern, as well as its disappearance after protective vaccination, can be also found for the mRNA production of inducible nitric oxide synthase (iNOS), arginase, and different nuclear receptors including pregnane X receptor in the liver (Krücken et al., 2009).

In mice infected with a non-lethal strain of *P. yoelii*, the liver has been also shown to produce mRNAs of both pro-inflammatory cytokines, i.e., IFNγ, IL-12 p40, IL-6, and TNFα, and anti-inflammatory cytokines such as IL-4 and IL-10 (Bakir et al., 2011). Both pro- and anti-inflammatory cytokines are highly expressed during precrisis of blood-stage infections. It has been suggested that the pro-inflammatory cytokines induce the immune system to take action, while the anti-inflammatory cytokines counteract the high levels of inflammatory cytokines (Bakir et al., 2011).

The liver-produced cytokines are not only important for the local response in the liver, but also have presumably an impact for the systemic response to blood-stage malaria. For instance, a rather complex and dynamic interplay has been evidenced for IL-6. This pleiotropic cytokine is produced not only by HCs, but also by KCs and other liver cells. The severity of blood-stage malaria correlates with circulating IL-6 levels. Increased levels have been described in patients suffering from malaria caused by *P. falciparum* and *P. vivax* (Kern et al., 1989; Jason et al., 2001; Lyke et al., 2004; Robinson et al., 2009), often associated with polyclonal B cell activation (Donati et al., 2004). Conversely, decreasing IL-6 levels are reported to be associated with decreasing parasitemia (Sarthou et al., 1997) and hyperpyrexia (Seoh et al., 2003), as well as after anti-malarial treatment (Hugosson et al., 2006). In experimental murine malaria, it has been shown that IL-6 trans-signaling, rather than classic IL-6 signaling, contributes to malaria-induced lethality (Wunderlich et al., 2012). Indeed, approximately 50% of IL-6Rα-deficient mice survive an otherwise lethal *P. chabaudi* blood-stage malaria. However, the lethal outcome is restored, when IL-6 trans-signaling is induced by injecting mouse recombinant sIL-6Ra in the IL-6Rα-deficient

mice. By contrast, inhibition of IL-6 trans-signaling through injecting recombinant sGP130Fc protein in wild type mice results in 40% survival (Wunderlich et al., 2012).

PHAGOCYTIC ACTIVITY

Intraerythrocytic stages of *Plasmodium* produce and store hemozoin in their phagosomes to detoxify the lytic ferriprotoporphyrin IX released during digestion of host hemoglobin by parasites (Olivier et al., 2014). Since ferriprotoporphyrin IX is also lytic for host cells including human RBCs (Orjih et al., 1988), the hemozoin is therefore cleared from the circulation as potentially dangerous waste material by phagocytosis of KCs to protect host cells.

On the other hand, KCs are also known to be endowed with the capacity to phagocytose and to remove senescent and aberrant erythrocytes including pRBCs from circulation (Otogawa et al., 2007; Lee et al., 2011) mediated by specific SRs (Terpstra and van Berkel, 2000). It has been therefore suspected that at least part of the intracellularly accumulated hemozoin in KCs is derived from internalized and decayed pRBCs. This view is supported by several findings. In rats with a high load of *P. berghei* blood-stage infections, KCs exhibit a markedly increased phagocytic activity, presumably due to an upregulation of scavenger activity for erythrophagocytosis (Nobes et al., 2002). Moreover, it has been shown in *P. berghei*-infected rats that the number of KCs is significantly increased and the phagocytic activity of KCs on a cell-by-cell basis is enhanced (Murthi et al., 2006). Furthermore, at peak parasitemia of *P. chabaudi*-infected mice, KCs are characterized by hyperplasia, and the massively enlarged KCs take on a "foamy" appearance detectable by light microscopy (Delic et al., 2010). These KCs contain, besides hemozoin, also regularly pRBCs inside the area of sectioned KCs thus supporting the view that KCs are indeed able to erythrophagocytose pRBCs and to destroy them in their phagosomes *in vivo*.

Several studies in non-KC macrophages have shown that ingestion of hemozoin by macrophages severely impairs macrophage activities upon diverse stimulations. For instance, hemozoin dramatically reduces the production of IL-6, but increases production of TNFα (Prada et al., 1995) and inhibits activities of NADPH-oxidase activity (Schwarzer and Arese, 1996) and protein kinase C (Schwarzer et al., 1993). Moreover, the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is inhibited (Schwarzer et al., 1993; Prada et al., 1996) and the expression of MHC II molecules, CD54 and CD11c is impaired (Schwarzer et al., 1998). However, hemozoin does not specifically affect expression of MHC I molecules, CD16 (low affinity Fc receptor for aggregated IgG), CD64 (high affinity receptor for IgG), CD11b (receptor for complement receptor iC3b), CD35 (receptor for complement components C3b and C4b), and CD36 (non-class-A SR; Schwarzer et al., 1998; Olivier et al., 2014). More recent data indicate that hemozoin can induce the production of IL-1β through the NOD-like containing pyrin 3 domain (NLRP3) inflammasome complex (Olivier et al., 2014). These data suggest that also *in vivo* activities of KCs may be negatively affected by ingested hemozoin. Indirect evidence for this view is provided by a magnetometric and electron microscopal study showing that ingestion and metabolism of *P. chabaudi*-infected RBCs by KCs *in vivo* decrease intracellular organelle motion in these KCs, which probably compromises host defense reactions (Bellows et al., 2011).

"PROTECTIVE" AUTOIMMUNITY

In humans, acquisition of natural immunity against blood-stage malaria is associated with the emergence of autoimmunity (Butcher, 2008). A number of studies with murine blood-stage malaria have substantiated the view that autoimmunity arises during blood-stage malaria and that the liver-inherent immune system is critically involved in the generation of this "anti-malaria" autoimmunity. This is presumably not mediated by conventional T and B cells, but rather by special populations of hepatic T and B cells of innate immunity (Abo et al., 2012). For instance, *P. yoelii* infections have been reported to induce severe thymic atrophy in mice, which ultimately arrests production of conventional T cells (Abo et al., 2012). Concomitantly, however, extrathymic IL-2Rb^{+} CD3^{int} T cells and its subset NK1.1^{-} CD3^{int} expand in the liver. Moreover, NK1.1^{-} TCR^{int} cells are present in the liver of athymic nude mice, which harbor neither conventional T cells nor NKT cells of thymic origin. When lymphocytes are isolated from the liver of athymic mice recovered from blood-stage malaria of non-lethal *P. yoelii*, these "immune" lymphocytes can confer protection from malaria to both irradiated euthymic and athymic mice (Mannoor et al., 2002). Furthermore, autoreactive extrathymic T cells have been described in the liver of malaria-infected mice, which react with both HCs and RBCs of malaria-infected mice (Abo et al., 2012).

In parallel with the expansion of extrathymic autoreactive T cells, the number of B220^{low} B-1 cells is also increased in the liver and is highest around peak parasitemia in mice infected with non-lethal *P. yoelii* (Kanda et al., 2010; Abo et al., 2012). These B-1 cells produce autoantibodies against nucleus, ss- and dsDNA (Kanda et al., 2014). In accordance, serum levels of both IgM- and IgG-type autoantibodies against denatured DNA increased during non-lethal *P. yoelii* infections in mice, both peaking around peak parasitemia (Bakir et al., 2011). These immune responses resemble those found in autoimmune diseases (Bakir et al., 2011). *In vivo* depletion of autoreactive B-1 cells and subsequent infection with normally self-healing *P. yoelii* result in death of 56% of mice (Mannoor et al., 2013). By contrast, adoptive transfer of such auto-antibody secreting B cells prior to infecting with *P. yoelii* leads to later appearance and inhibition of parasitemia (Mannoor et al., 2013). Auto-antibodies secreting B220^{low} B-1 cells have been suggested to target "abnormal self" cells, as, e.g., HCs and RBCs, in malaria-infected mice, and such autoantibody-labeled cells can be further processed and eliminated by macrophages and KCs (Kanda et al., 2014).

Previous authors have already suggested that autoimmunity plays a key regulatory role in protection from malaria (Jayawardena et al., 1979; Jarra, 1980). In accordance, it has been recently found that autoantibodies and sera from patients suffering from diverse autoimmune conditions, but not from malaria, inhibit the *in vitro* growth of *P. falciparum* in human RBCs (Bhatnagar et al., 2011; Brahimi et al., 2011). Especially that type of autoimmunity is apparently required for protection against blood-stage malaria (Daniel-Ribeiro, 2000), that is presumably directed

against autoantigens and parasite-induced neo-autoantigens on the surface of *Plasmodium*-infected RBCs (Wunderlich et al., 1988b,c; Fontaine et al., 2012). It is therefore not surprising that surface membranes of *Plasmodium*-infected RBCs can be used for protective vaccination of malaria-susceptible mice, which raises their survival from 0 to more than 80% and decreases peak parasitemia by about 30% (Wunderlich et al., 1988a; Krücken et al., 2009).

Collectively, the currently available information supports the view that the generation of autoimmunity and/or the enhancement of already existing low level "natural" autoimmunity in the liver may be an integrative—if not the essential—part of protective immunity against blood-stage malaria (Abo et al., 2012). Malaria-activated TLR8, possibly in cross-talk with TLR7, has been speculated to be involved in the generation and/or surveillance of this "protective" autoimmunity (Al-Quraishy et al., 2014).

LIVER INJURIES AND REPAIR

Blood-stage malaria causes severe injuries of the liver. Humans infected with *P. falciparum* or *P. vivax* have been described to suffer from hepatic dysfunctions and malarial hepatitis with jaundice and raised liver enzyme levels in the blood (Ananad et al., 1992; Kochar et al., 2003; Nautiyal et al., 2005). Liver injuries have been also found *postmortem* in fatal human malaria, as, e.g., KC hyperplasia, hemozoin accumulation within KCs, liver-necrosis, portal inflammation, steatosis, and cholestasis (Rupani and Amarapurkar, 2009). Similar histopathological injuries have been also observed in the liver of mice primarily infected with lethal *P. chabaudi* at peak parasitemia, besides increased serum levels of hepatic markers such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), bile acids, and bilirubin (Krücken et al., 2005; Delic et al., 2010). However, similar changes, though not as pronounced, also occur in the liver of mice when *P. chabaudi* infections take a self-healing course (Krücken et al., 2005; Delic et al., 2010).

Liver injuries are presumably not directly induced by the parasites, but largely indirectly as infection-induced overreactions of the liver-inherent immune system, as, e.g., increased oxidative stress induced by ROS/RNS produced during phagocytic activity of KCs, increased production of proinflammatory cytokines, increased release of perforin, granzymes, and lysosomal enzymes by LGL, autoreactive cytotoxic T cells, and "bystander killing" of liver cells due to cell-mediated cytotoxicity. For instance, hepatic CD1d-independent $DX5^+$ T cells have been shown to cause "bystander killing" in the liver of *P. berghei*-infected mice (Adachi et al., 2004). Hepatotoxicity can be also induced by hepatic CD1d-restricted NKT cells activated during blood-stage malaria through IL-12 secreted from KCs (Gonzalez-Aseguinolaza et al., 2000). Damages of liver cells have been suggested to be surveilled by DAMPs-activated TLRs (Adachi et al., 2001).

Even an apparent "non-immune" mechanism generating liver injury is very likely immune-mediated, as, for example, the almost doubling of the ammonia content in the blood plasma found at peak parasitemia during lethal *P. chabaudi* malaria (Delic et al., 2010). The increased systemic ammonia level signalizes severe local hepatic dysfunctions. Indeed, the liver metabolism is impaired, in particular the detoxifying capacity of the liver, evidenced as massive down-regulations of genes encoding enzymes involved in phase I–III metabolism (Krücken et al., 2005; Delic et al., 2010). However, similar changes in liver metabolism can be experimentally induced in mice only by injections of TNFα and IL-1β without any malaria infection (Kim et al., 2004, 2007). Increases in systemic ammonia levels presumably also cause dysfunctions of other organs. For instance, the brain is particularly sensitive to ammonia intoxication, which ultimately leads to hepatoencephalopathy (Butterworth, 2002; Häussinger and Schliess, 2008), that has been indeed reported to occur as a severe complication in *P. falciparum*-infected patients (Kochar et al., 2003; Whitten et al., 2011).

However, the liver also possess the capability for fast regeneration of destructed liver tissue. This capability also involves T cell-mediated repair mechanisms of the liver-inherent immune system. For instance, *P. chabaudi* blood-stage infections in mice have been described to induce an increase of liver-resident IL-17- and IL-22-producing $CD8^+$ T cells (Mastelic et al., 2012). In contrast to IL-17 producing T cells, the IL-22 producing T cells very likely protect from the potentially lethal effects of liver damages during primary *P. chabaudi* infections. Further work is required to disentangle those host mechanisms in the liver, which mediate repair and healing of liver tissue injured during blood-stage malaria, and those, which contribute to liver dysfunctions and ultimately to multiple organ failure as the reason for malaria mortality (Delic et al., 2010).

CONCLUDING REMARKS

The importance of the liver-inherent immune system as part of the host defense against blood-stage malaria is increasingly recognized, though still too hesitatingly. The liver-inherent immune system is able to develop several different effector responses against malarial blood stages. Development and efficacy of these blood-stage effectors may be modulated by those immune responses which are previously elicited against the pre-erythrocytic liver stages of the malaria parasites (Klotz and Frevert, 2008) and, conversely, the latter may be even boosted by the blood-stage effectors (Ocana-Morgner et al., 2003; Lau et al., 2014).

Overreactions of the effector responses directed against blood-stage malaria presumably lead to severe inflammatory liver injuries having systemic impact. Such overreactions are puzzling since the liver-inherent immune system is also known for its capability to generate tolerance, a state of immune hyporesponsiveness. The "tolerogenic" milieu of the liver may be considered to be beneficial for blood-stage parasites of different *Plasmodium* species including *P. falciparum* and *P. chabaudi* to escape anti-malaria effectors by intrahepatic sequestration (Medeiros et al., 2013; Brugat et al., 2014). Though intrahepatic sequestration mediated by CD36 has been reported to be fundamentally important for parasite growth *in vivo* (Fonager et al., 2012), parasite sequestration also occurs in other "non-tolerogenic" organs.

It is also puzzling how a tolerogenic hyporesponsive milieu as in the liver allows the generation of specific autoimmunity at all, that is presumably even an integrative—if not the essential—part of protective immunity against blood-stage malaria. This

autoimmunity is mediated by autoreactive extrathymic T cells and autoantibody-producing B-1 cells. One target of autoimmunity is the RBC, the host cell of the malaria blood stages. Remarkably, surface membranes of *Plasmodium*-infected RBCs, but not those of non-infected RBCs, can be used for protective vaccination of mice (Wunderlich et al., 1988a; Krücken et al., 2009). This raises survival of malaria-susceptible mice from 0 to more than 80%, decreases parasitemia and concomitantly attenuates overreactions of hepatic inflammation induced by blood-stage malaria of *P. chabaudi*. This vaccination model appears rather convenient to trace those immune and possible autoimmune mechanisms, which have to be activated and/or suppressed in the liver to generate efficient protection against blood-stage malaria. Such knowledge in turn will advance our understanding of how these mechanisms can be utilized for the design of an effective human anti-malaria vaccine.

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Priming of CD8$^+$ T cell responses to liver stage malaria parasite antigens

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While the role of malaria parasite-specific memory CD8$^+$ T cells in the control of exo-erythrocytic stages of malaria infection is well documented and generally accepted, a debate is still ongoing regarding both the identity of the anatomic site where the activation of naive pathogen-specific T cells is taking place and contribution of different antigen-presenting cells (APCs) into this process. Whereas some studies infer a role of professional APCs present in the lymph nodes draining the site of parasite injection by the mosquito, others argue in favor of the liver as a primary organ and hepatocytes as stimulators of naïve parasite-specific T cell responses. This review aims to critically analyze the current knowledge and outline new lines of research necessary to understand the induction of protective cellular immunity against the malaria parasite.

Keywords: *Plasmodium*, malaria, liver stage antigens, antigen presentation to T cells, dendritic and liver cells as APC

INTRODUCTION

It is currently accepted that priming of CD8$^+$ T lymphocytes by antigen-presenting cells (APCs) takes place in the secondary lymphoid organs such as spleen and lymph nodes [reviewed in Ref. (1)]. Multi-photon-based intravital microscopy revealed that the first contact between naïve CD8$^+$ T cells and APC takes place in the periphery of draining lymph nodes (DLN) shortly after infection and mainly occurs in the subcapsular sinus or the interfollicular regions enriched with pathogen-derived antigens (2, 3). Depending on the pathogen's nature, the rapid relocation of naïve T cells to the periphery of the draining lymph node can be either antigen-specific (2) or antigen-independent, associated with decreased local levels of chemokines and the drastic alteration of the lymph node architecture by the pathogen (3, 4). Data from mice infected with vesicular stomatitis virus demonstrated that, though CD169+ macrophages (5) residing in the subcapsular sinus were the major cell population bearing virus-derived antigens (2), dendritic cells (DCs) served as the primary APC triggering antigen-specific naïve CD8$^+$ T cells. The ability of immature dendritic cells to acquire exogenous antigens followed by their proteolytic processing and presentation on the MHC class I molecules, commonly referred to as "cross-presentation," is believed to be the major requirement for the generation of primary antigen-specific CD8$^+$ T cell responses against pathogens (6–9).

Upon the initial encounter of naïve T cells with APC, a heterogeneous progeny of antigen-specific CD8$^+$ T cells including short-lived effector cells (SLEC) and memory precursor effector cells (MPEC) [reviewed in Ref. (10, 11)] is generated. It is still not clear whether the SLEC versus MPEC differentiation is enforced by the asymmetric segregation of transcription factors and protein degradation machinery already at the first cell division (12–14) or it reflects the differential exposure to inflammatory and co-stimulatory "help" signals received from APCs by antigen-specific CD8$^+$ T cells during the expansion phase [reviewed in Ref. (10, 11)]. While generation of primary CD8$^+$ T cell responses to non-inflammatory antigens requires CD4$^+$ T cell help, induction of primary CD8$^+$ T cells responses to *Listeria*, LCMV, and influenza virus is CD4$^+$ T cell-independent and results from direct activation of APCs by the pathogen (15–17). Moreover, CD4$^+$ T cell help can be replaced by the CD40 triggering on the DCs, which prime antigen-specific naïve CD8$^+$ T cells (18, 19). Thus, the exact nature and requirements for "help" signals necessary for the initial triggering and subsequent expansion of primary antigen-specific CD8$^+$ T cell responses vary among different pathogens and sites of primary infection. In this report, our objective is to present and discuss the published data regarding CD8$^+$ T cell activation in *Plasmodium* infection, and suggest experiments to better understand the antigen presentation process.

Malaria infection is initiated through the bites by *Plasmodium*-carrying female Anopheles searching for blood to support egg development. As the mosquito probes the host environment under the skin for the presence of blood vessels, it injects salivary gland proteins both prior and during blood feeding to inhibit blood coagulation. Parasites deposited into the skin can also traverse surrounding cells and enter the circulation with subsequent infection of liver cells. Studies performed with parasites injected intradermally or intravenously show that the resulting liver parasite load is similar (20). In addition, transfer of parasites from the skin sites to DLN occurs (21).

Identification of the anatomical site and the type of APC, which orchestrate the induction of primary CD8$^+$ T cell responses against a particular antigen, represents an essential step in rational design of CD8$^+$ T cell-based vaccination strategies. Whereas the research on the effector phase of CD8$^+$ T cell response against malaria has been quite extensive (22–25), a rather limited number

of studies attempted to dissect the issue of liver stage-specific $CD8^+$ T cell priming in the infected host.

ROLE OF DIFFERENT ORGANS IN ANTIGEN PRESENTATION

In this respect, the study by Chakravarty et al. (21) appears to be one of the most comprehensive and systematic up to date. The authors concluded that extrahepatic lymphoid tissues, in particular the DLN and spleen are the most important sites contributing to the generation of the effector T-cell pool in the liver. In agreement with these data, Obeid and colleagues demonstrated that strictly subcutaneous immunization with irradiated sporozoites led to induction of sterile immunity against pre-erythrocytic malaria with T cell priming occurring in skin-draining lymph node (26). It was proposed that parasite-specific $CD8^+$ T cell priming depends on cross-presentation of malaria antigens (21). This indicates that professional APC, rather than infected hepatocytes, trigger priming of naïve $CD8^+$ T cells directed to liver stage antigens.

Several lines of experimental evidence were presented in support of these conclusions. Thus, IFNγ production by adoptively transferred circumsporozoite protein (CSP)-specific naïve transgenic T cells was first detected in the skin-DLN as early as on day 2 after mouse immunization by microinjection or mosquito bites, whereas no detectable T cell activation was detected in other organs including spleen. Hence, Chakravarty and co-authors suggested that these temporal differences in the onset of parasite-specific T cell activation could reflect the hierarchical order of T cell priming initiated in the DLN that could be followed by migration of primed $CD8^+$ T cells to other organs, including the spleen and the liver. However, removal of lymph nodes draining the site of parasite injection prior to the adoptive transfer of parasite-specific $CD8^+$ T cells, though resulted in a 60% reduction of activated $CD8^+$ T cells in the liver, did not affect the frequencies of primed $CD8^+$ T cells in the spleen where the first signs of T cells activation were documented only 24 h later than in DLN and at the same time point as in the liver. These data indicate that temporal differences in the onset of T cell activation used as a parameter for identification of the $CD8^+$ T cell priming site should be carefully reconsidered in future studies and further strengthen the importance of the spleen as a site of induction of primary $CD8^+$ T cell responses in animal models of the infection. The latter is in agreement with the data by Sano et al. (27) demonstrating that spleens of infected mice support priming of parasite-specific naïve $CD8^+$ T cells following intravenous injection of sporozoites.

At the same time, several lines of evidence presented by Chakravarty and colleagues (21) do not firmly support the essential role of the spleen in the parasite-specific $CD8^+$ T cell priming.

First, DCs isolated from the spleens 60 h after injection of sporozoites were unable to trigger proliferation of parasite-specific $CD8^+$ T cells, whereas DCs isolated from the DLN efficiently induced T cell proliferation and, presumably, presented the antigen. Since no data with liver-resident DCs were generated, a direct role of intrahepatic professional APCs in priming of parasite-specific $CD8^+$ T cells still needs to be addressed. In addition, as the first signs of activation of parasite-specific T cells in DLN were detected at day 2 post immunization, it is not completely clear whether DCs from DLN had a greater capacity to prime $CD8^+$ T cells as compared to spleen and liver-resident DCs at time points earlier than 60 h.

Second, animals subjected to simultaneous lymphadenectomy and splenectomy prior to the adoptive transfer of CSP-specific $CD8^+$ T cells followed by immunization with sporozoites and subsequent challenge with viable parasites 10 days later had similar load of parasites in the liver as non-immunized mice, indicating that either DLN or/and spleen are required for CSP-specific $CD8^+$ T cell priming. At the same time, splenectomy alone did not affect inhibition of parasite development in the liver, prompting the authors to conclude, that DLNs are the priming site of protective $CD8^+$ T cell responses.

Interestingly, as shown by Chakravarty and co-authors (21), removal of both DLNs and the spleen prior to immunization with sporozoites, though drastically reduced the activated T cell pool in the liver, failed to abrogate it completely, suggesting that at least a proportion of parasite-specific $CD8^+$ T cells found in the liver had been primed outside the DLN and the spleen. These findings could reflect the process of parasite-specific $CD8^+$ T cell triggering in the liver and define it as the organ essential for the parasite development. On the other hand, animals treated with FTY720, a drug, which inhibits lymphocyte egress from lymph nodes (28, 29), had substantially less IFN gamma producing parasite-specific $CD8^+$ T cells at day 7 post injection with irradiated sporozoites. Based on this observation, the authors concluded that systemic distribution of $CD8^+$ T cells, at least in part, contributes to the intrahepatic pool of parasite-specific $CD8^+$ T cells (21). It still needs to be seen, if treatment with FTY720 (30) inhibits the development of "early-primed" parasite-specific $CD8^+$ T cells in the liver and spleen, previously noted by the authors already 72 h after mosquito bite. In addition, effect of FTY720 on the protection of animals from subsequent challenge with live sporozoites has to be addressed in this model. Noteworthy, the time course of the parasite-specific clonal T cell activation in the lymph nodes, liver, and other organs is only slightly delayed (by 24 h) while it is known that activated T cells egress from the lymph nodes 4–5 days after antigen encounter (31, 32). The latter suggests that either activation of parasite-specific T cells may take place simultaneously in various organs, or unusually rapid egress from the lymph node after priming is an intrinsic feature of T cells in this specific experimental model.

ROLE OF INFECTED HEPATOCYTES IN ANTIGEN PRESENTATION

The role of infected hepatocytes in direct priming of naïve parasite-specific $CD8^+$ T cells is still a subject of controversy. Early study by Renia et al. demonstrated that intrasplenic injection of infected hepatocytes induced protective T cell-mediated immunity against infection with *Plasmodium yoelii* and *P. berghei* sporozoites (33). Leiriao et al. demonstrated that apoptotic hepatocytes infected with irradiated sporozoites are phagocytosed by DCs and merely serve as a source of *Plasmodium* antigens for the initiation of the protective immune responses via cross-priming (34). In contrast, Renia and collaborators argued against apoptotic infected hepatocytes as a source of antigens and suggested that liver DCs could be activated upon uptake of parasite antigens directly from

viable infected hepatocytes (35) as previously seen in other experimental models (36, 37). However, data from Chakravarty et al. implied that though cross-priming is required, it takes place in the DLNs and not in the liver (21). In agreement with these data, Jung et al. demonstrated that mice subjected to chemical depletion of $CD11c^+$ DCs fail to induce $CD8^+$ T cell responses to infection with *Plasmodium yoelii* (38). Neither of these studies considered hepatocytes as an APC subset capable of initiating the primary parasite-specific T cell responses.

A recent study by Balam et al. (39) focused on two questions: can infected hepatocytes directly prime naïve parasite-specific T cells and does stimulation of already primed $CD8^+$ T cells protect mice against parasite challenge? Administration of $CD8^+$ CSP-specific T cells but not an irrelevant T cell clone injected into TAP-deficient MHC class I mismatched recipient mice, simultaneously with infected hepatocytes bearing MHC haplotype relevant for parasite-specific T cells, resulted in 100% protection of mice from subsequent challenge with live sporozoites (39). As the observed protection was not due to a bystander effect or a continuous cytokine secretion by parasite-specific $CD8^+$ T cells, these data demonstrate that infected hepatocytes are capable of presenting the antigen to $CD8^+$ T cells, reactivating resting CSP-specific $CD8^+$ T cells and inducing protection.

Importantly, more than 60% of naïve BALB/c mice injected with irradiated sporozoite-infected hepatocytes were also protected from subsequent live parasite challenge, suggesting that infected hepatocytes could contribute to the priming of endogenous naïve T cell. However, T cell depletion experiments are required to confirm that protection is T cell-mediated. Finally, to formally exclude contamination with other APC potentially present in the hepatocyte preparations and capable of presenting CSP and priming the naïve $CD8^+$ T cells, isolation of pure hepatocyte population devoid of cells bearing markers of DCs, macrophages, and stellate cells should be done by flow cytometry using fluorescent transgenic parasites. On the other hand, arguing against the sole role of professional APC in priming of naïve immune responses to malaria parasites, mice depleted of DCs by treatment with cytochrome *c* were still protected from the challenge with live sporozoites in spite of significantly lower frequencies of endogenous parasite-specific T cells primed by the immunization with irradiated sporozoites (39). These data do not fully support the previously discussed role of dendritic cell function in induction of primary malaria liver stage-specific T cell responses (21, 38).

OTHER CONSIDERATIONS

The quality of hepatocytes as APCs capable of triggering T cells responses had been recently dissected by Ma et al. (40). It had been demonstrated that *P. berghei* and *P. falciparum* infected human hepatocytes retain largely unaltered expression of multiple molecules of the MHC class I pathway until very late stages of parasite development (40). Moreover, infected cells exhibited no obvious defects in the capacity to upregulate expression of different molecular components of the MHC class I machinery in response to pro-inflammatory lymphokines or trigger direct activation of allo-specific as well as peptide-specific human $CD8^+$ T cells (40). At the same time, it is not known whether or not the characteristic features of professional APC believed to be important for efficient T-cell priming, i.e., co-stimulatory molecules B7.1 and B7.2 ("signal 2"), as well as production of cytokines essential for the survival and maintenance of primed T cells ("signal 3") are possessed by the primary human hepatocytes *in vivo* and/or induced upon infection.

Current literature dissecting the ability of primary hepatocytes to specifically prime naïve $CD8^+$ T cells is scarce. Bertolino et al. demonstrated that purified primary murine hepatocytes were able to induce activation and proliferation of antigen-specific naive $CD8^+$ T cells *in vitro*, even in the absence of exogenously added cytokines as well as CD80 and CD86 co-stimulatory molecules (41). Moreover, the magnitude of T cell proliferation induced by primary hepatocytes was comparable to that induced by DCs. Naïve T cell priming by hepatocytes did not require $CD4^+$ T cell help and induced expression of early T cell activation markers and transient $CD8^+$ T cell effector activity followed by rapid cell death of activated T cells. Thus, primary hepatocytes were able to prime naïve T cells but failed to sustain productive antigen-specific $CD8^+$ T cell responses (41). In agreement with these data, *in vivo* experiments using endogenous expression of alloantigens under hepatocyte-specific promoters demonstrated that activation of primary T cells by hepatocytes as antigen-presenting cells leads to T cell apoptosis rather than formation of antigen-specific memory T cell pool (42–44). It was further demonstrated that T cells activated by hepatocytes died "by neglect" and lack of IL-2 and low expression of pro-survival genes due to insufficient co-stimulation during the priming phase (45). Hence, taking into account the inability of primary hepatocytes to provide appropriate co-stimulation during T cells priming along with the immunosuppressive microenvironment created by multiple subsets of the liver-resident APC [reviewed in Ref. (46, 47)], it may appear unlikely that hepatocytes infected with malaria parasites play a major role in the generation of effective parasite-specific $CD8^+$ T cell memory responses. However, it does not preclude the possibility that $CD8^+$ T cells specific to malaria antigens could be primed and activated, at least shortly, by hepatocytes supporting development of exo-erythrocytic forms. Indeed, given proper stimuli, such T cells can be rescued to full immunological competence and longer survival (48, 49). In the case of malaria, proper activation stimuli could be induced by *Plasmodium* infection leading to activation of numerous genes in hepatocytes (50, 51) including those involved in native immunity and antigen presentation. Since no transcriptional analysis has been performed in Kupffer cells traversed by sporozoites so far, it would be important to understand whether or not liver-resident macrophages change their immunomodulatory properties in the site of malaria infection.

At this point, a word of caution should be expressed to the fact that all animal studies discussed above were based on a single mouse strain, BALB/c, as well as a single $CD8^+$ T cell epitope derived from the CSP. Future studies on the induction of primary T cell responses to exo-erythrocytic forms of malaria need to be extended to other protective $CD8^+$ T cell epitopes including responses, which appear later in the liver stage by using either radiation attenuated (RAS) or genetically attenuated (GAS) sporozoites or sporozoites combined with chloroquine chemoprophylaxis (CPS) (52).

FIELD STUDIES

It is still unclear to what extend animal models dissecting induction of primary T cell responses to malaria as well as human studies involving vaccinated volunteers reflect the acquisition of natural cellular immune responses in malaria endemic areas. An acquisition of a sterile immune protection following immunization with RAS, GAS, or using CPS regime in animals and humans sharply contrasts with the situation in the field, where, in spite of frequent (up to 30 per month in certain areas) biting by infected mosquitoes (53, 54), no sterile protection is usually obtained in both adults and children in response to natural infection under drug treatment or intermittent preventive treatment (IPT). Several hypotheses could be proposed to explain this discrepancy: (1) sporozoite "charge" (and, as a result, supply of parasite antigens) is too small in the field as compared to that given under experimental conditions; (2) down-regulation of the parasite-specific $CD8^+$ T cell responses by the content of mosquito salivary glands delivered together with sporozoites to the site of T cell priming; and (3) excessive and/or preceding induction of immune responses to salivary gland proteins. As for the latter, given the fact that only a fraction (0–25% depending on the seasons and location) of mosquitoes are infected (54–56), a memory T-cell pool specific for salivary gland antigens is most likely established prior to parasite infection. As a result, secondary T cell responses directed to mosquito antigens could be preferentially activated at the expense of parasite-specific T-cell activation via, for example, competition for IL-2, homeostatic niche, or by active secretion of inhibitory molecules (57–64). If this is the case, the efficacy of sporozoite-based pre-erythrocytic vaccines may turn out to be low in endemic areas due to even subtle contamination with the salivary gland proteins. Both the second and third hypotheses may explain the low frequency of parasite-specific helper and $CD8^+$ T cell found in humans from malaria endemic regions, as well as the general failure [with the exception for a single donor so far (65)] to obtain stable human T-cell clones specific to malaria liver stage antigens.

FINAL REMARKS

In conclusion, existing experimental data obtained from animal models suggest that: (1) both DCs and hepatocytes can prime naïve malaria parasite-specific $CD8^+$ T cells, at least those directed to epitopes derived from CSP and (2) either DCs or hepatocytes are sufficient to induce protective CSP-specific T cell responses if the parasite load is not excessive. Identification of the essential site for priming of malaria liver stage-directed $CD8^+$ T cell responses of broader antigen specificity as well as mimicking the conditions of the natural exposure to the uninfected mosquito vector will pave the way for the optimal design of T cell-based vaccines. We hope that experimental approaches suggested above in the context of the reviewed original data (21, 27, 34, 39) will prompt further studies on the induction and maintenance of protective T cell responses against exo-erythrocytic stages of malaria infection.

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Novel approaches to identify protective malaria vaccine candidates

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Efforts to develop vaccines against malaria have been the focus of substantial research activities for decades. Several categories of candidate vaccines are currently being developed for protection against malaria, based on antigens corresponding to the pre-erythrocytic, blood stage, or sexual stages of the parasite. Long lasting sterile protection from *Plasmodium falciparum* sporozoite challenge has been observed in human following vaccination with whole parasite formulations, clearly demonstrating that a protective immune response targeting predominantly the pre-erythrocytic stages can develop against malaria. However, most of vaccine candidates currently being investigated, which are mostly subunits vaccines, have not been able to induce substantial (>50%) protection thus far. This is due to the fact that the antigens responsible for protection against the different parasite stages are still yet to be known and relevant correlates of protection have remained elusive. For a vaccine to be developed in a timely manner, novel approaches are required. In this article, we review the novel approaches that have been developed to identify the antigens for the development of an effective malaria vaccine.

Keywords: malaria, vaccine, library, antibodies, screen

INTRODUCTION

Malaria is an infectious disease caused by the protozoan parasite *Plasmodium* and transmitted by the *Anopheles* mosquitoes. Malaria is a major public health problem, leading to high mortality and morbidity. Nearly half the world's population is at risk of contracting malaria (CDC, 2012). There are 207 million cases of clinical malaria and approximately 627,000 deaths in WHO (2012). There is currently no available vaccine. Age and host immune status are high risk factors for malaria, with young children under the age of five, pregnant women and travelers or migrants who lack immunity to the disease being most susceptible. Other risk factors include the infectivity and the transmission dynamics of the parasite strain (Doolan, 2011).

The *Plasmodium* parasite has a complex life cycle. Following an infected mosquito bite, sporozoites are inoculated into the dermis of the mammalian host (Vanderberg and Frevert, 2004; Amino et al., 2006). The sporozoites travel to the liver *via* the bloodstream and infect the hepatocytes (Amino et al., 2006). During this phase in the hepatocytes, sporozoites develop into schizonts over 2–14 days, depending on the species. Merosomes, merozoites containing vesicles, eventually bud out from infected hepatocytes to release merozoites, which then infect erythrocytes (Sturm et al., 2006; Baer et al., 2007). Some of the blood stage parasites undergo sexual differentiation into male and female gametocytes that can be taken up by a feeding Anopheline during a blood meal. Ookinetes, which results from gametocyte fusion, develop into oocysts in the midgut of the mosquito. Upon oocyst maturation, newly formed sporozoites migrate to the salivary gland of the mosquito, awaiting the next blood meal (Moorthy et al., 2004).

Symptoms of malaria include fever, headache, chills, sweating, and vomiting. Recurrent fever is one of the hallmarks of clinical malaria. This is a consequence of the release of malarial toxins into the bloodstream following repetitive rupture and re-invasion of erythrocytes. With disease progression, the red blood cell counts decreases and severe anemia might occur. Malarial infected red blood cells, such as those of *Plasmodium falciparum*, can also sequester in deep tissues, causing cerebral malaria, and organ failure. These severe pathologies can eventually lead to death.

IMMUNE RESPONSES TO A MALARIA INFECTION

Protective immunity against malaria requires a timely and coordinated interplay between the innate and adaptive immunity. This involves dendritic cells, NK cells, B cells, $CD4^+$ and $CD8^+$ T cells (Stevenson and Riley, 2004).

Sporozoite-specific antibodies can block sporozoites from migrating to the liver or from invading into hepatocytes, arresting disease progression (Rathore et al., 2005; Finney et al., 2014). Antibody-mediated immunity has been thought to be the central effectors of parasite clearance in the peripheral blood as MHC class I/II molecules are absent on the surface of infected red blood cells (Langhorne et al., 2008). The importance of antibodies was first demonstrated by Cohen et al. (1961), showing that passive transfer of immunoglobulins from immune adults into naïve, infected children resulted in rapid reductions of parasite density and resolution of clinical symptoms (Cohen et al., 1961). Merozoite-specific antibodies can prevent merozoites from invading erythrocytes (Michon et al., 2000; Dutta et al., 2005; Jiang et al., 2011) and mediate clearance of infected red blood cells by phagocytic cells *via* antibody-dependent cellular inhibition (Marsh and

Kinyanjui, 2006). Pathogen-specific antibodies secreted by B cells with $CD4^+$ T helper cells enhancement are essential for clearance of parasitemia in the later stages of the infection (Langhorne et al., 2008).

In addition to the humoral arm of the adaptive immunity, cell-mediated immune responses are also crucial for protection against malaria. $CD8^+$ and $CD4^+$ T cells kill infected hepatocytes through diverse mechanisms (Renia et al., 1993; Doolan and Hoffman, 2000; Frevert et al., 2009; Trimnell et al., 2009; Cockburn et al., 2013) and induce sterile protection (i.e., no blood stage infection) in mouse models. Recent work has revealed an important role for IFNγ-secreting $CD8^+$ T cells in preventing chronic *P. chabaudi* blood stage infection in mice (Horne-Debets et al., 2013). In human, sterile protection has been observed in experimental sporozoite challenge experiments following vaccination with whole sporozoites (Hoffman et al., 2002; Roestenberg et al., 2009; Seder et al., 2013). Both sporozoite-specific antibodies and T cells were induced.

VACCINE DEVELOPMENT AGAINST MALARIA

The rationale for vaccine development to protect against malaria stems from observations where naturally acquired immunity to malaria can protect individuals living in malaria-endemic regions against malaria in an age-dependent and exposure-dependent manner (Gupta et al., 1999; Schofield and Mueller, 2006; Crompton et al., 2010). Although the protection is not sterilizing and is not always ensured for all chronically exposed individuals, passive transfer of sera from some chronically exposed individuals reduced strongly parasite levels in infected individuals (Cohen et al., 1961; Bouharoun-Tayoun et al., 1995). This demonstrated that antibodies can offer protection against the blood phase of the malarial infection.

More rationally, the typical Pasteur approach where attenuated parasites were used as vaccines has further demonstrated the feasibility of vaccination as protection against malaria. Immunization with irradiation-attenuated sporozoites has shown to confer sterile immunity against sporozoites challenge in animal models and in humans (Richards, 1966; Nussenzweig et al., 1967; Clyde et al., 1973; Rieckmann et al., 1974; Hoffman et al., 2002). The protective immune response involving antibodies and T cells was shown to target the pre-erythrocytic stages (Nussenzweig et al., 1967; Overstreet et al., 2008). In recent years, immunization with sporozoite or blood parasite under drug cover was also shown to confer strong protective immunity involving antibodies and T cells (Belnoue et al., 2002; Renia et al., 2006; Roestenberg et al., 2009; Friesen et al., 2010). Other approaches have used genetically attenuated parasites. Although these approaches have led to sterile immunity in mice (Mueller et al., 2005; Butler et al., 2012), they have yet to prove their efficacy in humans (Spring et al., 2013).

After decades of efforts spent on developing vaccines against malaria, no strong vaccine candidate has emerged as yet. Most vaccine development efforts are focused on only four antigens (WHO, 2012). The most clinically advanced candidate, RTS,S, conferred ∼50% protection from clinical *P. falciparum* malaria in children aged 5–17 months, and ∼30% protection in children aged 6–12 weeks (Agnandji et al., 2011, 2012). Vaccine efficacy was undetectable 3 years after vaccination (Bejon et al., 2013). This level of protection is suboptimal and too low to achieve malaria eradication. No single molecular signature, key cellular determinant or immune mechanism of naturally acquired or vaccine-induced immunity has been unequivocally associated with protection (Offeddu et al., 2012). This impediment toward vaccine development for malaria is multi-factorial. The *Plasmodium* parasite has a diverse protein repertoire, and a complex life cycle involving both vertebrate and invertebrate hosts. There are antigenic polymorphisms and variations across parasite strains and species, and the parasite has developed sophisticated strategies to evade the host immunity.

With more reports of drug resistance and insecticide resistance in some endemic regions (Trape et al., 2011; Phyo et al., 2012; Ashley et al., 2014), management of the malarial disease has been increasingly difficult. Identification of new malarial antigens for vaccine development is critical. Here, we reviewed the different techniques that have been used to identify antigens recognized by antibodies induced during natural infections or after vaccinations with whole parasites.

PRE-GENOMIC SCREENING METHODS FOR PROTECTIVE ANTIGENS

The advent of new molecular biology techniques in the 1980s led to the discovery of many *Plasmodium* antigens. DNA libraries, consisting of cDNA or genomic DNA, were constructed in bacteriophage vectors for expression in *Escherichia coli* (Kemp et al., 1986). Antigens such as the circumsporozoite protein (CSP), the S-antigen, the ring erythrocyte surface antigen (RESA) were the first to be identified using mouse monoclonal antibodies (Ellis et al., 1983), sera from infected humans (Kemp et al., 1983; Stahl et al., 1984) or sera from infected mice or monkeys (Anders et al., 1984; Brown et al., 1984; Ardeshir et al., 1985). All the antigens discovered using these approaches were immunodominant or possessed immunodominant regions made of repeats (Kemp et al., 1987). Although immunodominant antigens induce very strong antibody responses, recent studies have shown that they did not offer adequate protection when tested as subunit vaccines in clinical trials (Spring et al., 2009; Doolan, 2011; Sheehy et al., 2012), suggesting that these antigens might be used by the *Plasmodium* parasite for immune evasion (Anders, 1986; Rodriguez et al., 2008). The CSP, an antigen of pre-erythrocytic stage malaria and the first malaria antigen to be cloned (Ellis et al., 1983), is one such example. Despite being immunodominant, the Phase III vaccine efficacy was suboptimal, with 55% reduction in the frequency of malaria episodes during the 12 months of follow-up in children 5–17 months of age and 35% in children 6–12 weeks old at first immunization (Agnandji et al., 2011). The protection waned to a mere 16.8% after 4 years of follow-up, indicating the lack of long lasting protection which declined with time and exposure to the parasite (Olotu et al., 2013). Similarly, while immunization with the apical membrane antigen-1 (AMA-1), an antigen expressed both at the pre-erythrocytic and erythrocytic stages and strongly immunodominant during the erythrocytic stage, elicited functional antibodies with *in vitro* inhibition against homologous blood parasites, volunteers developed parasitemia upon challenge with controlled human malaria infection (infection by mosquito

bites) without a significant reduction of peak parasitemia nor delay to patency (Spring et al., 2009).

The cDNA library approach is advantageous with a good representation of a stage-specific *Plasmodium* proteome, depending on the stage of the parasite at which the RNA was extracted from. However, the repertoire of the *Plasmodium* proteome the cDNA library represents is not entirely comprehensive and does not include antigens expressed by the parasite in the other stages of its life cycle. Nevertheless, it has successfully identified more than a hundred malaria antigens. Recently, Raj et al. (2014) created a *P. falciparum* blood stage cDNA library constructed in bacteriophage and expressed in *E. coli*. The antigen library was screened against sera from either infection-resistant or – susceptible chronically exposed individuals living in malaria-endemic regions. 2 out of 3 antigens identified were novel antigens. Antibody to one of the identified novel antigens, PfSEA-1, inhibited parasite growth and blocked schizont egress. Using the *P. berghei* ANKA strain ortholog of PfSEA-1, the authors demonstrated reduced parasitemia and longer survival in mice vaccinated with PbSEA-1. They further validated PfSEA-1 as a promising vaccine candidate through epidemiological studies, where anti-PfSEA-1 antibodies were strongly associated with lesser incidence of severe malaria in Tanzanian children and lower level of parasitemia in Kenyan adults.

SCREENING METHODS TO IDENTIFY NEW ANTIGENS IN THE ERA FOLLOWING GENOME SEQUENCING

At the turn of the 20th century, whole genomes of many *Plasmodium* strains were sequenced. These genome sequencing initiatives, by The Wellcome Trust Sanger Institute and The Institute for Genomic Research, have provided a wealth of data for the identification of protective antigens (Carlton et al., 2002; Gardner et al., 2002). In this review, we discuss the novel approaches (summarized in **Table 1**) to screen libraries of malarial antigen using sera from protected versus unprotected individuals to identify antigens associated with protection, in search for new protective antigens for vaccine development against malaria.

NEW APPROACHES TO SEARCH FOR ANTIGENS INVOLVED IN PROTECTION

The availability of whole genome sequences has enhanced the understanding of the parasite biology. Protein functions can be predicted and validated. Potential antigens could be screened for specific characteristics such as surface expression. Comparative genomics between different parasite strains allows identification of antigens that have limited variation, with the promise of greater vaccine coverage and hence efficacy in the field (Kooij et al., 2005).

Transcriptomics and proteomics has provided critical information on the expression profiles of malarial proteins during the parasite's life cycle (Florens et al., 2002; Le Roch et al., 2002; Bozdech et al., 2003; Hall et al., 2005) – the mosquito stage (Lindner et al., 2013), the liver stage (Tarun et al., 2008), and the sexual stage (Lasonder et al., 2002; Khan et al., 2005). *In silico* analysis of stage-specific transcription pattern identifies antigens that are differentially expressed for specific targeting to a specific stage of the parasite's life cycle and also antigens with conserved expression across the different stages for cross-stages targeting (Florens et al., 2002).

SCREENING RECOMBINANT PROTEIN MICROARRAYS AGAINST IMMUNE SERA FROM VACCINATED HUMAN INDIVIDUALS

Using the published *P. falciparum* and *P. vivax* genomes, a set of selected genes was targeted for protein expression using an *in vitro* transcription/translation system (Doolan et al., 2008; Tsuboi et al., 2008; Crompton et al., 2010; Barry et al., 2011; Trieu et al., 2011; Molina et al., 2012; Baum et al., 2013; Lu et al., 2014). These recombinant proteins were then printed onto microarray chips and probed with immune sera that were obtained from human volunteers naturally exposed to malaria infections or immunized with radiation-attenuated sporozoites. Doolan et al. (2008) generated a protein microarray with 250 *P. falciparum* proteins and used it to profile antibody responses in sera from four groups of individuals: (1) protected, (2) non-protected individuals following vaccination with radiation-attenuated sporozoites, (3) partially protected individuals due to natural exposure, and (4) non-exposed individuals. The same group then expanded their library to include 1204 *P. falciparum* proteins (representing 23% of the *P. falciparum* genome; Crompton et al., 2010; Trieu et al., 2011; Baum et al., 2013) and also 91 *P. vivax* proteins (Molina et al., 2012). 22–29% of the screened proteins were found to be serodominant in immune sera, demonstrating a broad and varied response to many antigens. Antibody titres against current vaccine candidates such as CSP, atypical membrane antigen-1, liver stage antigen-3, merozoite surface protein-1 did not differ between immune and non-immune individuals (Crompton et al., 2010; Trieu et al., 2011).

Richards et al. (2013) expressed *P. falciparum* merozoite proteins, reported to have a role in erythrocyte invasion and/or localized on the merozoite surface or in the invasion organelles of the merozoites, in either bacterial or wheat germ cell-free expression systems. The recombinant proteins were tested for IgG immunoreactivity using sera from malaria-exposed Papua New Guinea children in ELISA. Forty six proteins were selected based on their ability to coat ELISA plates and their immunoreactivity. Sera from older children had higher immunoreactivity to most of the 46 proteins as compared to the younger children, indicating an acquisition of antibody responses with age, presumably due to prolonged exposure to the parasite. While merozoite surface proteins had higher seropositivity compared to the rhoptry and micronemal proteins, the antigens strongly correlated with protection were the rhoptry and micronemal proteins. This is consistent with other studies, suggesting that the key protective malarial antigens are the non-immunodominant antigens (Doolan et al., 2003, 2008; Crompton et al., 2010; Trieu et al., 2011; Baum et al., 2013). The authors proposed that a combinational vaccine consisting of non-immunodominant antigens, such as EBA, PfRh2 and PfRh4, is more likely to offer greater protection (Richards et al., 2013).

One of the main drawbacks with protein array is the extensive efforts needed to generate the soluble recombinant malarial antigens in the library (Doolan et al., 2008; Tsuboi et al., 2008). Cell-based expression systems have met with many difficulties. *P. falciparum* genes have a high A/T content and a substantial number

Table 1 | Approaches taken to identify protective malarial antigens for vaccine development.

	Type of antigen library	Antigen expression system	Size of library	Type of screening	Sera/antigens that library is screened against	Reference
Pre-genomic era	cDNA	*E. coli* cell-based		*In vitro* immunoscreen	Mouse monoclonal antibodies	Ellis et al. (1983)
	cDNA	*E. coli* cell-based	10 000 clones	*In vitro* immunoscreen	Sera from infected human individuals	Kemp et al. (1983), Brown et al. (1984), Stahl et al. (1984)
	cDNA	*E. coli* cell-based	10 000 clones 10 000 clones 9 000 clones	*In vitro* immunoscreen	Sera from infected mice, mice and rabbits, monkeys	Brown et al. (1984) Anders et al. (1984) Ardeshir et al. (1985)
Post-genomic era	cDNA	*E. coli* cell-based	1 250 000 clones	*In vitro* immunoscreen	Sera from infection-resistant and –susceptible chronically exposed human individuals	Raj et al. (2014)
	Recombinant proteins	*E. coli* cell-free *in vitro* translation	250 antigens	*In vitro* immunoscreen	Sera from protected and non-protected individuals following vaccination with radiation-attenuated sporozoites	Doolan et al. (2008)
	Recombinant proteins	*E. coli* cell-based and wheat germ cell-free system	46 antigens	*In vitro* immunoscreen	Sera from malaria-exposed children	Richards et al. (2013)
	Antigens expressed on cell surface	Mammalian cell-based	80 antigens	*In vitro* immunoscreen	Sera from protected and non-protected individuals following vaccination with live sporozoites	Chia et al. (2014)
	Whole parasite lysates	*P. yoelii* parasite	Whole proteome	*In vitro* immunoscreen	Affinity-purified IgG from immune mice that naturally survived a lethal infection	Kamali et al. (2012)
	DNA (exons)	–	19 genes	*In vivo* screen in mice for protection	–	Haddad et al. (2004)
	Recombinant proteins	Mammalian cell-based	51 antigens	*In vitro* protein–protein interaction screen	Malarial antigen, PfRh5	Crosnier et al. (2011)

of the genes encode stretches of repeat sequences (Gardner et al., 2002), hindering protein expression in cell-based expression system. Only 30% out of 1000 genes investigated by Mehlin et al. (2006) can be expressed in *E. coli* and a mere 6.3% of the proteins are soluble. The alternative is the wheat germ cell-free expression system. Tsuboi et al. (2008) were able to express 93 out of 124 *P. falciparum* genes as soluble proteins using the wheat germ cell-free system. Rui et al. (2011) reported greater immunogenicity of wheat germ proteins (as opposed to identical proteins produced in *E. coli*) in mice. The wheat germ cell-free expression system could be more suitable for producing antigens for vaccine development.

SCREENING CELL-ASSOCIATED ANTIGEN LIBRARIES AGAINST IMMUNE MOUSE SERA

We recently cloned a library of 80 malarial antigens into a mammalian surface expression vector pDisplay (Invitrogen; Chia et al., 2014). They transfected these expression vectors into mammalian

cells and the antigens were expressed on the cell surface, creating a library of antigen-presenting cells. The library was screened against immune and non-immune sera obtained from mice immunized with live sporozoites or blood parasites under drug cover (Belnoue et al., 2008). Similarly, all immunized volunteers in the study were protected from sporozoites challenge. The antibody repertoire of immunized volunteers was extremely broad and varied. MAEBL was found to be strongly associated with protection. MAEBL has been implicated in invasion into hepatocytes by sporozoites and merozoites into erythrocytes (Kappe et al., 1998, 2001). It was previously demonstrated that anti-MAEBL antibodies to inhibit *P. yoelii* sporozoites invasion *in vitro* in primary mouse hepatocytes, suggesting that MAEBL is a promising attractive vaccine candidate (Singh et al., 2004).

This approach removes the difficulty of expressing and purifying antigens as soluble recombinant proteins. However, the library size in this study represents a small representation of the entire *Plasmodium* genome (∼1%) and antigens screened were mainly restricted to those with reasonably good transfection efficiency.

SCREENING WHOLE PARASITE LYSATES AGAINST AFFINITY-PURIFIED IgG FROM NATURALLY SURVIVING MICE

Due to ethical and technical restrictions, identification of protective immune correlates in humans can only be evaluated in the peripheral blood component. This hinders the study of a vital stage of the parasite's life cycle, the liver stage. It also limits the examination of the host immune responses in the secondary lymphoid organs such as the spleen, which are also important sites for the induction of protective immunity.

The use of comparative genomic analysis, through the construction of genome-wide synteny maps, has identified other non-human models of malaria (Carlton et al., 2002; Tachibana et al., 2012). Mouse models have played a vital role in the understanding of the immunobiology of malarial infections. The mouse malaria parasite, *P. yoelii*, has been used as a model for malaria research due to the substantial similarity to the human parasite *P. falciparum* (Hall et al., 2002). In addition to being cheaper to maintain and easier to handle, the mouse also has a well characterized immune system, hence offering great advantages over non-human primate models (Wykes and Good, 2009; Taylor-Robinson, 2010). Infection studies of rodent malaria species and their hosts have provided information on parasite biology and pathogenicity. Identification of orthologs allows preclinical validation of new chemotherapies and vaccine candidates. The lethal *P. yoelii* model, which causes death in BALB/c mice, is an excellent model for investigating the vaccine efficacy of the candidates *in vivo* (Langhorne, 1994). Kamali et al. (2012) observed that 20% of ICR mice infected with the lethal strain of *P. yoelii* cleared the infection and these naturally surviving mice had boosted immunity following a second challenge. They affinity-purified the IgG from these mice and probed against whole parasite lysates. Using MALDI-TOF analysis, the antigenic specificities of the protective IgG were Heat shock protein 70 (HSP70), protein disulphide isomerase, plasmepsin and a 39 kDa subunit of eukaryotic translation initiation factor 3. Although the authors did not validate the protective potential of the identified antigens, the approach used in the study identified novel antigens (with the exception of PfHSP70.1 (Haddad et al., 2004). Antibodies against PfHSP70.1 have been shown to eliminate *in vitro* liver stage parasites through antibody-dependent cell-mediated cytotoxic mechanisms (Renia et al., 1990).

SCREENING FOR PROTECTIVE DNA VACCINE CANDIDATE IN MOUSE CHALLENGE MODEL

New molecular technologies such as Gateway cloning make it easy to clone large numbers of genes into plasmids. Using this technology, Haddad et al. (2004) produced an expression library of 182 *Plasmodium* exons coding for pre-erythrocytic antigens in the mammalian immunization vector VR1012. They immunized mice with 19 out of the 182 cloned vectors, either singly or in combination, and assessed vaccine efficacy following sporozoites challenge by examining the ability of the immunized mice to reduce liver stage parasite load. The most promising DNA vaccine candidate identified was Py01316, annotated as a Qa-SNARE protein in PlasmoDB, which gave a 68–79% reduction in parasite load.

The approach taken by Haddad et al. (2004) has the benefit of generating a library of DNA vaccine candidates, targeting many *Plasmodium* genes with great ease, and does not require the laborious process of generating recombinant proteins. It also provides information on the immunogenicity of the DNA constructs and *in vivo* effectiveness of the induced immune responses to reduce parasite load. However, screening for protective DNA vaccine targets in mice is laborious and requires a large number of mice. As a result, the authors screened a total of 19 out of the 182 cloned plasmids, which limited the number of malarial antigens that can be validated for protective efficacy.

TARGETED SCREENING TO IDENTIFY MALARIAL ANTIGENS AND THEIR RECEPTORS

Crosnier et al. (2011) expressed a library of erythrocyte surface proteins with the mammalian expression system. Using the avidity-based extracellular interaction screen (AVEXIS), they screened recombinant PfRh5 protein (the bait protein), which is critical for erythrocyte invasion by merozoites, against the library of erythrocyte surface proteins (the prey protein). The PfRh5 protein was found to interact with only one erythrocyte surface protein, basin. The anti-basin monoclonal antibodies were highly effective at blocking *P. falciparum* invasion into erythrocytes *ex vivo* and these blocking effects were efficacious with 15 other culture-adapted and field strains of *P. falciparum*. These data have implications for novel therapeutics. While this does not directly identify new protective malarial antigens for vaccine development, the identification of basin as the binding partner of PfRh5 and perhaps further characterisation studies on the expression levels of basin by the target population would provide critical information on the vaccine efficacy of PfRH5, should PfRh5 be developed as a vaccine candidate. Understanding how basigin binds to PfRh5 could also facilitate rational design of drug compounds to block *P. falciparum*'s invasion into erythrocytes.

One of the shortcomings of AVEXIS is the generation of the library of recombinant proteins, which is laborious. This approach also requires the prior identification of a known bait protein, which is difficult for identifying novel host–parasite interacting antigen

pairs. However, the AVEXIS assay is advantageous in its ability to detect direct low-affinity protein interactions, which might not be detectable in other screening methods. More recently, this type of library has shown to be a powerful tool to identify new protective antigens for potential vaccine target development. Using sera from a longitudinal study in a cohort of Kenyan children, Osier et al. (2014) identified 10 antigens (PF3D7_1136200, MSP2, RhopH3, P41, MSP11, MSP3, PF3D7_0606800, AMA1, Pf113, and MSRP1) that were associated with protection against clinical episodes of malaria. While the AVEXIS approach has been used to identify blood stage antigens in this study, the study could be extended to include antigens for other stages.

IDENTIFICATION OF PROTECTIVE PRE-ERYTHROCYTIC ANTIGENS

Pre-erythrocytic antigens have been attractive targets for vaccine development. This is mainly due to the demonstration that sterile immunity against malaria is achievable in experimental sporozoite challenge experiments in humans following vaccination with whole sporozoites (Hoffman et al., 2002; Roestenberg et al., 2009; Seder et al., 2013).

Most of the above described approaches for identification of protective antigens have used sera from chronically exposed individuals living in endemic regions to screen for protective malarial antigens. Although the approaches have identified mainly blood stage antigens, some of the identified blood stage antigens, such as AMA1 (Silvie et al., 2004), TRAP (Robson et al., 1995), EBA175 (Gruner et al., 2001b), PfEMP3 (Gruner et al., 2001a), and HSP70.1 (Renia et al., 1991), which are also expressed during the liver stage.

To screen for protective pre-erythrocytic antigens, it is essential to have the appropriate sera sets against which the library of malarial antigens will be screened. While sera from chronically exposed individuals living in endemic regions are relatively easier to obtain, they are more likely to have an antibody repertoire that is predominantly specific for blood stage antigens, hence might not be suitable for the identification of liver stage antigens. Marchand and Druilhe (1990) and Gruner et al. (2003) were the first to show in human that chloroquine prophylaxis protected against blood stage infection and use the protected sera to screen for protective antigens. This led to the identification of liver-specific (LSA1; Guerin-Marchand et al., 1987) or cross stage-specific antigens stages (LSA-3, STARP, and SALSA; Fidock et al., 1994; Bottius et al., 1996; Daubersies et al., 2000). Recently, the studies by Trieu et al. (2011) and Chia et al. (2014) involved the use of sera from animals immunized with sporozoites. Trieu et al. (2011) identified 16 previously uncharacterized pre-erythrocytic antigens (Trieu et al., 2011), while the pre-erythrocytic MAEBL antigen was identified by Chia et al. (2014).

CONCLUSION

Historically, vaccine development efforts have been focused on immunodominant antigens as vaccine candidates such as merozoite or sporozoite surface proteins. However, the success has been limited thus far.

More recently, through the use of new technologies, immunoscreens have become more comprehensive, and has revealed the strong association of non-immunodominant malarial antigens with protection. Antigen libraries, expressed in bacterial, mammalian, or wheat germ cell-free expression systems, are created either as DNA libraries, recombinant proteins or on cell surface as antigen-presenting cells. In the screening of these antigen libraries for protective antigens, immune sera are an important tool. The type of immune sera chosen for the screening is critical. Carefully planned vaccination trials with an experimental challenge provide differential groups of sera from protected versus non-protected vaccinated individuals to identify protective antigens. Sera from chronically exposed individuals living in malaria-endemic regions have been also used in studies to screen for protective antigens. While sera from these individuals inform about naturally acquired immunity, it is essential to differentiate between the susceptible and the resistant individuals in these naturally exposed individuals. These differential sera allow the exclusion of immunodominant antigens, and inclusion of the non-immunodominant antigens that are associated with protection.

Taken together, the current consensus is protection against malaria is attributed to robust humoral responses directed against a panel of various non-variant antigens instead of only a single or a few immunodominant antigens. Studies to validate the feasibility of these minor non-immunodominant antigens as vaccine candidates should be prioritized for vaccine development against malarial infections. In addition, understanding antigen recognition is an essential step for the establishment of key immune correlates of protection against malarial infections, which would aid greatly in validating vaccine efficacy.

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APC licensing and CD4+ T cell help in liver-stage malaria

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Malaria parasites spend a critical phase of their life cycle inside hepatocytes, in an environment with complex and distinctive immunological features. Here I will discuss how the immunological features of the liver and the adaptations of malaria parasites interact, resulting in defective CD8+ T cell immunity. These processes are explored with a focus on the mechanism by which CD4+ T cells deliver help to CD8+ T cells, and specifically through their interaction with antigen-presenting cells (APCs), resulting in "licensing" of the APCs and enhanced capacity to optimally activate CD8+ T cells. Synthesis of the available evidence supports a model in which the parasite-mediated manipulation of programmed cell death in infected hepatocytes impairs the capacity of the liver's immune system to successfully license APCs and fully activate T cell immunity.

Keywords: liver, hepatocyte, malaria parasite, immunity, CD4+ T cell help, CD8+ T cell, antigen-presenting cells, licensing

INTRODUCTION

Malaria parasites are vulnerable to the mammalian immune system from the time they penetrate the skin as sporozoites, during the passage of these pre-hepatic forms through the blood to the liver, during their residence inside hepatocytes, and after their emergence, when they parasitize erythrocytes. In this review, I consider only immunity to the hepatocellular stage of the parasite. Liver-stage malaria parasites gain access to hepatocytes by crossing the liver sinusoidal endothelium, and both direct and indirect evidence suggests that they may exploit Kupffer cells, the liver's large resident macrophage population, as portals to gain access to the underlying Space of Disse, and thus to hepatocytes (Frevert et al., 2005; Baer et al., 2007).

Once inside the hepatocyte, the malaria parasite establishes a vacuole, the membrane of which contains both host-derived proteins, and proteins secreted by the parasite. This parasitophorous vacuole separates the parasite from the host cell cytoplasm, and acts as the interface through which the parasite subverts cellular processes to protect it from host defense and to provide a supply of nutrients. The parasite's interactions are highly adapted and successful. Thus, young children infected for the first time with malaria show little resistance, but over time and with repeated antigen exposure, immunity to the blood-stage gradually established sufficient control over parasite growth that the severity of clinical illness is reduced. However, it is not clear that repeated infection engenders any immunity against the liver-stage itself. Therefore, to study immunity to the liver-stage requires the use of animal models.

While natural immunity to the liver-stage is difficult to detect, artificial immunization protocols clearly generate such immunity, manifest both as reduced parasite load in the liver and as a delay, or the prevention of blood-stage infection. One approach is to infect a murine or a human subject with radiation-attenuated parasites (Hoffman et al., 2002). This approach results in sterilizing immunity in both species, and in mouse models a variety of host defenses have been implicated, including innate and antigen-specific immune cells (NK cells, CD4+ T cells, CD8+ T cells) and cytokines (IL-12, IFN-gamma; Doolan and Hoffman, 2000). The development of techniques to modify *Plasmodium spp.* genes has led to an alternative approach: the development of genetically attenuated parasites (GAPs). While human trials are only beginning (Spring et al., 2013), GAP can produce solid protection in mouse challenge models (Jobe et al., 2007; Trimnell et al., 2009; Vaughan et al., 2010). Such immunity correlates well with the capacity of T cells to secrete IFN-gamma, and with CD8+ T cells to kill parasitized hepatocytes *in vitro* (Trimnell et al., 2009; Epstein et al., 2011). In principle, a CD8+ T cell can manifest either or both of these effector functions *in vivo*; for example in viral hepatitis B, the major anti-viral effect appears to be due to IFN-gamma secretion, rather than cytotoxic killing (reviewed in Guidotti and Chisari, 2001). However, a recent *in vivo* study shows that GAP immunization, followed by boosting with a DNA vaccine, results in host defense that can destroy *in situ* hepatocytes that express malaria-encoded antigens (Chen et al., 2014). These data support the working hypothesis that the central effector mechanism against liver-stage malaria parasites is cytotoxic killing of the infected cells by CD8+ cytotoxic T lymphocytes (CTLs), rather than cytokine-driven intracellular cure of the infection.

THE IMPORTANCE OF HELP

The fundamental immunological problem in liver-stage malaria is to account for the failure of effective immunity during natural malaria infection, but the capacity of irradiated sporozoites and GAP to induce such immunity. The role of CD4+ T cells in such immunity is complex. Direct antibody-mediated depletion of CD4+ T cells at the time of challenge does not impair anti-malaria immunity to the liver-stage, implying that at the effector level, CD8+ CTL are sufficient (Tarun et al., 2007). Yet experiments in animals that congenitally lack CD4+ T cells are consistent with the concept that CD4+ T cells are required to prime effective

immunity (Overstreet et al., 2011). In terms of the biology of CTL maturation and memory, this makes complete sense. In host defense against intracellular bacteria, in anti-viral immunity, and particularly in CD8+ T cell responses against non-inflammatory antigens such as a minor histocompatibility antigens, CD4+ T cell help is essential, but the nature of the requirement for help depends on the nature of the antigen. Thus, minor histocompatibility antigens require CD4+ T cells to induce an effective CD8+ T cell response, while other antigens need CD4+ T cells to ensure either the long-term survival, or the memory effector function of CD8+ T cells (Prlic et al., 2007). There is a rough inverse correlation between the extent to which the antigen is accompanied by accessory signals, including pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), and the extent to which CD4+ T cell help is essential for a CD8+ T cell response. In viral hepatitis C, the main cause of immune failure and chronic infection appears to be the lack of a CD4+ T cell response, resulting in CD8+ T cells that become exhausted over time (Thimme et al., 2001; Grakoui et al., 2003; Radziewicz et al., 2007; Mueller et al., 2010). This same exhausted phenotype in CD8+ T cells is a major feature of immune failure in many potentially immunogenic cancers (Pardoll, 2012). These diverse diseases illustrate the central importance of CD4+ T cells in most CD8+ CTL responses; but their precise role in immunity to liver-stage malaria has yet to be defined.

As a complex parasite, *Plasmodium spp.* encode diverse non-mammalian proteins and synthesize many other non-mammalian molecules that could serve as PAMPs, but during the liver-stage these molecules could be sequestered inside the parasitophorous vacuole. However, this is clearly not the case for all CTL target antigens, since in the context of immunity induced by either irradiated sporozoites or GAP, infected hepatocytes can be killed. There is evidence that *Plasmodium spp.* may actively manage DAMPs, particularly those associated with cell death. Thus, infection of mouse hepatocytes with *Plasmodium yoelii* results in decreased p53, a mediator of G1 cell cycle checkpoint arrest, and a drug that sustained p53 expression reduced infection (Kaushansky et al., 2013a). The death of hepatocytes infected with wild-type (WT) parasites was driven by mitochondria and impaired by Bcl-2, while such control of host cell death appeared to be linked to the presence of a parasitophorous vacuole (Kaushansky et al., 2013b). Thus it seems that the parasite first delays the division of infected stressed hepatocytes, then inhibits their death, and finally allows them to die via the mitochondrial pathway of apoptosis. All of these maneuvers may sequester both DAMPS and PAMPs, eliminating pathways of immune activation.

Together with accessory signals from PAMPs and DAMPs, maturation of an effective CTL response with protective memory depends on CD4+ T cell help. In the case of malaria liver-stage antigens, the parasites reside in a cell type that expresses major histocompatibility complex (MHC) class I, but not MHC class II. Since CD4+ helper T cells are activated by antigen-presenting cells (APCs) that express MHC class II, it is clear that for CD4+ T helper cells to develop, malaria-encoded antigens must be presented by a different cell type, a mechanism termed cross-presentation; the T cell activation that follows is cross-priming (Bevan, 2006). Cross-presentation may occur by the uptake of cellular fragments or soluble proteins, or by the transfer of complete MHC-antigen complexes (termed: cross-dressing; Wakim and Bevan, 2011). In the liver, many cell types have the potential to take up antigen and engage in cross-presentation. Thus, there are liver-resident and circulating dendritic cells (DCs), Kupffer cells, liver sinusoidal endothelial cells (LSECs) and potentially also hepatic stellate cells (Ebrahimkhani et al., 2011). Among these, liver DCs and LSECs have the strongest credentials (Berg et al., 2006; Sumpter et al., 2007). Caveats concerning their role as APCs for liver-stage malaria antigens will be considered below.

LICENSED VERSUS UNLICENSED HELP

Once a CD4+ T helper cell is activated, help may be delivered to a CD8+ T cell by several known mechanisms (Lee et al., 2003). Licensing of the APC occurs when the CD4+ T cell recognizes antigen presented by an MHC class II+ APC, and delivers activating signals through co-stimulatory pathways, involving such receptors as CD80, CD86, and CD40 on the APC and their counter-receptors on the T cell. These signals activate the APC, resulting it its capacity to deliver enhanced signals to any CD8+ T cell that recognizes antigen on the same APC (Smith et al., 2004). This mechanism, though elegant and precise, requires that two relatively rare cells, the antigen-specific CD4+ T cell and the antigen-specific CD8+ T cell, should find the same APC, but it does not require that they should do so at the same time. While recent advances in *in vivo* imaging clearly reveal sustained contact between both CD4+ T cells and CD8+ T cells with DCs, clear evidence for simultaneous clusters of all three cells is not a striking feature of active immune responses in lymph nodes (Germain et al., 2012). Licensing also clearly requires that the APC express MHC class I and MHC class II, while hepatocytes do not. Abundant evidence suggests that hepatocyte can act as APC and induce primary activation in naïve CD8+ T cells, both *in vitro* and *in vivo* (Bertolino et al., 1998; Klein and Crispe, 2006), but the lack of MHC class II expression means that a hepatocyte is ***an APC that cannot be licensed***, even in principle. Therefore if anti-malaria CD8+ T cells were to be primed directly on hepatocytes, they could only receive help through an alternative mechanism.

Two known possibilities exist to bypass the need for licensing. First, and most simply, the CD4+ T cell may secrete growth factors such as Interleukin-2 (IL-2) that promote CD8+ T cell proliferation and maturation. This model is supported by experiments in which CD8+ T cells, selectively deprived of the ability to respond to IL-2, fail to undergo full differentiation (Williams et al., 2006). The alternative mechanism through which CD4+ T cells can deliver help to CD8+ T cells is through a direct interaction that depends on CD40 on the CD8+ T cell, and appears to be a direct T–T interaction involving on CD40L on the CD4+ T cell (Isogawa et al., 2013). This mechanism of help allows for the CD8+ and the CD4+ T cell to interact with different APCs, and so could overcome the limitations of a CTL target antigen that is not readily cross-presented.

To understand how fully effective, long-lived CD8+ T cells might arise in response to *Plasmodium*-infected hepatocytes, it is important to know whether these CD8+ T cells are primed directly on hepatocytes, or via cross-presentation of antigen

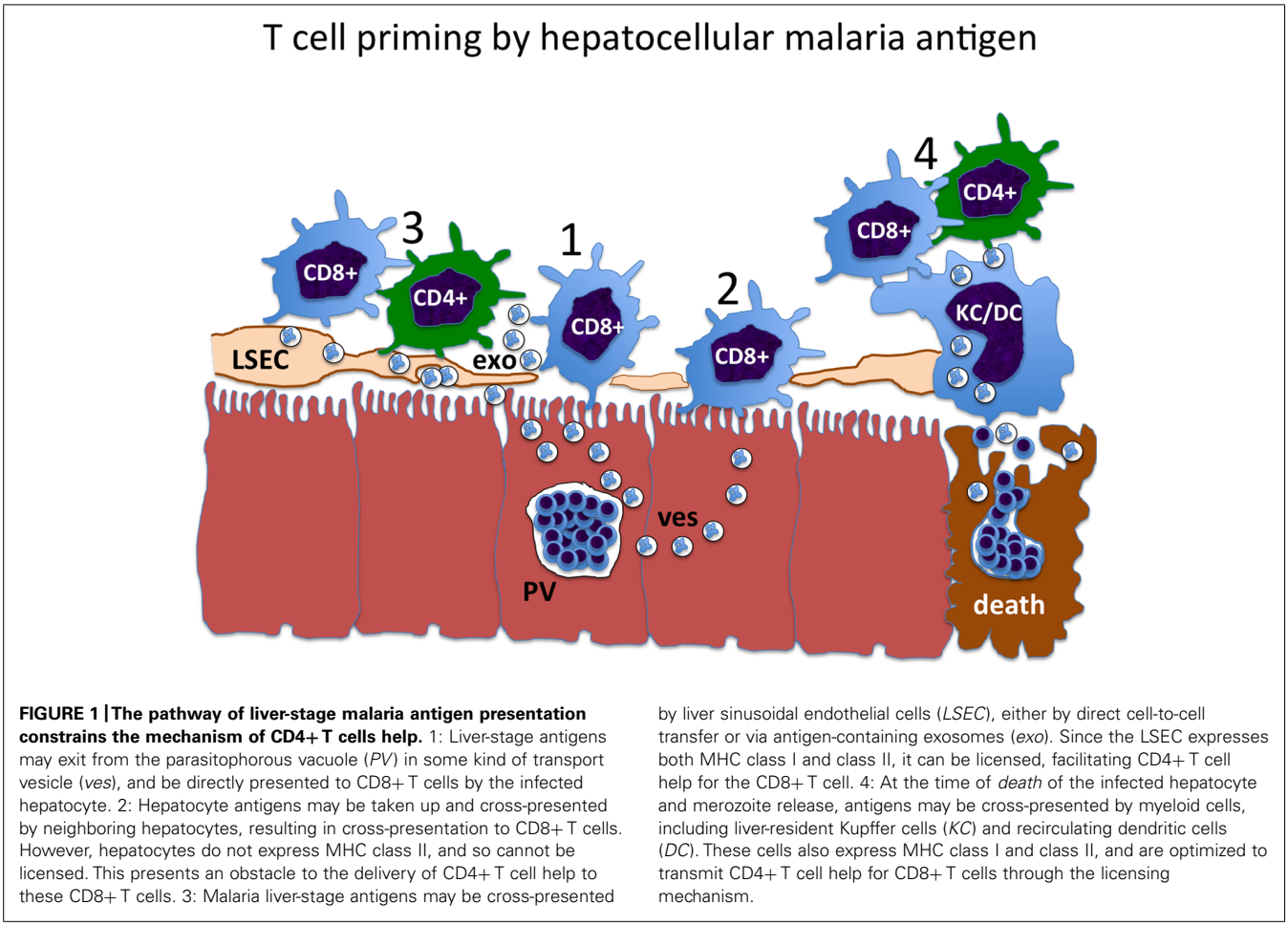

FIGURE 1 | The pathway of liver-stage malaria antigen presentation constrains the mechanism of CD4+ T cells help. 1: Liver-stage antigens may exit from the parasitophorous vacuole (*PV*) in some kind of transport vesicle (*ves*), and be directly presented to CD8+ T cells by the infected hepatocyte. 2: Hepatocyte antigens may be taken up and cross-presented by neighboring hepatocytes, resulting in cross-presentation to CD8+ T cells. However, hepatocytes do not express MHC class II, and so cannot be licensed. This presents an obstacle to the delivery of CD4+ T cell help to these CD8+ T cells. 3: Malaria liver-stage antigens may be cross-presented by liver sinusoidal endothelial cells (*LSEC*), either by direct cell-to-cell transfer or via antigen-containing exosomes (*exo*). Since the LSEC expresses both MHC class I and class II, it can be licensed, facilitating CD4+ T cell help for the CD8+ T cell. 4: At the time of *death* of the infected hepatocyte and merozoite release, antigens may be cross-presented by myeloid cells, including liver-resident Kupffer cells (*KC*) and recirculating dendritic cells (*DC*). These cells also express MHC class I and class II, and are optimized to transmit CD4+ T cell help for CD8+ T cells through the licensing mechanism.

by an APC that can be licensed. There is some circumstantial evidence that cross-presentation of hepatocellular antigens is constrained. First, in experimental delivery of antigen to hepatocytes using an adeno-associated virus (AAV) vector, the activation of antigen-specific CD8+ T cells was exclusively via non-bone marrow-derived APC, consistent with direct-priming (Wuensch et al., 2010). Second, in a liver transplant patient infected with Hepatitis C, new CTL appeared that were restricted to the human leukocyte antigen (HLA) of the liver donor, implying that they were primed on a solid tissue and not on bone marrow-derived DCs (Lauer, 2005). Third, when hepatocytes undergo apoptotic death, a major pathway of their disposal is phagocytosis by other hepatocytes (Dini et al., 1992; Dalton et al., 2003), which would keep any pathogen-encoded antigens within the hepatocyte pool. These considerations render it plausible that hepatocellular antigens are limited in their susceptibility to cross-presentation.

However, these experiments are subject to the caveat that LSECs are non-bone marrow-derived APCs that are not replaced with host-derived cells after a liver transplant. They are efficient in antigen uptake, and fully competent to cross-present both hepatocyte antigens and cancer cell-derived antigens *in vitro* (Berg et al., 2006; Ebrahimkhani et al., 2011). Furthermore these cells express both MHC class I and MHC class II, and so could potentially be licensed. *In vitro*, the activation of CD8+ T cells by LSECs typically leads to tolerance, but this could be in their un-licensed state; antigen recognition by a CD4+ T cell on an LSEC could result in licensing, with a changed outcome and complete activation when a CD8+ T cell interacts with the same LSEC. Kupffer cells occupy a rather similar position, since they are MHC class I+ II+ macrophages, and a subset of them termed sessile Kupffer cells are both radiation-resistant, and persistent after a liver transplant (Kennedy and Abkowitz, 1997; Klein et al., 2007). *In vitro*, Kupffer cell also induce abortive T cell activation leading to tolerance, but explicit experiments to license these cells via CD4+ T cell help, and then test their capacity to prime CD8+ T cells, have not been attempted.

THE ROOTS OF HELPLESSNESS

The mechanisms of direct versus cross-presentation, CD4+ T cell priming and the delivery of help via licensed APCs or otherwise collectively define the problem with respect to liver-stage malaria vaccine strategies (**Figure 1**). Any of the constraints that limit effective delivery of CD4+ T cell help to CD8+ T cells could apply in the context of natural malaria infection, but be circumvented by irradiated sporozoites or GAP vaccines.

First, viable malaria parasites could facilitate the death of the infected hepatocyte by a mechanism that favors the containment

of PAMPs and DAMPs. Either irradiated sporozoites, or GAP that act as live-attenuated vaccines, may lose this ability. This would have the effect of rendering malaria antigen-specific CD8+ T cells highly help-dependent due to the limited PAMP and DAMP signals. Second, the hepatocytes that are infected by wild-type parasites could undergo death in a manner that facilitates uptake by neighboring hepatocytes. Hepatocytes are surprisingly phagocytic. They can take up activated CD8+ T cells and destroy them in a mechanism of tolerance termed suicidal emperipolesis (Benseler et al., 2011); and they can take up apoptotic hepatocytes through a mechanism that depends on the asialo-glycoprotein receptor (McVicker et al., 2002; Dalton et al., 2003). Such uptake would tend to contain parasite-encoded antigens within an APC that can engage CD8+ T cells, but cannot be licensed, resulting in impaired susceptibility to CD4+ T cell help. It is then possible that irradiated sporozoites or GAP result in hepatocyte death from a different mechanism, such as pyroptosis or necroptosis, that facilitates cross-presentation by other cell types. So far these issues have been considered in cancer, where many anti-cancer drugs kill cancer cells by diverse mechanism that may influence antigen presentation (Guo et al., 2014); but the principle is likely to apply also to infectious disease, in particular malaria parasites in hepatocytes.

In addition to regulation at the levels of antigen recognition and APC licensing, T cell immune responses, including the responses to many hepatocellular pathogens, are regulated through multiple classes of inhibitors molecules, including co-inhibitory signals such as PD-L1 and Galectin-9 that interact with counter-receptors (PD-1, and Tim3 respectively) on the T cells; and immune suppressive small molecules such as kynurenine, the product of tryptophan breakdown by the enzyme indoleamine 2,3-dioxygenase (IDO), and prostaglandin-E2, which is made in immunosuppressive macrophages by an enzyme cascade involving cyclo-oxygenase type -2 (COX2; reviewed in Crispe, 2014). Several of these mechanisms have already been implicated in resistance to blood-stage malaria infection (Butler et al., 2012), but their significance in regulating immunity to the liver-stage had not been documented.

Such co-inhibitory signals should not be seen as an alternative explanation to the limitations placed on CD4+ T help by constraints of cross-presentation and APC licensing. One striking example comes from virus hepatitis research, where the "exhausted" state of anti-hepatitis B virus (HBV) CD8+ T cells could be reversed by providing CD40, a key component of the licensing mechanism (Isogawa et al., 2013). Typically, CD8+ T cells in chronic HBV and HCV display all the features of exhaustion, and in both cases there is a lack of CD4+ T cell activation. An inclusive view is that the display of PD-1, Tim3, and Lag3 on the exhausted CD8+ T cell may be a downstream consequence of the inability to deliver CD4+ T cell help. In malaria, as in other globally important liver pathogens, it will be most important to understand whether this results from a failure of APC licensing, or the failure of those mechanisms that have evolved as back-ups in case licensing fails.

CONCLUSION

Liver-stage malaria parasites inside hepatocytes occupy a cell type that can actively present antigen to MHC class I-restricted CD8+ T cells, but can neither directly activate CD4+ T cells, nor become licensed through interaction with CD4+ T cells. Since CD4+ T cell help is essential for full CD8+ T cell activation, function and protective memory, it is critical that both non-specific danger signals (DAMPs) and pathogen-associated innate immune activators (PAMPs) converge on an APC that can both cross-present antigen to CD4+ T cells, and be licensed to fully activate CD8+ T cells. In WT malaria infection, the parasite deftly subverts these mechanisms, in part through exerting control over the timing and mode of death of the infected cell. Successful experimental malaria vaccine strategies involve liver-attenuated parasites that furnish antigens, DAMPS and PAMPs, but fail to exert full control over the death of the infected cell. More detailed understanding of these mechanisms may lead to techniques to optimally deliver practical subunit vaccines, bringing CD4+ T cell help to anti-malaria CD8+ T cells and resulting in long-lived protective immunity at the liver-stage.

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Antibody and B cell responses to *Plasmodium* sporozoites

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Antibodies are capable of blocking infection of the liver by *Plasmodium* sporozoites. Accordingly the induction of anti-sporozoite antibodies is a major aim of various vaccine approaches to malaria. In recent years our knowledge of the specificity and quantities of antibodies required for protection has been greatly expanded by clinical trials of various whole sporozoite and subunit vaccines. Moreover, the development of humanized mouse models and transgenic parasites have also aided our ability to assess the specificity of antibodies and their ability to block infection. Nonetheless, considerable gaps remain in our knowledge – in particular in understanding what antigens are recognized by infection blocking antibodies and in knowing how we can induce robust, long-lived antibody responses. Maintaining high levels of circulating antibodies is likely to be of primary importance, as antibodies must block infection in the short time it takes for sporozoites to reach the liver from the skin. It is clear that a better understanding of the development of protective B cell-mediated immunity will aid the development and refinement of malaria vaccines.

Keywords: malaria, *Plasmodium*, B cells, antibodies, sporozoites, pre-erythrocytic stages

INTRODUCTION

The generation of protective antibodies underpins the success of almost all of our current vaccines (Plotkin, 2008, 2010). While there is no licensed vaccine available for malaria, one indication that a vaccine may be achievable comes from the seminal finding that immunization with radiation attenuated sporozoites (RAS) results in protection against live parasite challenge in both mice and humans (Nussenzweig et al., 1967; Clyde et al., 1973; Hoffman et al., 2002; Seder et al., 2013). Moreover, complete protection against malaria has also been demonstrated in volunteers immunized with low numbers of infectious bites under chloroquine prophylaxis (Roestenberg et al., 2009), also known as DAP immunization. Protective responses induced by RAS appear to be principally based on $CD8^+$ T cells and antibodies (Schofield et al., 1987; Weiss et al., 1988), with contributions from $CD4^+$ T cells, gamma-delta T cells and natural killer (NK) cells also proposed (Tsuji et al., 1994; Doolan and Hoffman, 1999; Oliveira et al., 2008). Antibodies could block infection at the pre-erythrocytic stages in several ways, either by neutralizing sporozoites directly, opsonizing sporozoites for phagocytosis or blocking invasion of parasites into hepatocytes. There is also some evidence that antibodies can block liver stage development, though the mechanism for this is unclear (Chatterjee et al., 1996). In this minireview we will examine the evidence that antibodies can play important roles in protection, evaluate the targets of those antibodies and determine what needs to be known to advance our knowledge of antibody and B cell immunity to sporozoites, and potentially contribute to vaccine development.

Abbreviations: AMA-1, apical membrane antigen 1; CSP, circumsporozoite protein; DAP, drug arrested parasites; LLPC, long-lived plasma cell; LSA-1, liver stage antigen 1; mAb, monoclonal antibody; STARP, serine threonine and asparagine rich protein; TRAP, thrombospondin repeat anonymous protein; TSR, thrombospondin repeat.

ANTIBODY MEDIATED PROTECTION AGAINST SPOROZOITE CHALLENGE

EARLY STUDIES ON HUMORAL IMMUNITY: THE IDENTIFICATION OF THE CIRCUMSPOROZOITE PROTEIN AS A TARGET OF PROTECTIVE IMMUNITY

Shortly after the discovery of protective immunization with RAS, it was found that RAS immunized mice rapidly cleared the sporozoite inoculum from circulation, suggesting the presence of neutralizing antibodies (Nussenzweig et al., 1972). Later transfer experiments showed that immunoglobulin G (IgG) and T cells acted synergistically to confer sterile immunity to sporozoites, (Schofield et al., 1987). More recently, sera from individuals immunized with DAPs was found to reduce infection of humanized mice infected with *Plasmodium falciparum* (Behet et al., 2014). Other early evidence for a role of antibodies in protection came from the identification of mAbs capable of inducing the precipitation of material from the surface of human and rodent malaria sporozoites – a phenomenon known as the circumsporozoite reaction (Yoshida et al., 1980). These mAbs were shown to be capable of blocking infection *in vitro* (Nardin et al., 1982), and *in vivo* (Potocnjak et al., 1980). Subsequently the target of these antibodies was cloned and identified as the CSP (Ellis et al., 1983; Dame et al., 1984; Enea et al., 1984)

CSP is a GPI-anchored protein consisting of a conserved domain structure with N- and C-terminal domains separated by an asparagine-rich repeat region. The C-terminal domain contains a conserved TSR, which is important for the recognition and binding of hepatocytes (Cerami et al., 1992; Frevert et al., 1993). The N-terminal domain acts by masking the TSR of the C-terminal domain, and has to be cleaved to allow the parasite to invade hepatocytes (Coppi et al., 2011). In contrast, the role of the repeat region, which in *P. falciparum* consists of $(NANP)_n$

repeats with a few NVDP repeats interspersed at the beginning, is unknown. Nonetheless, this region was identified early on as an important target of protective immunity, and contains the epitopes recognized by all the original anti-CSP mAbs reported (Zavala et al., 1983, 1985). In terms of protective immunity, much less work has been done to investigate antibody responses to the N- and C-terminal domains despite their functional importance. Several studies have shown that immunization with N-terminal peptides can induce invasion-blocking antibodies (Rathore et al., 2005; Bongfen et al., 2009). Interestingly, a correlation between the presence of antibodies to this region with a reduction in malaria morbidity has also been observed (Bongfen et al., 2009).

PROTECTION MEDIATED BY ANTI-CSP ANTIBODIES IN HUMANS: LESSONS FROM VACCINE TRIALS

Perhaps, the strongest evidence that anti-CSP antibodies can be protective comes from trials of the CSP-based RTS,S vaccine (Stoute et al., 1997). RTS,S is a virus-like particle consisting of 19 NANP repeats and C terminal domain of the CSP fused to the Hepatitis B Surface antigen. RTS,S is currently in Phase III clinical trials in a formulation with AS01, a proprietary adjuvant consisting of a mixture of liposomes, monophosphoril lipid A and saponin (Casares et al., 2010). In experimental challenges of malaria-naïve adults, RTS,S gives short-lived sterile protection in around 50% of volunteers (Kester et al., 2001, 2009). In phase III clinical trials in endemic areas, RTS,S gave 56% protection against clinical malaria among 5–17 month old children (Agnandji et al., 2011b), and 31% efficacy among younger infants (Rts et al., 2012). While there is some evidence of reduced numbers of infections in the field (Guinovart et al., 2009), the main effect of the vaccine appears to be on disease severity, which is surprising as CSP is not expressed in the pathogenic blood stages. The data are however similar to the findings of Bongfen et al. (2009; described above) showing protection against disease correlating with high titres of N-terminus specific antibodies. One explanation for these results is that the vaccine might lower the initial inoculum of parasites and thus the number of blood stages emerging from the liver, buying time for the immune system to control infection. It may also be that while the vaccine does not block all infectious bites, the breakthrough infections are less likely to be genetically complex or highly virulent (Moorthy and Ballou, 2009). Importantly, these data rebut one of the traditional objections to pre-erythrocytic stage vaccines, namely that they would be ineffective if parasites do breakthrough and establish blood stage infection. Nonetheless, this does not negate the importance of developing vaccines targeting other life cycle stages in tandem with pre-erythrocytic approaches.

The mechanisms of protection by RTS,S are poorly understood, with different trials measuring different immunological parameters. Most studies report ELISA titres of total IgG responses against the $(NANP)_n$ repeat, but some use μg/ml while other report titres as ELISA Units (EU). Only in early studies were antibodies segregated by subclass, with no association reported between subclasses and protection (Stoute et al., 1997; Kester et al., 2001). Antibodies to the C-terminal domain have been little studied and were not found to be associated with protection (Kester et al., 2001). Finally only one study reports the number of CSP-specific B cells in vaccinated individuals (Agnandji et al., 2011a). Nonetheless direct evidence that RTS,S induced antibodies can protect comes from a recent study in which human mAbs targeting the CSP repeat were shown to block *P. falciparum* infection of humanized mice (Foquet et al., 2014).

Clinical trials of the vaccine both in naïve individuals and in the field also provide strong evidence of a role for anti-CSP antibodies. Mathematical modeling of a Phase IIb trial in which malaria naïve volunteers were given the RTS,S vaccine formulated in either AS01B or AS02A (Kester et al., 2009), suggested that the bulk of protection comes from high levels (100–200 μg/ml) of anti-repeat antibodies, aided by robust $CD4^+$ T cell responses (White et al., 2013). Importantly, in this study protection did not correlate with titres of Hepatitis B antibodies – which are also induced by the vaccine – suggesting that the CSP antibodies were mediating protection and were not merely a correlate of vaccine "take" (Kester et al., 2009). In endemic areas, mathematical modeling based on a meta-analysis of all Phase II trials has suggested a threshold for infection blocking immunity around 51 EU/ml among children and infants, which is probably similar to the levels required for protection in naïve volunteers (White et al., 2014).

ANTI-CSP ANTIBODIES: OUTSTANDING QUESTIONS

Regardless of the exact measurement used, it is clear that very high titres of antibody are required for protection. While it is difficult to compare directly with other vaccine regimens, the protective cutoff for vaccines to *Haemophilus influenzae* and *Pneumococcus* are <1 μg/ml (Plotkin, 2008), which leads us to ask, why so much antibody is required for protection against sporozoites? One possible answer is that a high amount of antibody is required to eliminate every sporozoite in the short time it takes for the parasites to exit the skin and migrate to the liver. Nonetheless it is not clear how many of the antibodies measured by ELISA are functional: it may be that only the highest affinity antibodies are capable of sporozoite neutralization. Interestingly, in trials of RAS or DAP, *in vivo* protection and *in vitro* infection blocking is obtained at quite low anti-CSP titres (Seder et al., 2013; Behet et al., 2014; Finney et al., 2014). This may be because these whole parasite vaccines stimulate a broad range of protective immune responses, but it is also possible that they induce antibodies that are better able to recognize the native conformation of CSP. Finally there has been no biophysical or structural characterization of the binding of anti-CSP antibodies. These data would give an idea of the necessary affinity required for sporozoite neutralization and show how antibodies bind to the repeat region, which is likely to be somewhat disorganized (Plassmeyer et al., 2009) – it seems likely that these antibodies may have to stabilize the CSP structure and thus pay a high "entropic cost" in binding.

THE ROLE OF OTHER ANTIGENS IN PROTECTION

The limited success of CSP-based recombinant vaccines relative to whole parasite approaches has led to a search for other targets of anti-sporozoite antibodies. Immunity to CSP is probably not absolutely required for protection in rodent models as shown by experiments in which mice immunized with wild type *P. berghei*

RAS were fully protected against challenge with *P. berghei* sporozoites expressing CSP from either *P. yoelii* or *P. falciparum* (Gruner et al., 2007; Mauduit et al., 2009). Nonetheless, it is unclear how much of this CSP-independent protection is mediated by antibodies rather than T cells.

A variety of potential protective antigens have been identified in the sporozoite and liver stages by classical approaches (Duffy et al., 2012). However, while T cell responses to many of these antigens such as LSA-1 and TRAP are relatively well studied (Duffy et al., 2012; Offeddu et al., 2012), the evidence that antibodies to these proteins may be protective is limited (**Table 1**). Combined antibody titres to CSP, LSA-1 and TRAP have been correlated with reduced incidence of clinical malaria among Kenyan children (John et al., 2005), while antibodies to LSA-1 alone have been associated with protection from reinfection following treatment (Domarle et al., 1999). Early reports suggested that antibodies targeting STARP could have even stronger invasion blocking activity than anti-CSP antibodies, there has been little further examination of this molecule (Fidock et al., 1997). Nonetheless when TRAP and LSA-1 were formulated as vaccines in combination with the AS01 or AS02 adjuvant, they were unable to elicit protection (Cummings et al., 2010; Kester et al., 2014).

More recent studies have used protein microarrays to examine the diversity of antibody responses induced after RAS or DAP immunization of humans (Trieu et al., 2011; Felgner et al., 2013). Antibody profiling of RAS vaccines by protein microarray revealed strong antibody responses to two other established vaccine candidates (AMA1 and TRAP) in addition to CSP and a large number of proteins that had not previously been associated with protection. Many of the remaining proteins were hypothetical or involved in cell cycle functions. Protein microarray analysis of DAP immunized volunteers revealed a different antibody profile with CSP and LSA-1 being the only antigens recognized by all protected individuals. Overall, these studies suggest that no single molecule is the key to protection, rather protective antibody responses consist of broad responses to numerous antigens.

B CELL RESPONSES TO *Plasmodium* SPOROZOITES

While vaccination studies in particular have provided insight into protective antibody responses to sporozoites, sporozoite-specific B cell memory and plasma cell formation is poorly understood. Recognition of a foreign antigen by a B cell receptor leads to the proliferation and differentiation of the activated B cell resulting in the formation of short-lived plasma blasts, "early memory" B cells (that do not enter germinal centers) and germinal center B cells (Zotos and Tarlinton, 2012). Germinal center B cells are generally considered the precursors of LLPCs that can maintain antibody titres (**Figure 1**). Knowledge of the development and maintenance of LLPCs would be of particular interest as the rapid transit of sporozoites from skin to liver offers little or no opportunity for anamnestic responses (memory B cells) to contribute to protection. To the best of our knowledge, no work has been

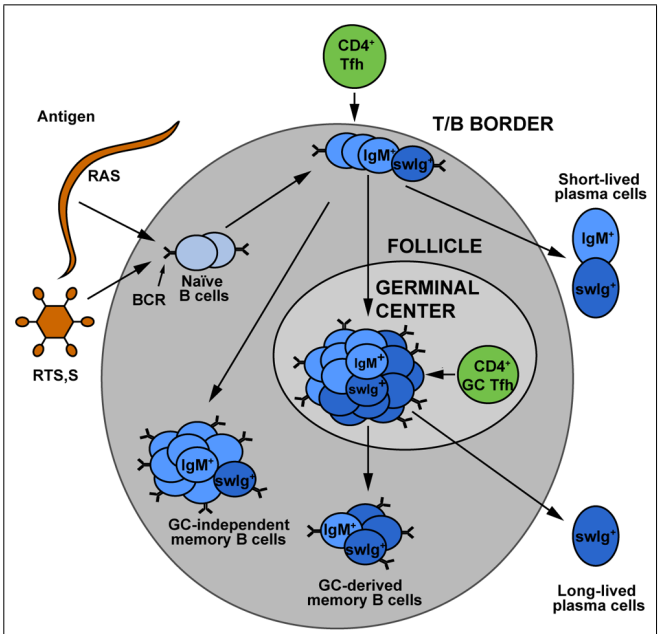

FIGURE 1 | The development of memory B cell subsets. Upon encounter with either sporozoite antigen or vaccines, naïve B cells can undergo a variety of different fates. Some develop into short-lived plasmablasts, which give an immediate antibody response to infection. Others may become "early memory" which is germinal center independent, or may enter germinal centers where theirB cell receptors undergo somatic hypermutation and affinity maturation. The germinal center B cells are believed to be the major precursors for long-lived plasma cells, which maintain the circulating antibody pool. Memory cells may be class switched or they may retain the IgM^+ B cell receptor. The relative contributions of these different memory populations to long term protection against malaria remains an area for further investigation. Figure is based on Taylor et al. (2012). Used with permission from Elsevier.

Table 1 | Potential non-CSP targets of anti-sporozoite antibodies.

Antigen	Evidence	Reference
TRAP/SSP2	Antibodies associated with protection from infection in endemic area	John et al. (2005)
	Mouse monoclonal antibodies display modest infection blocking activity *in vitro*	Charoenvit et al. (1997)
LSA-1	Antibodies associated with protection from infection in endemic area	John et al. (2005)
	LSA-1 repeat antibodies correlate with protection from reinfection in a drug treated cohort	Domarle et al. (1999)
STARP	Affinity purified antibodies from exposed individuals block sporozoite invasion *in vitro*	Fidock et al. (1997)
MB2	Rabbit polyclonal sera inhibit sporozoite invasion *in vitro*	Nguyen et al. (2009)
	Antibody levels correlate with protection in RAS immunized volunteers	Nguyen et al. (2009)

performed in animal models to examine the development and longevity of sporozoite specific B cells and plasma cells. There are however a number of observations of sporozoite specific B cells in naturally exposed and DAP or RTS,S vaccinated individuals. A study in Thailand reported very low levels of B cell responses following natural exposure with only 1/33 adults having CSP-specific B cells (Wipasa et al., 2010). More robust responses are seen following immunization: CSP specific cells accounted for 1% of circulating IgG secreting B cells following RTS,S vaccination and 0.25% of circulating IgG secreting B cells after DAP treatment (Agnandji et al., 2011a; Nahrendorf et al., 2014). These studies are limited however, in that they rely on restimulation enzyme-linked immunospot (ELISPOTs) and therefore cannot provide information on cell phenotype, they report over relatively short timescales and they investigate only IgG antibody secreting cells. Many of the shortcomings of the ELISPOT approach could be overcome by the use of fluorescently labeled antigens to detect rare B cell populations by flow cytometry, however while this has been performed previously for blood stage antigens (Muellenbeck et al., 2013), no such studies have yet been performed with sporozoite antigens.

THE ACTIVATION OF SPOROZOITE SPECIFIC MEMORY B CELLS

A key outstanding question is how are B cells primed by sporozoites? This is particularly important given that immunization with RAS and DAP represent our most successful immunization approaches. It has been shown that after mosquito biting, a large proportion of parasites remains in the skin and a subset of these migrate to the draining lymph node (Amino et al., 2006). The skin draining lymph node appears to be an important location for the induction of protective immunity to sporozoites: not only is it the first location where sporozoite specific $CD8^+$ T cells are detected, it has also been shown that the removal of this lymph node along with the spleen completely abrogates RAS-mediated protection (Chakravarty et al., 2007). By extension it seems likely that the first interactions of B cells with sporozoite antigens occur at this site.

The role of $CD4^+$ T cells, and in particular T follicular helper cells in providing help for antibody responses is also a neglected area. Sporozoites can induce $CD4^+$ T cells and numerous $CD4^+$ epitopes have been identified in the CSP proteins of both mice and human malaria strains (Nardin and Nussenzweig, 1993). Moreover, immunization studies with multiple antigen peptides have shown that B cell responses to the NANP repeat are enhanced by the inclusion of T cell epitopes (Tam et al., 1990). CSP-specific $CD4^+$ T cells expressing various effector functions area are also associated with protection by RTS,S (White et al., 2013). Nonetheless the extent to which these cells are acting as direct effectors or through help to antibody responses is unclear.

THE INFLUENCE OF BLOOD STAGE MALARIA ON B CELL RESPONSES TO SPOROZOITES

One critical factor that may affect the maintenance of sporozoite specific immunity, and immunity to vaccines in general, is malaria infection itself (Urban et al., 1999; Ocana-Morgner et al., 2003). In mouse models, the impact of blood stages on both bystander and malaria-specific immune responses has been examined. *P. yoelii* infection induces apoptosis of memory B cells and plasma cells specific for the blood stage antigen MSP-1 (Wykes et al., 2005); interestingly however, *P. yoelii* infection also induced apoptosis of bystander plasma cells. A similar effect has been reported for Influenza A-specific plasma cells following infection with *P. chabaudi* (Ng et al., 2014). This suggests that blood stage infection may cause a generalized loss of plasma cells and memory B cells irrespective of their specificity (Wykes et al., 2005). It is reported that this apoptotic effect is the result of decreased levels of B cell survival factor (BAFF) expression by conventional dendritic cells in infected mice (Liu et al., 2012). In humans, BAFF expression was found to increase during acute malarial infection and is associated with more severe disease rather than less (Nduati et al., 2011). The rise in soluble BAFF is also correlated with a general proliferation of B cells in volunteers given a controlled malaria challenge (Scholzen et al., 2014).

Blood stage malaria infections in humans have also been associated with high levels of so-called atypical memory B cells (Weiss et al., 2009, 2011; Portugal et al., 2012; Illingworth et al., 2013). Atypical memory B cells, characterized by low expression of CD21 and CD27, have also been described in HIV-infected viremic patients and display exhausted/anergic behavior (Moir et al., 2008). Although they exhibit an 'exhausted' phenotype in malaria infection by displaying decreased *in vitro* ability to differentiate upon stimulation into plasma cells (Weiss et al., 2009), atypical memory B cells isolated from asymptomatic semi-immune donors appear to be functional and may secrete anti-*P. falciparum* IgG (Muellenbeck et al., 2013). It would be desirable to know if sporozoite-specific B cells are driven to form atypical memory, either as bystanders to blood stage infection or due to continued antigen exposure – e.g., from frequent biting, or cross reactivity with blood stage antigens.

CONCLUDING REMARKS

Together the available data tell us that anti-sporozoite antibodies can protect and should be a major component of a pre-erythrocytic vaccine. Beyond CSP, however the targets of protective immunity are unknown. Further vaccine development is also hampered by a lack of basic knowledge on such issues as the optimal fine specificity, affinity and subclass required for protection by anti-sporozoite antibodies. Finally a successful vaccine will have to induce longer-lived plasma cell and memory responses than existing candidates. To understand these issues a better understanding of the immunology of anti-sporozoite B cell responses seems essential; such knowledge may enable the development of new subunit approaches, or enable us to optimize whole parasite vaccines.

AUTHOR CONTRIBUTIONS

Johanna N. Dups and Ian A. Cockburn jointly wrote the first draft of this manuscript. Marion Pepper reviewed the manuscript and contributed significantly to the final draft. All authors read and approved the final manuscript.

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Commentary on: Antibody and B cell responses to Plasmodium sporozoites

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A commentary on

Antibody and B cell responses to *Plasmodium* sporozoites
*by Dups JN, Pepper M, Cockburn IA. Front Microbiol (2014) **5**:625. doi: 10.3389/fmicb.2014.00625*

Dups et al. (1) assess how antibody and B cell responses to *Plasmodium* sporozoites may function as protective immune responses. They cover immune induction quite extensively but discuss virtually nothing on effector mechanisms of antibody responses against sporozoites. Because their review should be balanced by reference to studies describing effector mechanisms, this commentary will confine itself to recounting some of these.

In vitro exposure to serum from immunized mice results in an antibody-mediated precipitant projecting from sporozoites (2). Because of the striking way in which immune serum deformed sporozoites, this "CSP" reaction was postulated to be the basis for a humoral component of immunity against sporozoites. *In vitro* inhibition of sporozoite motility by serum from immune animals subsequently suggested that anti-sporozoite antibodies can function by blocking sporozoite motility (3). Further *in vitro* studies showed that monoclonal antibodies against the CS antigen blocked *in vitro* invasion by sporozoites into target cells (4, 5). Subsequent intravital studies revealed *in vivo* inhibition of sporozoite motility and inhibition of sporozoite invasion of dermal blood vessels by antibodies (6). And an association between high levels of CS antibodies and protection was shown in the first successful human immunization with radiation-attenuated sporozoites (7).

Anti-sporozoite antibodies act even earlier during challenge by mosquito bite, by inhibiting release of sporozoites from the mosquito proboscis into skin (8). This appears to be the initial manifestation of functional immunity against pre-erythrocytic malaria parasites. Analysis of kinetics of *Plasmodium berghei* sporozoites injected by mosquitoes into sporozoite-immunized vs. non-immunized mice showed significantly fewer sporozoites were deposited in immune mice (8). CS protein is released by sporozoites into media *in vitro* (9) and into saliva within salivary glands of infected mosquitoes (10, 11). Thus, CS protein released into mosquito saliva together with sporozoites is injected into hosts while mosquitoes probe for blood meals. This mosquito-injected CS protein appears to interact with homologous anti-CSP antibodies within immunized hosts at sites of saliva release; the interaction produces immune complexes that interfere with free release of sporozoites by mosquitoes *in vitro* and *in vivo* (8).

Some sporozoites injected by mosquitoes into immunized hosts may successfully escape into the blood. Indeed, sporozoites that bypass skin by being injected intravenously by syringe into mice passively immunized with immune serum are cleared effectively from the circulation (12), indicating a functional role for antibodies against "break-through" sporozoites that have reached the blood.

Conceivably, antibody-coated sporozoites may be blocked during attempted passage through Kupffer cells prior to hepatocyte invasion (13, 14). *In vitro* studies have documented that opsonized sporozoites are prone to phagocytosis (15–17), suggesting that Kupffer cells can eliminate opsonized sporozoites *in vivo*. Furthermore, antibodies also inhibit invasion and traversal through hepatocytes, events that rely on sporozoite motility (18).

Thus, anti-sporozoite antibodies can act sequentially against sporozoites at different stages of sporozoite invasion from the skin to invasion of dermal blood vessels, to passage from Kupffer cells into hepatocytes, and subsequent traversal of sporozoites through a series of hepatocytes.

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Plasmodium attenuation: connecting the dots between early immune responses and malaria disease severity

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Sterile attenuation of *Plasmodium* parasites at the liver-stage either by irradiation or genetic modification, or at the blood-stage by chemoprophylaxis, has been shown to induce immune responses that can protect against subsequent wild-type infection. However, following certain interventions, parasite attenuation can be incomplete or non-sterile. Instead parasites are rendered developmentally stunted but still capable of establishing an acute infection. In experiments involving *Plasmodium berghei* ANKA, a model of experimental cerebral malaria, it has been observed that several forms of attenuated parasites do not induce cerebral pathology. In this perspective we collect evidence from studies on murine malaria in particular, and attempt to "connect the dots" between early immune responses and protection from severe cerebral disease, highlighting potential parallels to human infection.

Keywords: malaria, attenuation, experimental cerebral malaria, early immune response

INTRODUCTION

Radiation-attenuated sporozoites, genetically attenuated parasites and sporozoites administered under chemoprophylaxis have all been shown to successfully induce long-lasting sterile protection against malaria infection, in both animal models and humans (Nussenzweig et al., 1967; Hoffman et al., 2002; Belnoue et al., 2004; Mueller et al., 2005; Roestenberg et al., 2009; Butler et al., 2011). Sterile protection is characterized by the elimination of the parasite through the pre-erythrocytic immune response before it is able to establish a blood infection, that can be detected microscopically and cause pathology. In contrast, protection from severe cerebral pathology does not necessitate the complete elimination of the parasite from the host. Instead, parasites transition from the liver into the blood but do not cause severe cerebral pathology.

This perspective focusses on attenuated, non-sterile infections, particularly those from studies on murine malaria, that result either intentionally or unintentionally from previous interventions such as immunization or genetic manipulation of parasite lines. Such interventions eventually limit or alleviate the murine severe disease outcome, experimental cerebral malaria (ECM). Moreover, we summarize evidence from both published and unpublished observations that suggest a critical role for early immune responses in influencing the development of cerebral pathology.

Experimental cerebral malaria develops when susceptible mouse strains are infected with specific strains of *Plasmodium*. For example, infection of C57BL/6 mice with *Plasmodium berghei* ANKA (*Pb*A) generates a severe cerebral syndrome generally considered to be analogous to human cerebral malaria (de Souza and Riley, 2002). Descriptions of ECM immunopathogenesis are multifaceted and largely hypothetical, involving an intricate series of interactions in space and time between both host and pathogen, but generally start from the onset of blood-stage infection (Renia et al., 2006). ECM pathology is generally marked by ataxia, fitting, coma, and eventually death (de Souza and Riley, 2002). Although subject to some debate (Carvalho, 2010; White et al., 2010; Craig et al., 2012), the close correlation between mice and humans in terms of immune responses and neuropathological processes (Hau and Van Hoosier, 2005; de Souza et al., 2010) has made ECM a generally accepted, if sometimes disputed, model (Hunt et al., 2010; Riley et al., 2010; Langhorne et al., 2011).

Few studies to date have addressed the potential for host immune responses directed against either the liver-stages or early blood-stages to modify the immunopathogenesis of ECM. Our experience is that the apparent absence of ECM following parasite manipulation is often considered irrelevant and hence not fully examined yet, for example during the functional characterization process of a transgenic parasite. Indeed, the absence of ECM following numerous experimental conditions is an accepted, anecdotally recounted but rarely published phenomenon.

In an attempt to shed light on a generally disregarded aspect of murine malaria, we have outlined several examples of malaria infection that share the same outcome of protection from severe cerebral complications, but in all probability do not share the same protective mechanism. It is our hope that highlighting and cataloging examples of this generally disregarded phenomenon will both draw attention to the field and help piece together

potential critical factors involved in protection against cerebral malaria.

INCOMPLETE PARASITE ATTENUATION AT THE LIVER-STAGE

Haussig et al. (2011) recently observed that targeting the apicoplast by disruption of a *Plasmodium*-specific protein that plays a role in liver merozoite formation (PALM) affected liver-stage development and the subsequent onset of blood-stage parasitemia. 30% of the *palm*(-)-immunized mice became patent following a delay of up to 4 days, of which the majority did not develop cerebral pathology (Haussig et al., 2011).

Another approach described an experimental vaccination regime consisting of sporozoite immunization applied concomitantly with either azithromycin or clindamycin drug cover. This permits full development of the malarial liver-stage, but inhibits the inheritance and biogenesis of the apicoplast, thus preventing the onset of blood-stage infection. While sterile protection was found to depend on IFN-γ producing $CD8^+$ T cells that exclusively targeted the intra-hepatic stages, it was dose-dependent and a reduction in the sporozoite numbers used for immunization led to breakthrough infections. However, all mice that developed blood-stage infecion featured a delay in the onset of patency and were apparently protected from ECM (Friesen et al., 2010).

Further it is known that sterile protection conferred by immunization under chloroquine (CQ) cover relies on a critical threshold of intra-hepatic parasites (Nganou-Makamdop et al., 2012). Comparable to Friesen et al. we have observed that a reduction in the sporozoite numbers used for immunization under CQ cover, does not confer sterile protection against a wild type infection, but delays the onset of blood-stages and protects against ECM (Pfeil et al., unpublished).

While the mechanism behind protection against severe cerebral symptoms in the models described above still remains elusive, preliminary data from another experiment suggests a role for an altered host immune response in the modulation of ECM outcome. Incomplete attenuation of *Pb*A parasites achieved via sub-therapeutic administration of a liver acting anti-malarial substance lead to the suppression of intrahepatic development, a delay in prepatency and subsequent abrogation of cerebral pathology. This effect was supported by a robust host immune environment involving a Th1 response and early T-cell activation in both liver and spleen (Lewis et al., unpublished).

A common motif between these observations is developmental impairment or attenuation during the transition from liver to the intraerythrocytic phase of the malaria parasite. It is conceivable that this altered transition results in a slow trickle of parasites into the bloodstream. How exactly this slow onset of blood-stage parasitemia modulates the immune response in a way that severe disease is prevented, remains unknown.

INCOMPLETE PARASITE ATTENUATION AT THE BLOOD-STAGE

The notion that parasite growth kinetics in the blood can be linked to cerebral malaria, while tenuous, is not entirely novel. In fact, several murine studies have documented early growth defects in the blood that caused an altered disease outcome. One such example was the oral administration of trioxane T-10 thioacetals to C57BL/6 mice 24 h after infection with *Pb*A-infected erythrocytes, which completely abrogated ECM in treated mice (Jacobine et al., 2012). Deletion of certain non-essential blood-stage antigens also achieves the same result. For example *P. berghei* parasites lacking the endogenous merozoite surface protein 7 (MSP7) remain viable, but are impaired in their multiplication rates in the blood (Tewari et al., 2005). A minor delay in parasite development *in vivo*, was attributed to enhanced reticulocyte preference but was sufficient to ablate ECM in C57BL/6 mice (Tewari et al., 2005; Spaccapelo et al., 2011). Similar virulence-attenuated phenotypes were also observed in experiments with parasites lacking plasmepsin 4 (Δ*pm4*) or a component of the PTEX, thioredoxin-2 (TRX2) Δ*PbTRX-2*. Protection from ECM in the case of Δ*pm4* parasites was associated with a growth defect in the blood (Spaccapelo et al., 2010). While Δ*PbTRX2* mutants displayed a marked delay in parasitemia resulting in abrogation of ECM in the majority of mice, variations in virulence were observed between Δ*PbTRX2* clones, which the authors hypothesized resulted from differences in the number of times the clones had been passaged (Matthews et al., 2013).

An inference drawn from the examples above suggests that chemical or genetic methods of attenuation modify parasite growth in the blood in a way that differs from a natural infection. This form of attenuation could potentially stall parasite development, thereby reducing the burden of viable parasites and the ensuing immunopathogenesis, thus resulting in the abrogation of ECM.

PARASITE ATTENUATION AND CLINICAL OUTCOME IN HUMANS

Although not directly comparable to the examples described above, similar observations have also been reported from human clinical trials. The partially protective effect against clinical and severe disease following immunization of individuals with the leading malaria vaccine candidate RTS,S represents a good example. The fact that a vaccine against pre-erythrocytic stages confers protection against severe malaria was suggested to stem from vaccine-induced immune responses that reduced the number of liver-stage parasites after natural infection. Such partial pre-erythrocytic immunity may result in the "leakage" of small numbers of parasites. This slow onset of blood-stage parasitemia might increase the time frame required to establish innate and adaptive immune responses that inhibit blood-stage growth and consequently limit severe disease (Guinovart et al., 2009). In a similar setting, long-term reduction in the risk of clinical malaria in Tanzanian children was observed following intermittent preventive treatment with the antimalarial sulfadoxine-pyrimethamine (SP). It was proposed that the long half-life and possibly anti-liver-stage acting properties of SP lead to low-dose blood-stage infections that effectively induce prolonged protection from clinical malaria (Schellenberg et al., 2001; Greenwood, 2007; Sutherland et al., 2007). Such clinical studies and many others that test vaccine efficacy or antimalarial drug potency, however, lack a detailed

understanding of the dowmstream effects on human cerebral malaria.

EARLY IMMUNE RESPONSES AND EVENTS THAT MAY AFFECT DOWNSTREAM IMMUNOPATHOGENESIS

Early immune responses and particularly elements and mechanisms of the innate immune system can influence downstream effector responses and consequently disease outcome (O'Garra and Murphy, 1994; Jankovic et al., 2001; Mitchell et al., 2005).

In vitro observations with *P. falciparum* and also murine studies have shown that infected red blood cells and parasite moieties such as glycosylphosphatidylinositol (GPI) and hemozoin can trigger innate pathways of the immune system, primarily through toll-like receptor signaling (Schofield et al., 1996; Coban et al., 2005). A study in the rodent model, that was published in 2007 identified TLR-2, -9 and MyD88-dependent signaling as mediators of ECM (Coban et al., 2007). However, subsequent studies showed that TLR-deficient mice still succumbed to ECM (Togbe et al., 2007; Lepenies et al., 2008), thus pointing out a controversial role for TLRs in the development of cerebral pathology.

Nevertheless, other components of the innate immune system have been implicated in the induction of ECM (Hansen et al., 2003, 2007; Maglinao et al., 2013; Palomo et al., 2013). For instance, Hansen et al. (2003) showed that susceptibility or resistance to ECM was dependent on CD1d-restricted NKT cells that modulated Th1/Th2 polarization. A subsequent study showed that NK cell depletion negated T cell recruitment to the brains of ECM-affected mice thus substantiating a role for NK cells in the regulation of adaptive immune responses that influence cerebral pathology (Hansen et al., 2007). Additionally, NK cells and γδ T cells, are also known as early sources of IFN-γ that could enhance parasite clearance mechanisms (Seixas and Langhorne, 1999; Artavanis-Tsakonas and Riley, 2002; Ing and Stevenson, 2009; Inoue et al., 2013).

Indeed, there is evidence that very early inflammatory responses are capable of altering downstream immunopathogenesis in a manner that involves $CD8^+$ T cells and IFN-γ (De Souza et al., 1997; Mitchell et al., 2005; Lewis et al., unpublished). ECM-susceptible mice, co-infected with *Pb*A and *Pb*K173 are completely protected from ECM and this protection was found to be associated with increased IFN-γ in the blood at 24 h post-infection and an increase in transcriptional abundance of IFN-γ, IL-10 and IL-12 in both the liver and spleen (Mitchell et al., 2005). In this model early production of IFN-γ was attributed predominantly to $CD8^+$ T cells that are known for their ability to rapidly produce this cytokine in a non-antigen-specific manner thereby contributing to innate immunity, e.g., in the early phase of bacterial infections (Berg et al., 2002, 2003; Kambayashi et al., 2003).

This is perhaps contradictory to the received wisdom that ECM is Th1 in nature and responsibility for pathology lies with IFN-γ, $CD8^+$ T cells (de Souza and Riley, 2002) and the Th1-biased C57BL/6 mouse (Locksley et al., 1987). The answer partly lies with the opposing roles of IFN-γ or TNF-α depending on the time of their production during infection, i.e., early expression correlates with protection from ECM while later expression promotes ECM (Grau et al., 1989; de Souza and Riley, 2002; Mitchell et al., 2005). One could speculate that an early inflammatory peak disrupts the delicate balance required for ECM immunopathogenesis. A possible explanation is that parasite elimination mechanisms are enhanced, thus preventing the critical antigen threshold required for the onset of immunopathogenesis (Howland et al., 2013).

Alternatively, early inflammatory responses could also induce early production of anti-inflammatory cytokines such as IL-10, a critical regulator in ECM immunopathogenesis (Kossodo et al., 1997; Couper et al., 2008; Niikura et al., 2010). Our preliminary data also indicates that an early acute systemic inflammation may provoke the production of IL-10 (Lewis et al., unpublished). IL-10 may then alleviate $CD8^+$ T cell activation, proliferation and down-regulate the expression of adhesion molecules on the vascular endothelium (Renia et al., 2006). Thus the timing and localization of the production of pro- and anti-inflammatory cytokines is crucial to the development of cerebral immunopathogenesis.

Although we are limited in our understanding of the impact of early immune responses on the development of cerebral malaria in humans, studies from mouse models have suggested that an ability to control the initial parasitemia permits the development of adaptive immune responses that support an early inflammatory response and enhance parasite clearance (Meding and Langhorne, 1991; Mohan et al., 1997; van der Heyde et al., 1997; Su and Stevenson, 2000). An early inflammatory response could in turn dampen the immunopathology that otherwise prevails during a natural infection.

Since ECM is likely caused by a series of immunopathogenic mechanisms that are interrelated but not necessarily sequential or reliant upon each other, the disruption of one mechanism in a given model may not necessarily translate into the same outcome in another.

Nevertheless, we propose the following mechanisms by which growth impairment might play a role in the abrogation of ECM.

A GROWTH DEFECT MAY AFFECT SEQUESTRATION IN PERIPHERAL ORGANS

Shortly after the onset of blood-stage infection, parasitized erythrocytes adhere to the peripheral tissues (Beeson et al., 2001), inducing the activation of monocytes, neutrophils, and DCs (Renia et al., 2006). The adherence of parasitized erythrocytes to the vascular endothelium has been shown to induce chemokine secretion and provoke an "activated" state in the brain endothelium. Leukocytes and parasitized erythrocytes bound to the endothelium interfere with the circulation and produce cytotoxic molecules. This damages the blood-brain-barrier and causes hemorrhages and oedema (de Souza et al., 2010). A blood-stage growth "defect" or "modification" may alter the kinetics of the replicating parasite, thereby altering the localization, severity or timing of parasite sequestration. In turn, this may modify the induced innate immune response.

GROWTH KINETICS COULD ALTER ANTIGEN PRESENTATION

Given the shared antigen repertoire between liver and blood-stage parasites (Belnoue et al., 2008; Tarun et al., 2008), it is

conceivable that altered or possibly prolonged presentation of shared antigens by late liver-stages directly influences the early immune response against erythrocytic stages, eventually altering disease outcome. Additionally, the timing and localization of parasite sequestration may also impact upon the adaptive immune response to malaria infection. $CD8^+$ T cells are a critical requirement for cerebral pathology and disruption of their chemotaxis to the brain can protect against cerebral pathology (Renia et al., 2006). A recent publication indicated that T cells specific for a malarial glideosome-associated protein may be responsible for ECM pathology due to cross-presentation of parasite antigen by the brain microvessels (Howland et al., 2013). Activated endothelial cells are capable of presenting antigen in an MHC-I context (Pober and Cotran, 1991) and are likely to phagocytose sequestered parasite material for presentation of antigen to $CD8^+$ T cells (Renia et al., 2006). The authors also demonstrated that the number of antigen-specific T cells does not increase in ECM models or upon the induction of cerebral pathology. Instead, the degree of cross-presentation of parasite moieties by the activated brain endothelium is reduced (Howland et al., 2013). A reduction in parasite sequestration may therefore reduce the priming of the immune events that would otherwise, like a line of dominoes, lead to the induction of cerebral pathology.

PRIMING IN THE SPLEEN COULD BE MODIFIED BY GROWTH IMPAIRMENT

As the organ responsible for blood filtration, the spleen is likely to be responsible for the priming and activation of T cells that subsequently migrate to the brain (Renia et al., 2006). Indeed, splenectomised mice infected with low doses of *Pb*K173 do not develop cerebral symptoms (Curfs et al., 1989; Hermsen et al., 1998). Activation occurs at an early timepoint post-infection and it is conceivable that modification of parasite growth kinetics may reduce the exposure of parasite moieties to macrophages and dendritic cells within the splenic tissues, modifying the phagocytosis, processing and presentation of antigen. It is known that $CD11c^{hi}CD8^+$ dendritic cells are responsible for ECM immunopathogenesis (Piva et al., 2012) by phagocytosing dying cells and processing antigen in an MHC-I restricted manner (den Haan et al., 2000). Disrupting this process may modify the priming of lymphocytes which, in turn, may reduce systemic inflammation and endothelial cell activation.

REGULATORY T CELLS

Although the role of regulatory T cells in malaria is controversial, it is conceivable that they play a role in the protection we observe in some models. ECM can be ablated in normal *Pb*A infection by the expansion of regulatory T cells (Haque et al., 2010). One possibility is that regulatory T cells temper the pro-inflammatory response (Riley et al., 2006), which is a key factor in the development of cerebral malaria. In fact, concomitant infection of mice with *Schistosoma japonicum* and *P. berghei* reduces ECM mortality by promoting a Th2 response that is supported by proliferating Tregs (Wang et al., 2013). Interestingly, protection from the severe symptoms of malaria in *P. falciparum* is also associated with the expansion of $CD4^+CD45RO^+FOXP3^-$ regulatory T cells (Walther et al., 2009).

CONCLUSION

The examples elaborated above substantiate a crucial role of early immune responses in influencing the immunopathogenesis of ECM. While a clear distinction cannot be drawn between the responses toward late liver-stages and those toward the early blood-stages, they both seem to exert an effect on the onset of parasitemia. Attenuated infections could serve as tools to improve our understanding of the mechanisms by which early immune responses regulate downstream adaptive immunity and consequently cerebral pathology. Elucidating these mechanisms could help refine future intervention strategies.

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The case for a rational genome-based vaccine against malaria

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Historically, vaccines have been designed to mimic the immunity induced by natural exposure to the target pathogen, but this approach has not been effective for any parasitic pathogen of humans or complex pathogens that cause chronic disease in humans, such as *Plasmodium*. Despite intense efforts by many laboratories around the world on different aspects of *Plasmodium* spp. molecular and cell biology, epidemiology and immunology, progress towards the goal of an effective malaria vaccine has been disappointing. The premise of rational vaccine design is to induce the desired immune response against the key pathogen antigens or epitopes targeted by protective immune responses. We advocate that development of an optimally efficacious malaria vaccine will need to improve on nature, and that this can be accomplished by rational vaccine design facilitated by mining genomic, proteomic and transcriptomic datasets in the context of relevant biological function. In our opinion, modern genome-based rational vaccine design offers enormous potential above and beyond that of whole-organism vaccines approaches established over 200 years ago where immunity is likely suboptimal due to the many genetic and immunological host-parasite adaptations evolved to allow the *Plasmodium* parasite to coexist in the human host, and which are associated with logistic and regulatory hurdles for production and delivery.

Keywords: malaria, vaccine, rational vaccine design, antigen discovery, genome-based, protective immunity

INTRODUCTION

Vaccines are the most efficient health care intervention for preventing morbidity and mortality and improving public health, except for water sanitation (World Health Organization [WHO], 2014a). The field of vaccinology originated in 1796 when Edward Jenner protected James Phipps against smallpox by inoculation with cowpox (Jenner, 1798; Baxby, 1999; Tuells, 2012). However, despite dedicated efforts to develop vaccines against a range of viral, bacterial, or parasitic diseases, approximately one third of all deaths (at least 15 million people each year) and 68% of deaths in children under 5 years of age (5 million children each year) are due to infectious diseases (World Health Organization [WHO], 2014b). Of these, three pathogens that cause chronic infections – the *Plasmodium* parasite, human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis* (TB); known by public health officials as the "big three" – are the major threats responsible for 10% of all deaths globally and more than half the global burden of infectious diseases. Moreover, although there is an extensive vaccine portfolio against viral and bacterial pathogens, there are no licensed vaccines for any parasitic infection of humans or for any chronic infections by complex pathogens (World Health Organization [WHO], 2006; Moorthy and Kieny, 2010). Indeed, there is only one therapeutic vaccine approved by the US Food and Drug Administration (FDA) or European Medicines Agency (EMA), for a metastatic hormone-refractory prostate cancer (Provenge®, DendreonCorp, USA) but this requires preparation of a personalized vaccine for each patient and so is expensive (~$US93,000) and has very poor uptake. New approaches for the development of vaccines against complex and chronic pathogens are urgently needed. Malaria is an excellent model for such approaches, being a complex pathogen which causes chronic infections and one of the "big three" public health targets.

The Malaria Vaccine Technology Road Map was published in 2006 as the result of a collective effort by the malaria vaccine community[1]. A comprehensive update to this roadmap was released in 2013 with the strategic goal to, by 2030, license vaccines targeting *Plasmodium falciparum* and *P. vivax* with a protective efficacy of at least 75% against clinical malaria with a duration of protection of at least 2 years and booster doses to be required no more frequently than annually[2]. In the intervening years, there has been a call for global malaria eradication, issued by Bill and Melinda Gates in October 2007 (Roberts and Enserink, 2007) and taken up the malaria community with a consensus community-based Malaria Eradication Agenda[3]. Many experts consider that vaccines will play a key role in the eradication process (Hall and Fauci, 2009; Plowe et al., 2009) and vaccines will certainly be important to sustain and improve on levels of control achieved by other interventions such anti-malarial drugs, insecticide spraying and insecticide-impregnated bed nets. Additionally, the Global Vaccine Action Plan 2011–2020 compiled by stakeholders from the global health

[1]http://www.malariavaccine.org/files/Malaria_Vaccine_TRM_Final_000.pdf
[2]http://www.who.int/immunization/topics/malaria/vaccine_roadmap/en/
[3]http://www.who.int/malaria/elimination/maleraupdate.pdf

community including the World Health Organization (WHO), the United Nations Children's Fund (UNICEF), the National Institute of Allergy and Infectious Diseases (NIAID), and the Bill & Melinda Gates Foundation (BMGF) included as a key indicator of progress towards one of its six strategic objectives, "proof of concept for a vaccine that shows greater or equal to 75% efficacy for HIV/AIDS, tuberculosis or malaria" by 2020 (World Health Organization [WHO], 2012).

However, despite intense effort for many decades by researchers throughout the world, the development of a malaria vaccine that meets the stated goals remains elusive (Sherman, 2009). Almost all efforts have focused on the pre-erythrocytic or asexual blood stages of *P. falciparum* although recent efforts have been also directed to the sexual stage to provide 'herd immunity' rather than confer individual protection as a consequence of the malaria eradication agenda, as well as *P. vivax* (Schwartz et al., 2012; World Health Organization [WHO], 2014b). An efficacious pre-erythrocytic vaccine is considered ideal since it would halt the development of the parasite in the liver stage and thereby prevent the blood stage of the life cycle which is the stage associated with the development of clinical symptoms, as well as the transmission of the disease which occurs in the sexual stage. The most advanced malaria vaccine candidate is RTS,S, a hybrid virus-like particle containing the C-terminus of the *P. falciparum* circumsporozoite protein (CSP) fused to hepatitis B surface antigen, expressed in *Saccharomyces cerevisiae* together with hepatitis B surface antigen (Ballou and Cahill, 2007). Recently, regulatory approval for RTS,S/ASO1 targeted at infants aged 6–14 weeks and administered through the routine Expanded Program on Immunization (EPI) has been submitted to the European Medicines Agency by GlaxoSmithKline following pivotal Phase III evaluation at 11 sites in sub-Saharan Africa; if the required regulatory approvals are obtained and the Phase III safety and efficacy data are satisfactory, the WHO has indicated that a policy recommendation for RTS,S may be granted in 2015[4]. Although modeling estimates predict that this vaccine should have a public health impact in terms of number of "deaths averted" (Brooks et al., 2012; Nunes et al., 2013), the Phase III efficacy of the vaccine for its intended outcome in the target age group is very low and is not sustained (Duncan and Hill, 2011; Olotu et al., 2013). Specifically, after 18 months of follow-up, efficacy against clinical malaria was only 27% among infants aged 6–12 weeks at the first vaccination; among children aged 5–17 months at first vaccination, vaccine efficacy was 46%; moreover, these results were achieved on top of existing malaria interventions including insecticide-treated bed nets used by 86 and 78% of trial participants aged 6–12 weeks or 5–17 months, respectively[5]. Although the RTS,S data are encouraging, this milestone has taken almost 30 years of extensive preclinical and clinical development by GSK in partnership with the US Army, supported by more than US$200 million from the Bill & Melinda Gates Foundation in addition to more than $350 GSK funds to date, with an additional $260 million investment anticipated[5] (Ballou and Cahill, 2007).

[4]http://www.malariavaccine.org/files/MVI-GSK-RTSSfactsheetFINAL-web.pdf

[5]http://www.malariavaccine.org/files/MVI-GSK-FAQ-FINAL-web.pdf

CHALLENGES FOR MALARIA VACCINE DEVELOPMENT

The development of an effective vaccine against malaria has been hindered by the complexity of the *Plasmodium* spp. parasite as well as the host response to the parasite. Recent evidence indicates that the *Plasmodium* parasite has co-evolved with the human host for 1000s of years (Liu et al., 2010) with evolutionary co-adaptation allowing for chronic persistence of the parasite in the human host, and recurrent infections (Pierce and Miller, 2009). The *Plasmodium* life cycle involves both invertebrate (mosquito) and vertebrate (mammalian) hosts, with multiple stages within the host (sporozoite, liver, asexual blood stage, sexual) and numerous intracellular and extracellular environments in which the parasite develops (Langhorne et al., 2008). Different host responses are required to target these distinct life cycle stages – primarily antibodies against the extracellular parasite stages exposed in the peripheral circulation (sporozoites, asexual blood stage merozoites) and T cells against the intracellular stages (hepatic/liver stages) which are not accessible to circulating antibodies. In addition, the *Plasmodium* spp. parasite has a large 23 megabase genome that contains an estimated 5,300 putative proteins, many of which are expressed in different stages of the life cycle (Gardner et al., 2002). Moreover, many of these protein may exhibit allelic polymorphism (more than one allele existing for certain regions of a protein, e.g., MSP1 or AMA1), antigenic polymorphism (point mutations at the nucleotide level, e.g., CSP) often localized to B cell or T cell epitopes, or antigenic variation (multi-copy variant surfaces antigens, e.g., var genes, rifins; Good et al., 1988; Takala and Plowe, 2009; Kirkman and Deitsch, 2012; Barry and Arnott, 2014). A vaccine must be effective against all antigenic or allelic variants responses, to ensure efficacy against all variant circulating strains in the field (Moorthy and Kieny, 2010). In addition to this selection by the parasite of mutations that avoid the host protective immune response and confer susceptibility to the disease, there is selection by humans of mutations that confer resistance. Indeed, malaria is considered to have had the greatest impact of all pathogens in shaping the human genome with evidence that a number of genetic polymorphisms within the human genome, including α-*thalassemia* and hemoglobinopathies such as the sickle-cell trait, have arisen due to evolutionary pressure exerted by the *Plasmodium* parasite (Mackinnon and Marsh, 2010; Taylor et al., 2013). The high mortality rate associated with malaria would be predicted to exert a powerful selective pressure on the human genome by positively selecting any genetic mutation that confers protection against death to allow the *Plasmodium* parasite to persist in the host and be transmitted (Pierce and Miller, 2009; Mackinnon and Marsh, 2010). Other sophisticated immune evasion strategies at the host-parasite interface include the ability of the parasite to modulate the host immune response, reviewed extensively elsewhere (Langhorne et al., 2008; Casares and Richie, 2009; Pierce and Miller, 2009). These complex host–parasite interactions at the genetic and immunological levels pose significant challenges for vaccine development.

FEASIBILITY OF VACCINATION AGAINST MALARIA

In spite of these challenges, there is growing evidence supporting the feasibility of developing an effective malaria vaccine. Field studies have demonstrated a decreasing incidence and density

of infection with age and exposure to natural infection, and reduced frequency and severity of clinical illness, indicative of the acquisition of anti-disease immunity (Baird, 1998; Doolan et al., 2009). Also, passive transfer of polyclonal sera or purified immunoglobulin from individuals with lifelong exposure to *P. falciparum* resulted in a significant reduction in blood-stage parasitemia and recovery from clinical symptoms (Cohen et al., 1961; Sabchareon et al., 1991). Those studies implicate antibodies directed against blood stage antigens as the key immune effectors in naturally acquired immunity. This protection is anti-disease immunity but not anti-parasite immunity since most individuals with long-term exposure in malaria endemic areas who have developed effective clinical immunity will nonetheless continue to experience low-density, asymptomatic infections (Okell et al., 2009). Longitudinal studies in malaria-endemic populations suggest that immune responses to the pre-erythrocytic stages probably have limited involvement in this anti-disease immunity and that immunity to the pre-erythrocytic stage is not naturally acquired (Owusu-Agyei et al., 2001; Tran et al., 2013). Nonetheless, epidemiological studies suggest that exposure to low numbers of sporozoites, although not sterilizing, can reduce the parasite load in the liver and lower blood stage parasitemia, since the intensity of exposure to biting infectious mosquitoes (entomological inoculation rate) has been significantly associated with the incidence and density (but not prevalence) of *P. falciparum* parasitemia in children (Doolan et al., 2009).

More convincing evidence for the feasibility of vaccination against malaria exists from studies focused on the experimental induction of protective immunity, which have established that sterile infection-blocking protective immunity directed against the pre-erythrocytic stage can be achieved in mice and humans. Considered for many years the "gold standard" for malaria vaccine development, sterile protection can be induced in mice, non-human primates and humans by exposure to the bites of radiation-attenuated *P. yoelii, P. berghei, P. knowlesi*, or *P. falciparum* infected mosquitoes, or by intravenous immunization with isolated irradiated sporozoites, provided that the dose of radiation is sufficient to attenuate the parasite such that it can invade the hepatocyte but not develop into the blood-stage (Nussenzweig et al., 1967; Gwadz et al., 1979; Hoffman et al., 2002; Weiss and Jiang, 2012). The parasite is arrested in early liver stage development, with each invading sporozoite giving rise to only a single hepatic parasite. Murine and non-human primate studies establish that the protective immunity induced by immunization with radiation attenuated sporozoites (RAS) is directed against the liver stage parasite and mediated primarily by $CD8^+$ T cells and IFN-γ (Schofield et al., 1987; Doolan and Hoffman, 2000; Tsuji, 2010; Weiss and Jiang, 2012). Recent studies have shown that protection in humans can be induced in a dose-dependent manner by intravenous but not intradermal immunization with radiation-attenuated, aseptic, purified, cryopreserved *P. falciparum* sporozoites (Epstein et al., 2011; Seder et al., 2013); five intravenous doses of 135,000 *Pf*SPZ (675,000 sporozoites in total) were required to achieve sterile immunity in 6/6 volunteers. $CD8^+$ IFN-γ-producing T cells in the liver were implicated as the primary immune effector based on non-human primate studies showing that high frequencies of $CD8^+$ T cells could be induced by intravenous but not intradermal routes of immunization, and mouse studies showing that these cells could protect (Epstein et al., 2011). These data are consistent with an earlier proposal (Langhorne et al., 2008) that sporozoites injected intravenously can enter the liver within seconds and be processed and presented by liver-resident antigen-presenting cells for induction of host immunity, whereas sporozoites inoculated intradermally via mosquito bite may take minutes to hours to enter the liver; or might may be taken up by a different type of antigen presenting cell such as the skin-derived $CD103^+$ dendritic cells (Bedoui et al., 2009).

Sterile immunity against *Plasmodium* sporozoite challenge can be also induced in mice by homologous immunization with infectious (live) wild type sporozoites while receiving a prophylactic regimen of chloroquine (Beaudoin et al., 1977; Orjih et al., 1982; Belnoue et al., 2004) or primaquine (Putrianti et al., 2009). This immunity is directed against the liver stage and mediated by $CD4^+$ and $CD8^+$ T cells, but not antibodies (Belnoue et al., 2004; Roestenberg et al., 2009). More recently, this observation has been translated to humans with the demonstration that human subjects exposed three times to the bites of 10-15 *P. falciparum* infected mosquitoes under the cover of chemoprophylaxis (ChemoProphylaxis and Sporozoites, CPS-immunization; also known as infection–treatment–vaccination, ITV) were sterilely protected against subsequent challenge with *P. falciparum* sporozoites, but not *P. falciparum*-infected erythrocytes (Roestenberg et al., 2009; Bijker et al., 2013, 2014). This protection was sustained for up to 2 years (Roestenberg et al., 2011) and was dose dependent: complete protection was obtained in 4/5, 8/9, and 5/10 volunteers immunized three times with bites from 15, 10, or 5 *P. falciparum*-infected mosquitoes, respectively, and CPS immunization is thus estimated to be about 20 times more efficient than RAS immunization (Bijker et al., 2014). Since chloroquine kills asexual blood stage parasites but not sporozoites or liver stage parasites, in the CPS-model parasite infection is aborted in the early phase of blood-stage infection allowing full liver-stage development of the parasite. Consequently, CPS immunization exposes the host to parasite antigens expressed in early and late liver stages as well as early blood stages (Bijker et al., 2013). However, the protective immunity appears to be directed primarily against the liver-stage of the parasite since CPS-immunized volunteers showed no evidence of protection against blood-stage challenge *in vivo* and IgG from CPS-immunized volunteers did not inhibit asexual blood-stage growth *in vitro* (Bijker et al., 2013). Moreover, this protection appears to be mediated by T cells since protected subjects had significantly higher proportions of $CD4^+$ T cells expressing the degranulation marker CD107a and $CD8^+$ T cells producing granzyme B after *in vitro* restimulation with *P. falciparum*-infected red blood cells (Bijker et al., 2014), and antibodies to nine antigens representing different stages of the *P. falciparum* life cycle did not predict protection (Nahrendorf et al., 2014) even though CPS-immunization induced functional antibodies against *P. falciparum* sporozoites which could inhibit sporozoite traversal through hepatocytes and liver-stage infection (Behet et al., 2014). The antigenic targets of the CPS-induced T cell mediated are not yet known but this is under investigation.

In a variation on the CPS approach, the induction of robust protective immunity by prophylactic administration of antibiotic drugs which specifically inhibit apicoplast biogenesis during exposure to intravenously or mosquito bite transmitted sporozoites was reported in the *P. berghei* murine model (Friesen et al., 2010). This approach was conceived to overcome limitations of ITV associated with drug resistance parasite populations. The correct choice of antibiotic in this model allows for continued liver-stage maturation and exponential expansion of attenuated liver-stage merozoites from a single sporozoite and subsequent release into the host peripheral circulation of merosomes (detached vesicles containing liver stage merozoites) which are incapable of infecting red blood cells, thereby halting the parasite life cycle prior to the asexual blood stage. This immunity appears to be targeted at the liver stage and mediated primarily by $CD8^+$ T cells and IFN-γ. This strategy is distinct from the RAS model where each sporozoite gives rise to only a single attenuated liver-stage parasite, and the primaquine chemoprophylaxis model where liver-stage development is aborted before the onset of nuclear divisions (Putrianti et al., 2009), and the chloroquine chemoprophylaxis model which targets the early blood-stage (Belnoue et al., 2004; Roestenberg et al., 2009).

Another area of active investigation is genetically attenuated parasites (GAP) generated via targeted disruption of genes essential for liver-stage or blood stage development (Mueller et al., 2005). These have been comprehensively reviewed elsewhere (Butler et al., 2011; Matuschewski et al., 2011; Nganou-Makamdop and Sauerwein, 2013) and include GAPs that arrest development early (Δp52/Δp36, ΔSAP1, ΔSLARP) or later (ΔUIS3/UIS4, ΔE1a, ΔE3, ΔFABI, ΔFABB/F, ΔFABZ, and ΔPKG) during liver stage development. In a first-in-human safety and immunogenicity clinical trial, 5/6 volunteers administered GAP sporozoites deleted of two *P. falciparum* pre-erythrocytic stage-expressed genes (P52 and P36) via mosquito bite did not develop blood stage parasitemia (Spring et al., 2013). However, the development of peripheral parasitemia in one volunteer showed that this double knockout GAP was incompletely attenuated. Although no breakthrough blood infections were observed in a study evaluating the *P. yoelii* Δp52/Δp36 GAP (Labaied et al., 2007), others observed developing *P. berghei* or *P. falciparum* liver stages *in vitro* culture with the respective Δp52/Δp36 GAPs and breakthrough blood infections in *P. berghei* Δp52/Δp36 GAP immunized mice, showing that the Δp52/Δp36 GAP was not adequately attenuated (Annoura et al., 2012). A minimal set of screening criteria has been proposed to assess the adequacy of genetically attenuation before advancing candidate GAPs into further clinical development (Annoura et al., 2012). Most recently, a triple gene deleted GAP (*Pf* Δp52/Δp36/Δsap1) had been shown to be completely attenuated in a humanized mouse model (Mikolajczak et al., 2014).

Vaccination with chemically attenuated parasites is also being pursued. In the original studies in mice, chemical attenuation of *P. yoelii* or *P. berghei* sporozoites with the DNA sequence-specific alkylating agent centanamycin conferred sterile immunity *in vivo* following one to three intravenous doses (50/20/20K) of centanamycin-treated *P. yoelii* or *P. berghei* sporozoites (Purcell et al., 2008a,b). The level of protection, parasite-specific antibodies, and IFN-γ-producing $CD8^+$ T cell responses induced by chemically attenuated sporozoites (CAS) were similar to those induced by RAS. In the blood stage, Good et al., (2013) have recently reported that a single immunizing dose of 10^6 *P. chabaudi* parasitized red blood cells chemically attenuated with centanamycin could protect against challenge with 10^5 homologous or heterologous (*P. vinckei* and *P. yoelii*) parasites in a $CD4^+$ T cell dependent manner (Good et al., 2013). Chemically attenuated *P. falciparum* parasitized red blood cells are currently being evaluated in the clinic (Good, personal communication).

Another whole organism based strategy directed at the blood stage of the parasite life cycle was designed to induce T helper 1 (Th1) cell mediated immunity in the absence of antibodies by immunizing with subpatent ultra-low dose parasitized erythrocytes followed by drug treatment (Pombo et al., 2002). This built on observations that parasites in high density could cause apoptosis of parasite-specific T cells (Hirunpetcharat and Good, 1998; Xu et al., 2002). Good et al. (2013) showed that malaria-naive humans deliberately infected four times with approximately 30 viable parasitized red blood cells followed by drug treatment developed robust T cell responses in the absence of antibody which prevented parasite growth in three of four individuals and delayed the onset of parasite growth which remained subpatent in the fourth individual (Pombo et al., 2002). Efficacy against a higher dose challenge post-immunization was not assessed, and the contribution of residual drug to this protection could not be excluded. This ultra-low dose immunization approach has not yet been repeated in humans or mice. However a subsequent study in the *P. chabaudi* model demonstrated that three intravenous infections with a relatively high dose of 100,000 *P. chabaudi* infected erythrocytes followed by drug cure after 48 h and before microscopic patency could protect mice against a 10-fold higher (10^6) parasite challenge; mice had robust cell-mediated immune responses and antibodies to merozoite antigens but variant-specific antibodies were not detectable (Elliott et al., 2005).

These proof-of-concept studies with whole organism based vaccines show that experimental induction of sustained protective immunity to *Plasmodium* spp. parasites is possible.

MALARIA VACCINE STRATEGIES

Evidence that immunity can be induced experimentally or acquired naturally with age and/or exposure suggests two fundamental approaches to vaccine development (Good and Doolan, 2010):

(1) Induce robust immune responses against a selected panel of antigens recognized as immunodominant in the context of natural infection.
(2) Induce a broad immune response against a large number of parasite antigens not necessarily recognized as immunodominant in the context of natural infection in order to mimic the immunity induced by the whole parasite.

Until recently, almost all malaria vaccine efforts have been directed at the former approach. Most of these subunit efforts have targeted only a very small number of target antigens, focusing almost exclusively on CSP for the pre-erythrocytic stage and MSP1 and AMA1 for the blood stage (Schwartz et al., 2012; World

Health Organization [WHO], 2014b) and investigating a variety of vaccine delivery systems. A major emphasis has been on purified recombinant proteins formulated with adjuvant, but viral vectored approaches have become of increasing interest, particularly for the pre-erythrocytic stage where induction of parasite-specific T cell responses is desirable. These have been reviewed extensively elsewhere (Bruder et al., 2010; Crompton et al., 2010b; Anders, 2011; Schwartz et al., 2012; Birkett et al., 2013). However, despite extensive efforts throughout the world spanning many decades and in contrast to the immunity induced by experimental immunization with variations of whole organism based vaccines, candidate subunit vaccines against malaria have been poorly efficacious (Schwartz et al., 2012; World Health Organization [WHO], 2014b). Indeed, it is not surprising that a vaccine based on a single antigen is unlikely to confer solid protection against a complex multi-lifecycle stage parasite expressing thousands of proteins that has co-evolved with the human host for millennia.

This marked lack of success in single-antigen subunit based vaccines, combined with the recognition that an effective malaria vaccine will likely need to be a multi-stage multi-immune response vaccine (Doolan and Hoffman, 1997) given the challenges described above, has caused a resurgence of interest in whole organism vaccine approaches, intended to reproduce the protective immunity induced by exposure to the parasite in experimental challenge models or naturally in the field. However, a number of challenges are associated with whole organism based vaccine strategies (Menard, 2005; Ballou and Cahill, 2007; Anders, 2011). Specific concerns with GAPs include potentially inadequate attenuation, as already demonstrated with the *Pf* Δ52/Δp36 GAP in the only human trial to date (Spring et al., 2013), and reversion to virulence of a parasite that has co-evolved with the human host for millennia if it is genetically modified in only one or a few regions of its genome. Other concerns include logistical challenges associated with manual dissection of sporozoites, route of administration, loss of viability upon cryopreservation, and cold-chain requirements. Additionally, antigenic variability of the parasite means that robust cross-protection from a single strain product is essential. Thus, although promising results have been obtained in preclinical models, it remains to be seen whether the many technical, logistical, and regulatory hurdles associated with large-scale production and field deployment of live-attenuated parasites can be overcome.

Even if these technical, logistical, and regulatory challenges can be overcome, a key question is whether whole parasite approaches will induce optimal immunity. Those approaches are essentially similar to the classical "identify, isolate, and inject" approach pioneered in the late 17th century by Edward Jenner, which has proved successful with a wide range of bacterial and viral pathogens, but not yet any parasitic pathogens or any chronic diseases (reviewed in Doolan et al., 2014). This could be attributed to the complexity of parasites as compared to viruses and bacteria, with larger genomes and multiple intracellular and extracellular life cycle stages. Additionally, it is now well established that microbial pathogens have evolved complex and efficient ways of counteracting and evading innate and adaptive immune mechanisms of the host (Zepp, 2010). Thus, logically, robust immunity against such pathogens would not be induced by strategies using the whole pathogen intended to mimic experimentally that immunity induced by natural exposure. Rather, effective vaccination would require that we do better than nature, by inducing responses that are quantitatively and/or qualitatively different immune response to that induced by natural infection.

Inherent in this approach is the cumulative effect of multiple potentially low level immune responses directed against a number of antigens which may or may not be dominant parasite antigens, which together exceed a response threshold sufficient to protect. We proposed this "threshold of immune response concept" over 15 years ago (Doolan and Hoffman, 1997). Specifically, we proposed that the intensity of an immune response will be determined by the sum of a number of signals received by a T cell (or B cell) with the appropriate receptor, and that although a single antigen (with one or more target epitopes) could be sufficient to generate a protective immune response if it is appropriately presented to the immune system, a wide repertoire of specificities at the epitope level (more antigens) should increase the probability of collectively inducing a protective host immune response. Experimental evidence that responses to a given antigen following protective immunization in mice with RAS are not as high as antigen-specific responses induced by vaccination with antigen-specific peptides, recombinant protein or live vectors which nonetheless fail to protect validates this concept. In an elegant series of studies in the *P. berghei* model, Harty and colleagues were able to define a threshold frequency of $CD8^+$ T cells that predicted long-term sterile immunity against sporozoite challenge, and showed that an extremely high frequency of $CD8^+$ T cells (exceeding 8% of all circulating $CD8^+$ T cells in BALB/c mice and 19% in outbred mice) was required for both a single-antigen subunit vaccination (Schmidt et al., 2008) and RAS immunization (Schmidt et al., 2010); this level greatly exceeding the number of memory $CD8^+$ T cells required for resistance to other pathogens.

A similar requirement for a protective threshold of antibody production for parasite clearance following lethal challenge has been demonstrated in a *P. chabaudi* model using MSP1-specific transgenic $CD4^+$ T cells in immunodeficient mice, where levels of MSP1-specific antibody and the speed of their production correlated with the time of resolution of infection (Stephens et al., 2005). In humans, in the field, a broad repertoire of antibody responses to multiple antigens has been associated with protection from clinical malaria (Osier et al., 2008; Crompton et al., 2010a).

These data suggest that to induce optimal protection against malaria, vaccination with the whole parasite is not required, and would likely be suboptimal. Rather, vaccination with only key components of the parasite that have been rationally selected in the context of relevant biological function would be preferable.

NOT ALL ANTIGENS ARE EQUAL

It is now generally recognized that not all antigens, or epitopes within a given antigen, are equal in the context of host immunity. The phenomenon whereby immune responses are mounted against only one or a few of the entire repertoire of peptide epitopes expressed on a given antigen, or antigens expressed by a given pathogen, is termed immunodominance (Sercarz et al., 1993; Akram and Inman, 2012). In theory, any of the proteins expressed by the parasite genome may be a target of protective

immune responses. However, many proteins expressed by the parasite genome do not elicit immune responses, and for many if not most of the subset which do elicit immune responses, the response is not protective. Also in theory, a robust and effective immune response directed against an accessible dominant target would be highly successful in eliminating the pathogen from the host. However, as noted above, in many cases immune evasion strategies have evolved to allow the pathogen to escape the protective host response. Factors that could influence immunodominance, and their importance in protection, have not been investigated in the context of a complex pathogen, although some hypotheses exist (Doolan et al., 2014).

Although immunization with whole organisms preferentially induces responses against immunodominant epitopes, responses to subdominant T cell epitopes can contribute to controlling infection (Friedrich et al., 2007; Kloverpris et al., 2009; Ruckwardt et al., 2010). Also, the ability to focus the immune response away from dominant antigens or epitopes and towards subdominant antigens or epitopes could be of value in chronic diseases where T cells directed against the immunodominant antigens or epitopes might be anergic but T cells specific for non-dominant epitopes might be reactive. Translating this to infectious diseases where the development of effective vaccines based on immunodominant antigens has thus far not been successful (Good and Doolan, 2010), one could speculate that the critical targets of protective immunity may be those that are not dominant in the context of the whole organism.

In support of this, although the CSP is the dominant sporozoite surface protein and represents a target of immune responses induced by immunization with radiation attenuated *Plasmodium* sporozoites, those responses are much weaker than responses induced by CSP-based subunit vaccines, and responses are also directed against other non-CSP antigens (Doolan et al., 1997, 2000, 2003; Kumar et al., 2006; Gruner et al., 2007; Trieu et al., 2011). Notably, sterile $CD8^+$ T-cell mediated immunity to sporozoite challenge could be induced by immunization with RAS in JHT transgenic mice that were tolerant to CSP, so this protection was directed against non-CSP antigens (Kumar et al., 2006). Sterile protection could be also induced by RAS or CPS immunization with transgenic *P. berghei* parasites in which the endogenous CSP was replaced by that of *P. falciparum* or *P. yoelii,* respectively, despite the absence of immune responses specific to the CSP expressed by the parasite used for challenge (Gruner et al., 2007; Mauduit et al., 2010). Also, human volunteers protected by immunization with RAS did mount $CD8^+$ and $CD4^+$ T cell and antibody responses to CSP but those responses were similar to, or lower than, those in immunized volunteers who were not protected against sporozoite challenge indicating that the RAS-induced protective immune responses are directed predominantly against non-CSP antigens (Doolan et al., 1997, 2000, 2003; Trieu et al., 2011). Recently, using protein microarrays, we have identified a signature of 19 mostly uncharacterized antigens which is strongly associated with RAS-induced protective immunity; reactivity to any individual antigen did not correlate with protection (Trieu et al., 2011).

Accumulating experimental data in preclinical and clinical studies of malaria thus indicate that in fact not all antigens are equal, that antigen selection is important, and that it is the cumulative response to a number of key antigens that is important, rather than a dominant response to a single antigen.

GENOME-BASED VACCINE DESIGN

The identification within the hierarchy of antigens (or epitopes) expressed by the pathogen that are targets of protective immune responses and that will stimulate effective immunity against that pathogen is a key component of rational vaccine design (Rueckert and Guzman, 2012). Cutting-edge technologies and screening strategies to mine genomic sequence information for state-of-the-art rational vaccine design, as well as genome-based rational vaccine design strategies, and recently reviewed elsewhere (Doolan et al., 2014; Schussek et al., 2014). The challenge, then, is how to select the key targets since there is no algorithm that can be applied to identify the important antigens and epitopes. Advances in the genomic era offer great potential, particularly when the genome of the target pathogen is large, and large-scale genomic, proteomic and transcriptomic datasets provide valuable resources to mine for antigen discovery.

In the case of malaria, the recent availability of large-scale genomic (**Table 1**), proteomic (**Table 2**), transcriptomic (**Table 3**) and comparative data from *P. falciparum* and other *Plasmodium* species provides an unprecedented opportunity to identify key targets antigens of protective immunity amongst the large repertoire of antigens expressed by the whole parasite. Since 2002, the genomes of seven *Plasmodium* parasites have been published, including that for the two major human parasites (*P. falciparum, P. vivax*) (Gardner et al., 2002; Carlton et al., 2008; Pain et al., 2008); two non-human primate parasites (*P. knowlesi*, *P. cynomolgi*; Pain et al., 2008; Tachibana et al., 2012), and three murine parasites (*P. yoelii* 17XNL, *P. berghei, P. chabaudi*; Carlton et al., 2002; Hall et al., 2005). Draft complete genomes are also available for the avian malaria parasite *P. gallinaceum*, non-human primate parasite *P. reichenowi* Denni, the lethal murine parasite *P. yoelii YM,* and three different strains of *P. falciparum* (HB3,Dd2 and IT; **Table 1**). Partial genome sequence is also accessible for 21 isolates of *P. falciparum* and four isolates of *P. vivax* from geographically distinct areas of the world, as well as low coverage draft genomes for the other two parasites infecting humans, *P. malaria* and *P. ovale*. With the advent of next-generation sequencing technology, the sequencing of genomes for an additional 105 *Plasmodium* species/strains/isolates have been proposed by the malaria community[6]. These parasites include 50 *P. falciparum* field isolates collected from patients in East Africa, America, and Asia; 24 *P. falciparum* parasites representing both contemporary or historical parasite strains, including strains used in drug and vaccine trials; 16 *P. vivax* isolates from Africa, America, and Asia; four non-human primate parasites (*P. reichenowi, P. cynomolgi*, *P. inui*, *P. coatneyi*, *P. fragile*); and complete sequence and closure of three murine parasites (*P. chabaudi*, *P. yoelii*, and *P. berghei* and two avian and reptile parasites (*P. relictum* and *P. mexicanum*). For some of these parasites, a first partial assembly is already available (**Table 1**).

[6]http://www.genome.gov/pages/research/der/pathogensandvectors/plasmodiumwhitepaperv8.pdf

Table 1 | *Plasmodium* genomic datasets.

Genome	Strain	Host	Submitter	Status	Reference
P. falciparum	*3D7*	Human	Genome Sequencing Consortium	High quality genome produced in 2002. Updated and reassembled version available on GeneDB (September 2011)	Gardner et al. (2002); GeneDB; PlasmoDB; Parasite Genomics Group (WTSI)
P. falciparum	*IT*	Human	WTSI*	Draft genome produced using Sanger sequencing and Illumina sequence-by-synthesis. Annotation version available on GeneDB (March 2013)	GeneDB; PlasmoDB, Parasite Genomics Group (WTSI)
P. falciparum	*HB3, Dd2*	Human	Broad Institute	Draft genomes available on NCBI[$] (September 2009)	NCBI
P. falciparum	*20 strains*	Human	Broad Institute	First-pass partial assemblies available on NCBI for *7G8, CAMP/Malaysia, Vietnam Oak-Knoll (FVO) D10, D6, FCH/4, IGH-CR14, K1, MaliPS096_E11, NF135/5.C10, NF54, Palo Alto/Uganda, RAJ116, RO-33, Santa Lucia, Senegal_V34.04, Tanzania, UGT5.1, VS/1, Brasil I.*	NCBI
P. vivax	*Sal-1*	Human	TIGR	Published in 2008. Updated and reassembled version (10x coverage) available on GeneDB (May 2013)	Carlton et al. (2008); GeneDB; PlasmoDB; Parasite Genomics Group (WTSI)
P. vivax	*India VII, Mauritiana, North Korean*	Human	Broad Institute	First-pass partial assembly available on NCBI (July 2012)	NCBI
P. malariae		Human	WTSI	Partial draft genome	Parasite Genomics Group (WTSI)
P. ovale		Human	WTSI	Low-coverage draft produced using Sanger sequencing, from multiple sources of *P. ovale* DNA (Nigeria I/CDC strain and LSHTM)	Parasite Genomics Group (WTSI)
P. knowlesi	*H*	Primate and Human	WTSI	Published in 2008. Updated and reassembled version (8× coverage) available on GeneDB (March 2014)	Pain et al. (2008); GeneDB; PlasmoDB; Parasite Genomics Group (WTSI)

(Continued)

Table 1 | Continued

Genome	Strain	Host	Submitter	Status	Reference
P. cynomolgi	*B*	Primate	Osaka University	Draft genome obtained using Illumina Sequence-by-synthesis technology. Updated contig sequence and annotation available on PlasmoDB (September 2013)	Tachibana et al. (2012); PlasmoDB, Parasite Genomics Group (WTSI)
P. reichenowi	*CDC Dennis*	Primate	WTSI	First-pass partial assembly produced using Sanger sequencing available on NCBI (May 2014)	Parasite Genomics Group (WTSI).
P. inui	*San-Antonio1*	Primate	Broad Institute	First-pass partial assembly available on NCBI (January 2014)	NCBI
P. coatneyi	*Hackeri*	Primate	NHGRI	First-pass partial assembly available on NCBI (July 2014).	NCBI
P. gaboni	*Pgk*	Chimpanzee	TIGR**	First-pass partial assembly available on NCBI (February 2014)	NCBI
P. gallinaceum	*8A*	Avian	WTSI	Low-coverage draft genome produced by Sanger sequencing. Updated high quality draft genome is being produced using Illumina Sequence-by-synthesis.	Parasite Genomics Group (WTSI)
P. yoelii yoelii	*17X and 17XNL*	Murine	WTSI	Low-coverage draft genome of 17XNL published in 2002. Updated and reassembled version of 17X available on GeneDB (May 2013)	Carlton et al. (2002), Hall et al. (2005); GeneDB; PlasmoDB; Parasite Genomics Group (WTSI)
P. yoelii yoelii	*YM*	Murine	WTSI	First-pass partial assembly available on GeneDB (January 2012)	GeneDB; Parasite Genomics Group (WTSI)
P. chabaudi	*chabaudi*	Murine	WTSI	Low-coverage draft genome published in 2005. Additional sequencing completed using Illumina Sequence-by-synthesis technology and available on GeneDB (March 2013)	Hall et al. (2005); GeneDB; PlasmoDB. Parasite Genomics Group (WTSI)
P. berghei	*Anka*	Murine	WTSI	Low-coverage draft genome published in 2005. Additional sequencing completed using Illumina Sequence-by-synthesis technology and available on GeneDB (March 2013)	Hall et al. (2005); GeneDB; PlasmoDB; Parasite Genomics Group (WTSI)
P. vinckei	*Petteri, Vinkei*	Murine	Broad Institute	First-pass partial assembly available on NCBI (2014)	NCBI

**WTSI, Wellcome Trust Sanger Institute; **TIGR, The Institute for Genomic Research; $NCBI, National Center for Biotechnology Information*

Table 2 | *Plasmodium* proteomic datasets.

Species/Strain	Parasite material	Method	Description	Reference
P. falciparum 3D7	Infected erythrocytes and gametocytes	MudPIT*	*P. falciparum* asexual blood stage and sexual stage proteomes. 2,415 parasite proteins identified.	Florens et al. (2002)
P. falciparum NF54	Trophozoites, schizonts, gametocytes, and gamete	LC-MS/MS**	*P. falciparum* asexual blood stage and sexual stage proteomes. 1,289 proteins detected: 714 in asexual blood stages, 931 in gametocytes and 645 in gametes.	Lasonder et al. (2002)
P. falciparum 3D7	Infected erythrocytes	MudPIT	*P. falciparum* parasite infected erythrocyte surface proteome. 423 proteins identified.	Florens et al. (2004)
P. falciparum 3D7	Infected erythrocytes	MudPIT	*P. falciparum* infected erythrocyte proteome. 802 proteins identified in the nuclear proteome.	Oehring et al. (2012)
P. falciparum 3D7 and P. vivax Sal-1	Infected erythrocytes	LC-MS/MS	Proteomic analyses of clinical isolates of early stages of *P. falciparum* and *P. vivax*. 100 proteins identified.	Acharya et al. (2009)
P. falciparum 3D7, F12	Trophozoites, gametocytes	LC-MS/MS	Quantitative comparative proteomics analysis of trophozoites and early gametocyte stages of *P. falciparum*. 1090 new proteins identified.	Silvestrini et al. (2010)
P. vivax Sal-1	Infected erythrocytes	MS/MS†	Elucidation of the *P. vivax* schizont proteome from clinical sample. 316 proteins identified.	Roobsoong et al. (2011)
P. falciparum 3D7 and P. berghei ANKA	Midgut and salivary glands Sporozoites	nLC-MS/MS	Proteomic comparison of sporozoites from oocysts and salivary glands. 127 proteins identified in oocysts, 450 in oocyst-derived sporozoites, and 477 in salivary gland sporozoites.	Lasonder et al. (2008)
P. falciparum 3D7 and P. yoelii 17XNL	Salivary gland sporozoites	LTQ Orbitrap Velos§, nLC-MS/MSi	Putative surface proteomes of *P. falciparum* and *P. yoelii* salivary gland sporozoites. 1991 *P. falciparum* sporozoite proteins and 1876 *P. yoelii* sporozoite proteins identified.	Lindner et al. (2013)
P. berghei ANKA	Mixed asexual blood stages, gametocytes, ookinetes, oocysts and salivary gland sporozoites	MudPIT	*P. berghei* oocysts and sporozoite proteomes. 1836 proteins identified: 1139 in blood stage, 1091 in ookinetes, 733 in gametocytes, 277 in oocysts and 134 in salivary gland sporozoites.	Hall et al. (2005)
P. berghei ANKA	Gametocytes	LC-MS/MS	Comparative proteomic analysis of male vs female gametocytes. 353 proteins in mixed-gametocyte proteome identified, 305 proteins in male gametocytes, and 170 proteins in female gametocytes.	Khan et al. (2005)
P. yoelii 17X	Infected hepatocytes	LC-MS/MS	*P. yoelii* liver stage proteome. 712 proteins identified.	Tarun et al. (2008)

**MudPIT, Multidimensional protein identification technology; ** LC-MS/MS, liquid chromatography tandem mass spectrometry; nLC-MS/M S, nano flow liquid chromatography mass spectrometry; † MS/MS, tandem mass spectrometry; §LTQ Orbitrap Velos, TQ Orbitrap Velos mass analyzer coupled to nano-liquid chromatography.*

Table 3 | *Plasmodium* transcriptomics datasets.

Species/Strain	Parasite material	Method	Description	Reference
P. falciparum 3D7	Nine different life cycle stages: mosquito salivary gland sporozoites, seven asexual erythrocytic stage time ponts, and sexual stage gametocytes	Microarray	Gene expression profiles of human and mosquito stages of *P. falciparum* life cycle. 43% of expressed genes were cell-cycle regulated; 1489 genes regulated in erythrocytic stages, and 746 genes differentially regulated in sporozoites and gametocytes.	Le Roch et al. (2003)
P. falciparum 3D7	Trophozoites and schizonts	Microarray	Gene-expression profile of the intraerythrocytic trophozoite and schizont stages. Revealed extensive transcriptional regulation of genes specialized for processes specific to trophozoites or schizonts.	Bozdech et al. (2003)
P. falciparum 3D7	Gametocytes stages I–V	Microarray	Transcriptomic analysis of high-purity stage I-V *P. falciparum* gametocytes. Identified a sexual development cluster of 246 genes exhibiting highly correlated, gametocyte-specific expression patterns.	Young et al. (2005)
P. falciparum 3D7, Dd2, HB3	Asexual erythrocytic stages	Microarray	Transcriptome of asexual intraerythrocytic developmental cycle (3D7, 6287; Dd2, 5294; HB3, 6415 genes). 60% of genome identified as transcriptionally active during erythrocytic stage. Transcripts profiles were well conserved amongst strains, except for surface antigens.	Bozdech et al. (2003) Llinas et al. (2006)
P. falciparum: 21 lines, from four subclonal groups (3D7A/3D7B, 7G8, D10, HB3A/HB3B)	Asexual erythrocytic stage at seven time points post-infection (10, 20, 30, 34, 37, 40, or 43 h)	Microarray	Asexual blood-stage transcriptome of 21 *P. falciparum* lines, from four subclonal groups. Transcriptional heterogeneity among essentially isogenic parasites was observed.	Rovira-Graells et al. (2012)
P. falciparum 3D7	Peripheral blood samples from infected patients	Microarray	*In vivo* gene expression profiles of parasites isolated from clinical samples. Expression profiles clustered into three distinct groups corresponding to distinct physiological states: glycolytic growth, starvation response, or general (non-nutritional) stress response.	Daily et al. (2007)
P falciparum 3D7 and NF54	Sporozoites and gametocytes (3D7 stages II, III, and V; NF54, stage V)	Quantitative PCR	Transcript profiles of *rif* and *var* genes. A single rif gene, PF13_0006, showed high transcript abundance in mature gametocyte stage V and in sporozoites.	Wang et al. (2010)
P. falciparum FcB1	Late schizont/merozoite stages and rings/trophozoites/early schizonts stages	ESTs	EST library of highly synchronized *P. falciparum* parasites to isolate genes selectively expressed during merozoite morphogenesis, using SSH*. Identified genes selectively expressed during the last hours of the erythrocytic cycle. Subsequently identified 243 protein coding genes, including 121 hypotheticals, by sequencing 22,125 clones from the SSH library.	Florent et al. (2004, 2009)

(Continued)

Table 3 | Continued

Species/Strain	Parasite material	Method	Description	Reference
P. falciparum 3D7	Asexual erythrocytic stage at 8 time points post-infection (5, 10, 15, 20, 25, 30, 35, and 40 h)	RNA-seq	RNA-seq analysis of the transcriptome throughout intraerythrocytic development. Variation in overall transcriptional activity with stage-specific regulation (low at early stages, peaking at trophozoite stage).	Bartfai et al. (2010)
P. falciparum 3D7	Asexual erythrocytic stage at seven time points post-infection	RNA-seq	Illumina-based RNA seq throughout intraerythrocytic development. Identified 107 novel transcripts and 38 pseudogenes, with many demonstrating differential expression over time.	Otto et al. (2010)
P. falciparum 3D7	Seven life cycle stages: two gametocyte stages (II and V), ookinete, and four asexual erythrocytic stages (ring, early trophozoite, late trophozoite, and schizont	RNA-seq	Transcriptomic analysis of asexual and sexual stages. Identified many unknown splicing junctions and stage specific gene expression including occyst-specific genes.	Lopez-Barragan et al. (2011)
P. falciparum 3D7	Parasites cultured *in vitro*, as well as two pools of field isolates from *Plasmodium*-infected pregnant women and children	NSR-seq	Transcriptome of *in vitro* and *in vivo* blood stages. Identified a subset of genes upregulated in parasite-infected pregnant women; and a subset of genes that differentiated parasites infecting children from parasites infecting pregnant women.	Vignali et al. (2011)
P. falciparum 3D7	Mixed asexual erythrocytic stages	ESTs	7,683 *P. falciparum* 3D7 ESTs were generated from mixed asexual stages.	Zhang et al. (2011)
P. falciparum 3D7	Asexual erythrocytic stages	SAGE	Transcriptional profile of erythrocytic stages in different studies.	Patankar et al. (2001), Gunasekera et al. (2003, 2004)
P. vivax clinical isolates	Peripheral blood samples from infected patients	Microarray	Complete transcriptional profile throughout the intraerythrocytic cycle of three clinical isolates from acute *P. vivax* patients. Identified distinct expression patterns genes predicted to encode proteins associated with virulence and host pathogen interactions.	Bozdech et al. (2008)
P. vivax Sal-1	Human and mosquito stages, including sporozoites, gametes, zygotes and ookinetes, and *in vivo* asexual blood stages	Microarray	Characterization of the *P. vivax* transcriptome. Distinct stage-specific expression profiles. Identified DNA sequence motifs upstream of co-expressed genes that are conserved across different species.	Westenberger et al. (2010)

(Continued)

Table 3 | Continued

Species/Strain	Parasite material	Method	Description	Reference
P. yoelii yoelii 17X	Infected hepatocytes at 3 timepoints post-infection (24, 40 and 50 hr); midgut-oocyst sporozoites and salivary gland sporozoites; and mixed blood stages and blood-stage schizonts	Microarray	Profile of genome-wide liver stage gene expression was compared with other life cycle stages. Identified 1985 genes active during liver stage development including 1000 upregulated genes and 174 genes that were more abundant or unique in the liver stage.	Tarun et al. (2008)
P. falciparum	cDNA from *P. falciparum* salivary gland sporozoites vs. sporozoites co-cultured with human primary hepatocytes for 1 h	Microarray	Transcriptome of salivary gland sporozoites was compared with that of sporozoites co-cultured with hepatocytes. 532 genes were up-regulated and 79 genes downregulated following co-culture, in comparison to non-exposed salivary gland sporozoites. Two proteins with temporal upregulation (PFD0425 [SIAP1] and Pf08_0005 [SIAP2]) implicated in both traversal and hepatocyte invasion.	Siau et al. (2008)
P. yoelii yoelii 17XNL	Midgut sporozoites and salivary gland sporozoites	Microarray	Comparative transcriptome study of *P. yoelii* oocyst sporozoites and salivary gland sporozoites. 124 genes were upregulated and 47 downregulated in salivary gland sporozoites. Similar transcription profiles of 11 *P.falciparum* orthologs confirmed by qPCR.	Mikolajczak et al. (2008)
P. yoelii yoelii 17X	cDNA library from *P. yoelii* liver stages laser-capture microdissected at 40 h post infection	EST	623 non-redundant genes were identified, of which 25% were unique to the liver stage.	Sacci et al. (2005)
P. berghei HPE and HP	Non-gametocyte producing clone (HPE) and gametocyte producing clone (HP); rings, young trophozoite, young schizont, young gametocyte, mature trophozoite, mature and mature gametocytes	Microarray	Comparative transcriptomes of non-gametocyte and p gametocyte producing clones of *P. berghei*. 215 and 355 genes were upregulated in the G1 and the S/M phases, respectively. 58% of the G1 proteins (125 genes) and 59.4% of the S/M proteins (199 genes) were also up-regulated in gametocytes.	Hall et al. (2005)
P. falciparum 3D7, P. vivax Sal-1, P. yoelii, P. berghei ANKA	Full-length cDNA libraries (all stages)	ESTs	Comparative transcriptomes of full-length cDNA sequences of *P. falciparum* (12,484 cDNAs), *P. vivax* (9,633 cDNAs), *P. yoelii* (11262), and *P. berghei* (1518 cDNAs).	Watanabe et al. (2007)

**SSH, suppression subtractive hybridization.*

In addition to this genomic data, the rapid development of high throughput technologies for profiling the transcriptome, proteome, metabolome, and interactome, including capillary liquid chromatography, tandem mass spectrometry (LC-MS/MS), Multidimensional Protein Identification Technology (MudPIT), microarray DNA chip, yeast two-hybrid (Y2H) screening and most recently RNA seq and NSR-seq (Winzeler, 2006) can be applied for the rational identification of potential vaccine candidate antigens.

In early studies, proteomes of four stages of the *P. falciparum* parasite life cycle (sporozoites, merozoites, trophozoites, and gametocytes) were revealed by MudPIT (Florens et al., 2002) as well as the proteome of the asexual blood stages (trophozoites and schizonts) and sexual stages (gametocytes and gamete) by LC-MS/MS analysis (Lasonder et al., 2002). This *P. falciparum* sporozoite proteome included a total of 1048 proteins of which almost half (49%) were unique to this stage. The proteomes of *P. berghei* oocysts and sporozoite were subsequently defined by MudPIT in 2005, resulting in the identification of 1836 proteins (Hall et al., 2005). Recently, nano-liquid chromatography (nanoLC) coupled high-resolution MS was applied to profile the proteome of highly purified salivary gland sporozoites from *P. falciparum* and *P. yoelii*, identifying a total of 1991 *P. falciparum* sporozoite proteins and 1876 *P. yoelii* sporozoite proteins (Lindner et al., 2013).The liver stage proteome was defined in the rodent host *P. yoelii* by LC-MS/MS resulting in the detection of 712 proteins in the liver stage schizont proteome, with 174 of them more abundant and/or detected only in the liver stage (Tarun et al., 2008).

A study of *P. falciparum* infected erythrocytes, fractionated through biotin-streptavidin interaction and analyzed by MudPIT, identified 164 proteins of the 423 proteins that were enriched the in biotin-labeled fractions and thus considered surface proteins. Among these were known secreted proteins, such as Exp-1 and Exp-2, and rhoptry proteins (RAP-1, -2, and -3, RhopH-2 and -3; Florens et al., 2004). Another LC-MS/MS study of early stages from *P. falciparum* clinical isolates detected 88 *P. falciparum* proteins in the peripheral circulation (Acharya et al., 2009). More recent analyses on the proteome of asexual trophozoites, early gametocytes, and mature gametocytes from *in vitro* culture by high accuracy (LC MS/MS) have identified over new 1000 parasite proteins, not previously identified (Silvestrini et al., 2010). Most recently, proteomic analysis by MudPIT of *P. falciparum* nuclei of ring, trophozoite and schizont stages has elucidated the nuclear proteome of *P. falciparum* during intra-erythrocytic development, consisting of over 800 proteins (Oehring et al., 2012).

Elucidation of the gametocyte sex-specific proteomes of *P. falciparum* by LC-MS/MS resulted in the identification of 305 unique proteins in the male gametocyte proteome and 170 unique proteins in the female gametocyte proteome (Khan et al., 2005). The identification of sex-specific proteins has brought new insight in understanding the role of these proteins during the sexual differentiation and thus proving the basis for identifying targets for the interruption of transmission, either by drugs or vaccines.

Other comparative LC MS/MS proteomic studies of *P. falciparum* and *P. berghei* have identified novel proteins in the pre-erythrocytic stages of the *Plasmodium* life cycle: 127 proteins in the oocyst proteome, 450 proteins in oocyst-derived sporozoites and 477 proteins in salivary gland sporozoites, for a total of 728 *Plasmodium* proteins, of which 250 were exclusively detected in the oocyst/sporozoite stages when compared to the *P. falciparum* blood stage proteome (Lasonder et al., 2008).

More recently, the proteome of *P. vivax* asexual schizonts has been defined by analyzing fresh parasite isolates from patients exposed to *P. vivax* by tandem MS/MS (Roobsoong et al., 2011).

Complementing the proteomic analyses, a number of transcriptomics studies have been undertaken, ranging from analysis of gene transcription using random clones selected from genomic DNA libraries to more recent global expression transcription profile using oligonucleotide microarray, RNA-seq or NSR-seq. Transcriptomic data are now available from multiple life-cycle stages or gene knock-out mutants of *P. falciparum* and *P. berghei* as well as multiple stages of *P. yoelii* (mosquito, erythrocytic and liver stages; **Table 3**). Specifically, transcriptomic data available for *P. falciparum* include genome-scale transcriptomic analyses of nine different life cycle stages (3D7 strain) including salivary gland sporozoite, early and late ring stage, early and late trophozoite, early and late schizont, merozoite, and gametocyte stages (Le Roch et al., 2003); the intraerythrocytic trophozoite and schizont stages (Bozdech et al., 2003); the intraerythrocytic developmental cycle of *P. falciparum* HB2, Dd2, and 3D7 strains (Llinas et al., 2006); as well as 21 other *P. falciparum* lines from four subclonal groups (3D7A/3D7B, 7G8, D10, HB3A/HB3B) during the asexual intraerythrocytic developmental cycle at seven time points post-infection (10, 20, 30, 34, 37, 40, or 43 h; Rovira-Graells et al., 2012) plus analysis of parasites derived directly from blood samples from 43 infected patients (Daily et al., 2007). The transcriptome of high-purity stage I-V *P. falciparum* gametocytes is also available (Young et al., 2005), as well as the transcript profiles of rif and var genes at different stage of gametocytogenesis (Wang et al., 2010).

In addition to genome-wide gene expression studies, RNA-seq analysis of the *P. falciparum* transcriptome is available for multiple time points during the intraerythrocytic developmental cycle (Bartfai et al., 2010; Otto et al., 2010; Lopez-Barragan et al., 2011); and two gametocyte stages (Lopez-Barragan et al., 2011). The transcriptional profile of two pools of field isolates from malaria-infected pregnant women and children has been also determined by NSR-seq (Vignali et al., 2011).

For *P. vivax*, transcriptomic data include genome-scale transcriptomic analyses throughout the intraerythrocytic cycle of three distinct *P. vivax* isolates (Bozdech et al., 2008) as well as sporozoites co-cultured with hepatocytes (Siau et al., 2008); and sporozoites, gametes, zygotes, and ookinetes, and asexual blood stages obtained from infected patients (Westenberger et al., 2010).

Plasmodium yoelii gene expression data are available for oocyst sporozoites and salivary gland sporozoites (Mikolajczak et al., 2008); three time points during the liver stage (24, 40, and 50 h post-infection), two time points during the mosquito stage (midgut-oocyst sporozoites and salivary gland sporozoites; Tarun et al., 2008), and two intraerythrocytic stages (Tarun et al., 2008). *P. berghei* expression data are available for rings, young

trophozoites, young schizonts, and mature schizonts (Hall et al., 2005).

Other transcriptomic datasets include EST data from cDNA libraries of *P. falciparum* (12,484 cDNA sequences), *P. vivax* (9,633 cDNAs), *P. yoelii* (11,262 cDNAs), and *P. berghei* (1,518 cDNAs) (Watanabe et al., 2007; Tarun et al., 2008), as well as 7,683 *P. falciparum* 3D7 ESTs generated from mixed asexual stages and SAGE data (Patankar et al., 2001; Gunasekera et al., 2003, 2004).

These genome-wide genomic, proteomic, and transcriptomics analyses (**Tables 1–3**) have revealed potential antigens expressed in the sporozoite and intrahepatic stages and novel proteins on the surface of malaria-infected erythrocytes that may play a role in pathogenesis and immunity, and that may represent potential new vaccine candidates.

Several novel parasite surface antigens have been discovered (Florens et al., 2002, 2004; Lasonder et al., 2002; Le Roch et al., 2004; Sam-Yellowe et al., 2004) but, so far, this wealth of data has yielded few new vaccine targets (**Table 4**; Duffy et al., 2012). In our opinion, translation of this wealth of information from the large-scale genomic, proteomic, and transcriptomic datasets into practical application, such as the identification of promising new target antigens for vaccine development, requires integrating this knowledge with functional outputs such as biologically relevant immune responses. Thus, in our laboratory, we are pursuing immunomics-based approaches which integrate the disciplines of genomics and immunology using biological samples from humans or animals with immunity to the disease of interest to identify the subset of pathogen-derived proteins or their epitopes that are recognized by the host immune system (Klysik, 2001; Doolan, 2011). No vaccines derived from immunomics have yet reached the stage of clinical testing but a number of promising candidate antigens have been identified by us in the malaria model using antibody based (Doolan et al., 2008; Crompton et al., 2010a; Trieu et al., 2011) or T-cell based (Doolan et al., 2003; Doolan, 2011). We are using peripheral blood mononuclear cells (T cells) and plasma/sera (antibodies) from individuals experimentally immunized with RAS or CPS or naturally exposed to malaria for proteome-wide immune screening assays using clinically relevant

Table 4 | *Plasmodium* antigens identified from genome-based datasets.

Antigen	Model	Main finding	Reference
Ag2/CelTOS	*P. falciparum* RAS T cell screening; *P. yoelii* and *P. berghei* murine immunization/challenge	One of four highly reactive *P. falciparum* proteins identified by T cell based screening of 27 putative proteins with RAS immunized volunteers; conferred cross-species protection against *P. yoelii* and *P. berghei* sporozoite challenge.	Doolan (in preparation), Doolan et al. (2003), Bergmann-Leitner et al. (2010)
Thirty-four pre-erythrocytic antigens	*P. yoelii* murine immunization/challenge	Only three antigens (P33p[*Py*52] [PY01340], Ag2 [*Py*CelTOS], and Ag5[PY00419] elicited $CD8^+$ T cell responses but none conferred protection.	Mishra et al. (2011)
PY03011, PY03424, and PY03661: pre-erythrocytic antigens	*P. yoelii* murine immunization/challenge	The combination of the three antigens (but not individual antigens) conferred sterile protection against *P. yoelii* sporozoite challenge in a high proportion of mice.	Limbach et al. (2011)
PyTmp21(PY06414): pre-erythrocytic antigen	*P. yoelii* murine immunization/challenge	*Py*Tmp21 elicited functional immunity that significantly reduced liver stage parasite burden following *P. yoelii* sporozoite challenge.	Chen et al. (2014)
PbS20 and PbTRAP pre-erythrocytic antigens	*P. berghei* murine immunization/challenge	Systematically evaluated H(2b)-restricted peptides predicted from genome-wide analysis, and identified two epitopes as targets of $CD8^+$ T cells induced by whole parasite vaccines; $CD8^+$ T cells specific for the *Pb*TRAP epitope but not the PbS20 epitope inhibited liver stage parasite development *in vivo*	Hafalla et al. (2013)
PyTAM: blood-stage antigen	*P. yoelii* murine immunization/challenge	*Py*TAM elicited functional immunity that conferred significant protection against mortality from lethal *P. yoelii* challenge infection.	Cherif et al. (2014)
Py01157: sexual and sexual stage antigen	*P. yoelii* murine immunization/challenge	*Py*01157: conferred partial protection against challenge with non-lethal *P. yoelii* 17XNL, but not against challenge with lethal *P. yoelii* 17XL.	Zhang et al. (2012)

selection criteria to identify the key antigens and their epitopes recognized by recall *Plasmodium*-specific immune responses in protective human models. Data generated to date establish proof-of-concept for both T cell and antibody based approaches to identify from genomic datasets the subset of antigens and epitopes which represent promising candidates for next generation malaria vaccine development (Doolan, 2011; Matuschewski et al., 2011; Doolan et al., 2014; **Table 4**).

In the initial proof-of-concept study (Doolan et al., 2008) for the application of protein microarray technology for antigen discovery, a protein microarray with 250 pre-erythrocytic *P. falciparum* proteins were probed with sera from individuals with sterile immunity or no immunity against experimental challenge following vaccination with radiation-attenuated *P. falciparum* sporozoites, partial immunity acquired by natural exposure, or no previous exposure to *P. falciparum*, to identify 72 highly reactive P. falciparum antigens. Subsequently, a larger protein microarray containing 2,320 *P. falciparum* proteins fragments (corresponding to 1200 *P. falciparum* proteins, ∼25% of the proteome) was screened with plasma from clinically divergent groups of individuals immunized with *P. falciparum* irradiated sporozoites and identified a signature of 16 antigens strongly associated with RAS-induced sterile protective immunity; responses to any individual antigen were not associated with protection (Trieu et al., 2011). Using this same protein microarray, plasma from children and young adults naturally exposed to malaria in an endemic area of Mali were screened and 46 novel proteins significantly associated with a reduction of re-infection in the subsequent malaria season were identified (Crompton et al., 2010a). One *P. falciparum* antigen identified by T cell based screening of RAS-immunized volunteers (Doolan et al., 2003), known as Ag2 or CelTOS (Kariu et al., 2006), has been shown to be a target of cross-species protection in the murine model (Bergmann-Leitner et al., 2010). Another *P. yoelii* antigen, PyTmp1, could induce immune responses that reduced liver stage parasite burden *in vivo* (Chen et al., 2014), and three other *P. yoelii* antigens in combination (but not individually) could induce sterile immunity in a high proportion of immunized mice (Limbach et al., 2011). Two *P. yoelii* blood stage antigens, PyTAM and Py01157, have been also associated with partial protection in the murine model (Zhang et al., 2012; Cherif et al., 2014; **Table 4**). These data support the potential of genome-based antigen discovery. Assessment of the protective capacity of promising new antigens discovered by genome-based screening is currently a bottleneck in the pre-clinical development pipeline. Development and refinement of a high throughput screening system would overcome this obstacle and allow the development of a rationally designed genome-based vaccine against malaria.

CONCLUSION

We advocate a modern genome-based approach to rational vaccine design which takes advantage of the wealth of genomic, proteomic, and transcriptomic datasets, using biological samples from humans or animals with immunity to the disease of interest and functionally relevant screening criteria to identify the key antigens and their epitopes targeted by protective immune responses. With this focus, we can improve on nature. In the case of malaria, validated human challenge models provide a valuable resource for genome-based antigen discovery. Moreover, this information can be integrated with other omic-based and cutting-edge immunological based approaches. For example, systems immunology could be applied to identify immune signatures that distinguish a protective immune response from a non-protective one. The many technological and intellectual advances in the omics era offer great potential for the development of rationally designed vaccines against malaria as well as other pathogens that have thus far proved elusive.

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Montanide, Poly I:C and nanoparticle based vaccines promote differential suppressor and effector cell expansion: a study of induction of CD8 T cells to a minimal *Plasmodium berghei* epitope

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The development of practical and flexible vaccines to target liver stage malaria parasites would benefit from an ability to induce high levels of CD8 T cells to minimal peptide epitopes. Herein we compare different adjuvant and carrier systems in a murine model for induction of interferon gamma (IFN-γ) producing CD8 T cells to the minimal immunodominant peptide epitope from the circumsporozoite protein (CSP) of *Plasmodium berghei,* pb9 (SYIPSAEKI, referred to as KI). Two pro-inflammatory adjuvants, Montanide and Poly I:C, and a non-classical, non-inflammatory nanoparticle based carrier (polystyrene nanoparticles, PSNPs), were compared side-by-side for their ability to induce potentially protective CD8 T cell responses after two immunizations. KI in Montanide (Montanide + KI) or covalently conjugated to PSNPs (PSNPs-KI) induced such high responses, whereas adjuvanting with Poly I:C or PSNPs without conjugation was ineffective. This result was consistent with an observed induction of an immunosuppressed environment by Poly I:C in the draining lymph node (dLN) 48 h post injection, which was reflected by increased frequencies of myeloid derived suppressor cells (MDSCs) and a proportion of inflammation reactive regulatory T cells (Treg) expressing the tumor necrosis factor receptor 2 (TNFR2), as well as decreased dendritic cell (DC) maturation. The other inflammatory adjuvant, Montanide, also promoted proportional increases in the $TNFR2^+$ Treg subpopulation, but not MDSCs, in the dLN. By contrast, injection with non-inflammatory PSNPs did not cause these changes. Induction of high CD8 T cell responses, using minimal peptide epitopes, can be achieved by non-inflammatory carrier nanoparticles, which in contrast to some conventional inflammatory adjuvants, do not expand either MDSCs or inflammation reactive Tregs at the site of priming.

Keywords: malaria, adjuvant, nanoparticle, CD8 peptide, Treg, MDSC

INTRODUCTION

Malaria affects over 200 million people annually, resulting in over half a million deaths with most mortality coming from infections with *Plasmodium falciparum,* and developing a malaria vaccine has become a major global effort (Arama and Troye-Blomberg, 2014). The most advanced malaria vaccine development focuses on the pre-erythrocytic stage, at which sporozoite parasites enter the circulation after a mosquito bite and then rapidly enter and infect hepatocytes. CD8 T lymphocytes, particularly those capable of producing interferon gamma (IFN-γ), can mediate effective sterile liver-stage immunity (Schneider et al., 1999; Doolan and Martinez-Alier, 2006; Krzych et al., 2014). Developing a CD8 T cell inducing liver-stage vaccine would be beneficial to further avoid the clinical symptoms of malaria, such as fever, associated with subsequent blood stages of infection, as well as preventing transmission and the sexual development of parasites (Arama and Troye-Blomberg, 2014). Whole irradiated sporozoites are effective CD8 T cell inducing vaccines (Doolan and Martinez-Alier, 2006), and immunity to a dominant circumsporozoite protein (CSP) CD8 T cell epitope of *P. berghei*, named pb9 (sequence SYIPSAEKI), can mediate protection in murine animal models (Schneider et al., 1999).

Unfortunately, synthetic and recombinant vaccines have been less effective at inducing CD8 T cells, particularly in humans (Arama and Troye-Blomberg, 2014). The choice of adjuvant and the delivery system for the selected antigens will play a major role in the ability of vaccines to induce CD8 T cell immunity. Minimal CD8 T cell peptide epitopes offer production, stability, and flexibility advantages in vaccine formulation (Plebanski et al., 2006). Herein we compare side by side two adjuvants with proven capacity to promote CD8 T cell responses, Montanide (a water in oil emulsion) and Poly I:C (TLR3 agonist). Both have been used in various clinical trials as adjuvants in human vaccines against specific diseases (Aucouturier et al., 2002; Bonhoure and Gaucheron, 2006; Trumpfheller et al., 2008; Longhi et al., 2009; Mbow et al., 2010). Given cerebral malaria pathology is associated with inflammation (Postels and Birbeck, 2013), the use of novel nanovaccine technologies which induce CD8 T cell immunity

without conventional pro-inflammatory signals also offers a conceptual advantage. Based on our previous studies, such inert nanoparticles coated with a target antigen of choice can promote high levels of immunity in the absence of inflammation or added extrinsic adjuvants, even to peptide based antigens (Fifis et al., 2004a,b; Xiang et al., 2013). Responses are as high as experimental gold standards for antibody production (e.g., Freunds adjuvant) and CD8 T cell induction [e.g., *ex-vivo* antigen pulsed dendritic cells (DCs)], and better than a range of conventional inflammatory experimental adjuvants (Fifis et al., 2004a).

The size of the nanoparticle is a key factor, with even small deviations away from the optimal size range of 40–50 nm causing major decreases in immunogenicity (Fifis et al., 2004a; Mottram et al., 2007). We herein compared Montanide and Poly I:C, representing two pro-inflammatory adjuvants, against such nanoparticle based vaccines for delivery of the minimal pb9 CD8 T cell epitope. Moreover, we speculated that inflammatory responses during the priming phase of immunity could further result in the activation of the immune-suppressive mechanisms that arise to control such inflammation, but may interfere with efficient CD8 T cell stimulation. In this context, it is known that enhancing cross-presenting DC frequency and function, and preventing myeloid derived suppressor cells (MDSCs) accumulation promotes antigen specific immune responses (Ohkusu-Tsukada et al., 2011). It has also been suggested that Poly I:C is capable of increasing antigen specific effector T cells over regulatory T cells (Treg), enhancing immunity (Perret et al., 2013). Hence, as well as comparing the magnitude of the CD8 T cell responses induced by the different adjuvants, this study evaluates the ability of Montanide, Poly I:C, and nanoparticles to promote the induction of inflammation reactive Tregs and the expansion of MDSCs, compared to effector T cells and stimulatory antigen presenting types such as DCs.

MATERIALS AND METHODS

MICE

Six to eight weeks old BALB/c mice were purchased from Monash Animal Services (MAS) Melbourne, VIC, Australia. The studies presented here were approved by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee, Melbourne, VIC, Australia.

NANOVACCINE FORMULATIONS

Conjugation of malaria peptide antigens to nanoparticles was based on the previous described method (Xiang et al., 2013) with a slight modification. Briefly, carboxylated polystyrene nanoparticles (PSNPs; Polysciences Inc, Warrington, PA, USA) of 40 nm (∼40–50 nm) at a final of 1% solids were activated in a mixture containing 2-N-Morpholino-ethanesulfonic acid (MES; 50 mM final, pH = 7), and 1-ethyl-3-(3-dimethylaminopropryl) carbodiimide hydrochloride (EDC; 4 mg/ml final). Malaria peptide SYIPSAEKI (KI; Mimotopes, Melbourne, VIC, Australia; 1 mg/ml final) was also added to the conjugation mix and together incubated on a rotary wheel at room temperature for approximately 4 h. Following antigen incubation, the conjugation reactions were then quenched by adding excess glycine (7 mg/ml final) and further incubated for 30 min. The free, unconjugated peptide antigens, and other excess conjugation agents, were removed by dialysis (10–14 kDa molecular weight cut-off (MWCO) membrane; Viskase, Darien, IL, USA) against phosphate buffered solution (PBS, pH = 7.2) at 4°C overnight. Conjugation efficiency and final sizes of the nanovaccine formulation (e.g., PSNPs-KI) were determined by Bicinchoninic acid assay (BCA; Thermo Fisher Scientific, Rockford, IL, USA) and dynamic light scattering instruments (Zetasizer; Malvern Instruments, Worcestershire, UK), respectively, following the manufacture's instruction.

OTHER VACCINE ADJUVANT AND IMMUNIZATIONS

Other vaccine adjuvants such as Montanide ISA 720 (70% v/v final, Tall Bennett Group, USA), Polyinosinic–polycytidylic acid sodium salt (Poly I:C; 25 μg/mouse final, Sigma Aldrich, St. Louis, MO, USA) were also used in this study. The adjuvant effect of these vaccine formulations were tested *in vivo* by immunization of mice and measuring for IFN-γ production by ELISpot assay (Xiang et al., 2006). Briefly, adjuvant mixed KI formulations (e.g., KI + Montanide; KI + PolyI:C; KI + PSNPs) at the desired final concentrations (∼25 μg KI/mouse) and nanoparticle conjugated KI (PSNPs-KI at 1% solid of PSNPs) formulations were injected into mice intradermally (i.d.) at the base of the tail. 14 days after the last immunization, mice were sacrificed and splenocytes were isolated and assayed for IFN-γ production via ELISpot assay.

ELISPOT ASSAY

Antigen specific CD8 T cell responses were evaluated by IFN-γ ELISpot assay (Xiang et al., 2006). Briefly, 96 well multiscreen filter plates (MSIP plates, Millipore, Billerica, MA, USA) were coated with 5 μg/ml final (100 μl/well) of anti-mouse IFN-γ (AN18, MABTech, Stockholm, Sweden) in PBS, and incubated overnight at 4°C. Following overnight incubation, the wells were washed and blocked with RPMI 1640 (Gibco, Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS; Gibco, Life Technologies), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 M Hepes, and 0.1 mM 2-mercaptoethanol, for a minimum of 1 h. Splenocytes from mice (immunized with or without vaccine formulations as listed above) were added in triplicate wells (1×10^7 cells/ml, 50 μl/well), along with the recall antigen (peptide SYIPSAEKI at different doses, 50 μl/well). Media alone control, or concanavalin A (ConA; Amersham Biosciences, Uppsala, Sweden; final 1 μg/ml) were also used, as negative or positive controls, respectively. All mice produced high levels of IFN-γ in response to ConA, with SFU often above the threshold for accurate counting (data not shown), indicating adequate cell viability and functionality. Cells with antigens were incubated in a 37°C incubator filled with 6% CO_2 for a minimum of 16 h. Plates were then washed five times in PBS, biotinylated detection antibody anti-IFN-γ (R4-6A2-Biotin, MABTech; 1 μg/ml in PBS 0.5% FCS, 100 μl/well) was added and followed by further incubation at room temperature for 2 h. Plates were then washed again, as above, and streptavidin-alkaline phosphatase enzyme conjugate (ALP; 1 μg/ml in PBS 0.5% FCS, 100 μl/well; MABTech) was added, followed by a further 1.5 h incubation at room temperature. After a final wash in PBS and followed by water, the spots were developed using a colorimetric AP kit (Bio-Rad, Philadelphia, PA, USA) following the manufacturer's instructions. Spots were counted by an

AID ELISpot Reader System (Autoimmun Diagnostika GmbH, Germany).

FLOW CYTOMETRY

For phenotypic analysis of cells by flow cytometry, inguinal lymph node cells were isolated 48 h after immunization with the adjuvants alone. 2×10^6 cells/sample were stained for 15 min at room temperature with 30 μl of antibody cocktails, including antibodies with different fluorochromes at different concentrations based on prior optimizations. Antibodies used in the present study include; anti-CD11c V450 (HL3), anti-CD11b PeCy7 (M1/70), anti-Gr-1 (Ly6C and Ly6G) PerCP Cy5.5 (RB6-8C5), anti-CD3 AF700 (500A2), anti-CD4 BV605 (RM4-5, Biolegend, San Diego, CA, USA), anti-CD8 BV650 (53-6.7, Biolegend), anti-CD25 PeCy7 (PC61), anti-FoxP3 APC (MF23), and anti-CD120b (TNFR2) PE (TR75-89). All antibodies were from BD Biosciences (NJ, USA) except where specifically indicated. Following incubation, cells were washed with 100 μl PBS/2% FCS (FACS buffer). Stained cells were fixed with 1% (v/v) paraformaldehyde (PFA, Sigma Aldrich) and acquired using an LSRII flow cytometer (BD Biosciences) located at the AMREP Flow Cytometry Core Facility (Melbourne, VIC, Australia). Data was analyzed using FlowJo software (version10, Treestar, USA).

STATISTICAL ANALYSIS

Statistical analysis was done by ANOVA analysis, with *post hoc* Tukeys multiple comparison tests or Fisher's LSD test, or unpaired *t*-tests, using Graphpad Prism software (version 6, San Diego, CA, USA). Statistical significance was determined as $p < 0.05$. Group sizes are indicated in the figure legends. All values are expressed as mean ± SD.

RESULTS

PEPTIDE COVALENTLY BOUND TO, BUT NOT MIXED WITH, PSNPs INDUCES CD8 T CELLS

Peptide delivery by nanoparticles (either mixed or conjugated) was compared for immunogenicity *in vivo* using BALB/c mice. To generate the conjugated nanovaccine, the immune-dominant CD8 T cell peptide epitope of the CSP protein, SYIPSAEKI (KI), from *P. berghei* was covalently attached to carboxylated polystyrene nanoparticles (PSNPs, 40–50 nm) using an optimized covalent conjugation protocol as previously described (Xiang et al., 2013). As shown in **Table 1**, the average size of the PSNPs-KI formulation was 47.97 ± 2.64 nm, and the polydispersity index (PdI) was very low (0.07 ± 0.03), indicating the successful formulation of a uniformly dispersed nanoparticle formulation with a narrow size distribution range (**Figure 1**). The antigen loading was 0.32 ± 0.09 mg/ml, which represented 1032.6 ± 147.8 peptide molecules per particle (**Table 1**). The number of peptide molecules per particle was comparable to previous studies with a model peptide antigen, SIINFEKL, where potent responses were observed at that loading (Xiang et al., 2013).

Table 1 | Characterization of SYIPSAEKI conjugation to PSNPs (PSNPs-KI) for size, polydispersity, and peptide loading*.

Formulation	Size (nm)	Polydispersity index (PdI)	Peptide molecules per particle
PSNPs-KI	47.97 ± 2.64	0.07 ± 0.03	1032.6 ± 147.8

**Data presented as mean ± SD, n = 4 repeated conjugations.*

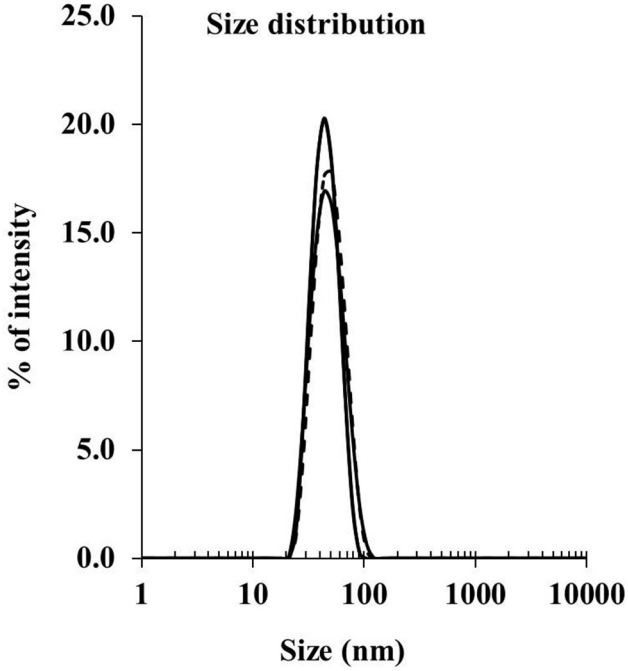

FIGURE 1 | Size distribution for PSNPs-KI formulations. SYIPSAEKI peptides were covalently conjugated to PSNPs, and the final sizes were measured by dynamic light scattering instruments (Zetasizer).

Mice were immunized twice, 14 days apart, with SYIPSAEKI peptide either alone (KI alone), mixed with the PSNPs (PSNPs + KI), or covalently conjugated to the PSNPs (PSNPs-KI) at the dosage of ∼25 μg of KI/mouse/injection. As results show in **Figure 2A**, neither the "KI alone" nor the "PSNPs + KI" treatment groups showed induction of KI specific CD8 T cell responses, assessed by IFN-γ ELISpot after two immunizations. However, when mice were immunized with KI conjugated to PSNPs (PSNPs-KI), significant ($p < 0.001$) levels of KI specific IFN-γ producing CD8 T cells were induced, even at the smallest amount of recall antigen concentration (0.25 μg/ml). Increasing the recall antigen concentration did not further enhance the overall antigen specific IFN-γ responses, suggesting the recall of high affinity T cells. These results also show that this specific malaria antigen peptide needs to be covalently conjugated to its carrier nanoparticles to induce potent immune responses. Moreover, it shows that this system can utilize minimal CD8 T cell epitopes, without added CD4 T cell epitopes, and still induce levels of immune responses previously associated with powerful 'Prime-boost' immunization modalities and sterile protection against sporozoite challenge (Plebanski et al., 1998).

Given the PSNPs-KI formulation induced potent immune responses with two immunizations; we further tested formulation potency in a single dose immunization regime. As shown

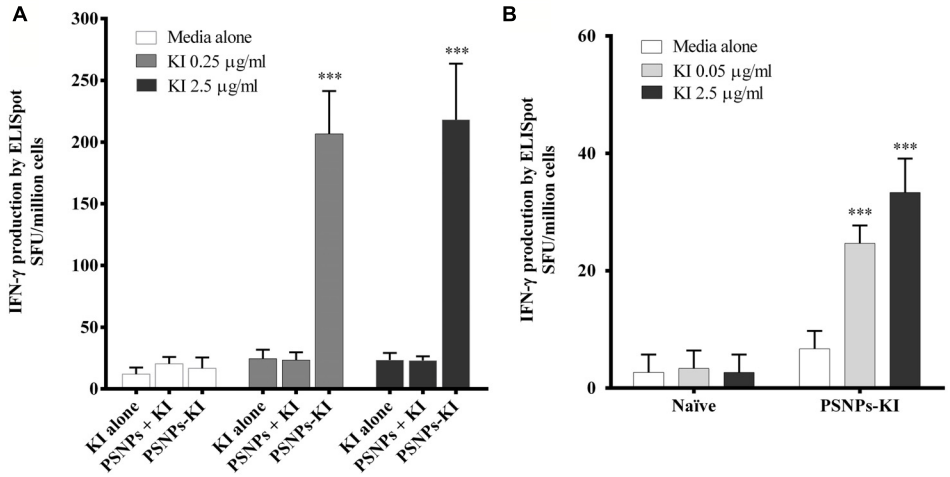

FIGURE 2 | Antigen specific CD8 T cell responses induced by SYIPSAEKI peptide vaccines in combination with PSNPs. Mice (BALB/c) were immunized with SYIPSAEKI peptide mixed with or conjugated to PSNPs intradermally at the base of tail. 14 days after the last immunization, spleen cells were collected and assessed for IFN-γ production by ELISpot assay. **(A)** KI peptides conjugated to, but not mixed with, PSNPs induced high levels of KI specific CD8 T cell responses after two immunizations (2 weeks apart). Data presented as mean ± SD of SFU/million cells from each group ($n = 3$ mice/group). **(B)** Immunogenicity of PSNPs-KI formulation after one immunization ($n = 4$ mice per group). Data presented as mean ± SD of SFU/million cells (pooled for each group) from the triplicated wells in ELISpot assay. Statistical analysis was performed via ANOVA, $***p \leq 0.001$.

in **Figure 2B**, after one immunization, PSNPs-KI formulations induced good antigen specific CD8 T cell responses, significantly higher than naïve controls ($p < 0.001$, **Figure 2B**). Recall T cells were elicited in ELISpot at both 2.5 and 0.05 μg/ml, suggesting high affinity T cells were induced already in the initial priming phase.

PSNPs-KI AND MONTANIDE INDUCE THE HIGHEST CD8 T CELL RESPONSES

To benchmark the immunogenicity of PSNPs-KI compared to other types of conventionally adjuvanted experimental formulations capable of inducing CD8 T cell responses, we further tested Montanide and Poly I:C with KI side by side with PSNPs-KI. Strong and comparable KI specific CD8 T cell responses were detected in mice immunized with "PSNPs-KI" and "Montanide + KI" formulations (**Figure 3**). The magnitude of the KI specific IFN-γ production by both these formulations was significantly higher ($p < 0.001$) than that from mice immunized with KI alone or "Poly I:C + KI" (**Figure 3**). Despite the literature indicating that Poly I:C is a potent CD8 T cell response inducer (Nordly et al., 2011), Poly I:C mixed with KI formulation didn't promote the induction of IFN-γ producing CD8 T cells above that induced with peptide alone, after two immunizations.

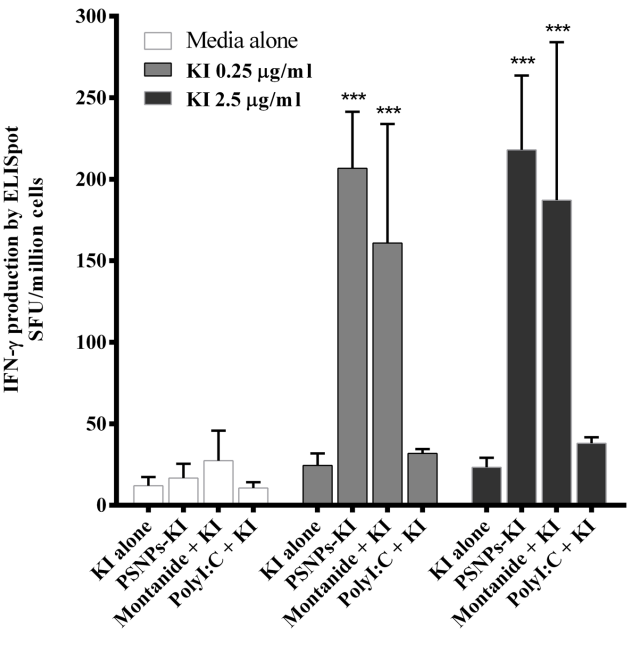

FIGURE 3 | Induction of SYIPSAEKI specific CD8 T cells by different adjuvants. BALB/c mice were immunized twice, intradermally, 2 weeks apart, with SYIPSAEKI peptides incorporated with respective adjuvants. 14 days after the last immunization, spleen cells were collected and assessed for IFN-γ production by ELISpot assay. Data presented as mean ± SD of SFU/million cells from each group ($n = 3$ mice/group). Statistical analysis was performed via ANOVA, $***p \leq 0.001$.

POLY I:C, BUT NOT PSNPs OR MONTANIDE, IS ASSOCIATED WITH A LACK OF DC MATURATION 48 H POST INJECTION WITH THE ADJUVANT ALONE

To further understand how the potent CD8 T cell responses could be induced by a single CD8 T cell epitope when conjugated to PSNPs in the absence of a CD4 T cell helper epitope, as well as to compare the non-specific action mode of other adjuvants alone in the induction of cell activation, we investigated the level of DC activation in the local draining lymph node (dLN). This was done by assessing the expression levels of MHCII, CD40, CD80, and CD86 on the $CD11c^+$ DCs, from the inguinal lymph node, after the injection of PSNPs, Montanide or Poly I:C *in vivo*, in the absence

of antigen. We hypothesized that there would be efficient CD86 induction on DC, making them highly capable of activating CD8 T cells (Clarke, 2000; Steinman et al., 2003; Maroof et al., 2009). The critical time-period for CD8 T cell expansion is between 48 and 72 h post priming, a period of repeated transient contact between T cells and DC (Henrickson et al., 2008). Increases in suppressor cells would be expected to follow initial inflammation induced by adjuvants, which usually peaks at 12–24 h post administration. Therefore, between 24 and 72 h immunosuppressive mechanisms would be expected to come into play. We assessed DC frequency and expression of co-stimulatory molecules in adjuvant dLN 48 h after injection with the adjuvants alone. Results in **Figure 4A** (gating strategy) and **Figure 4B** show that the overall frequency of DCs (Gr-1$^-$ CD11c$^+$ cells) remained the same in the dLN 48 h post injection with all three types of carrier/adjuvants. There was a significant increase in the expression of CD80 in the dLN DCs after treatment by both PSNPs and Montanide ($p < 0.001$ and $p < 0.05$, respectively, **Figure 4C**). Furthermore, there was a significant increase in expression levels of CD86 on CD11c$^+$ cells for all adjuvants tested ($p < 0.001$ compared to PSNPs, and $p < 0.01$ compared to Montanide and Poly I:C treatment, **Figure 4C**), implying DCs were potentially being activated even in the absence of a CD4 T cell helper epitope. CD11c$^+$ DCs in the Montanide group further showed an increase in the expression of CD40, compared to the naïve and PSNPs groups ($p < 0.05$, **Figure 4D**). However, surprisingly, DCs in the Poly I:C treated group showed significantly lower levels of expression of MHCII compared to all other treatment groups ($p < 0.05$ compared to naïve, $p < 0.01$ compared to PSNPs and $p < 0.001$ compared to Montanide treatment, **Figure 4D**), suggesting these DCs were at a different state of maturation, and/or activation, upon treatment with Poly I:C.

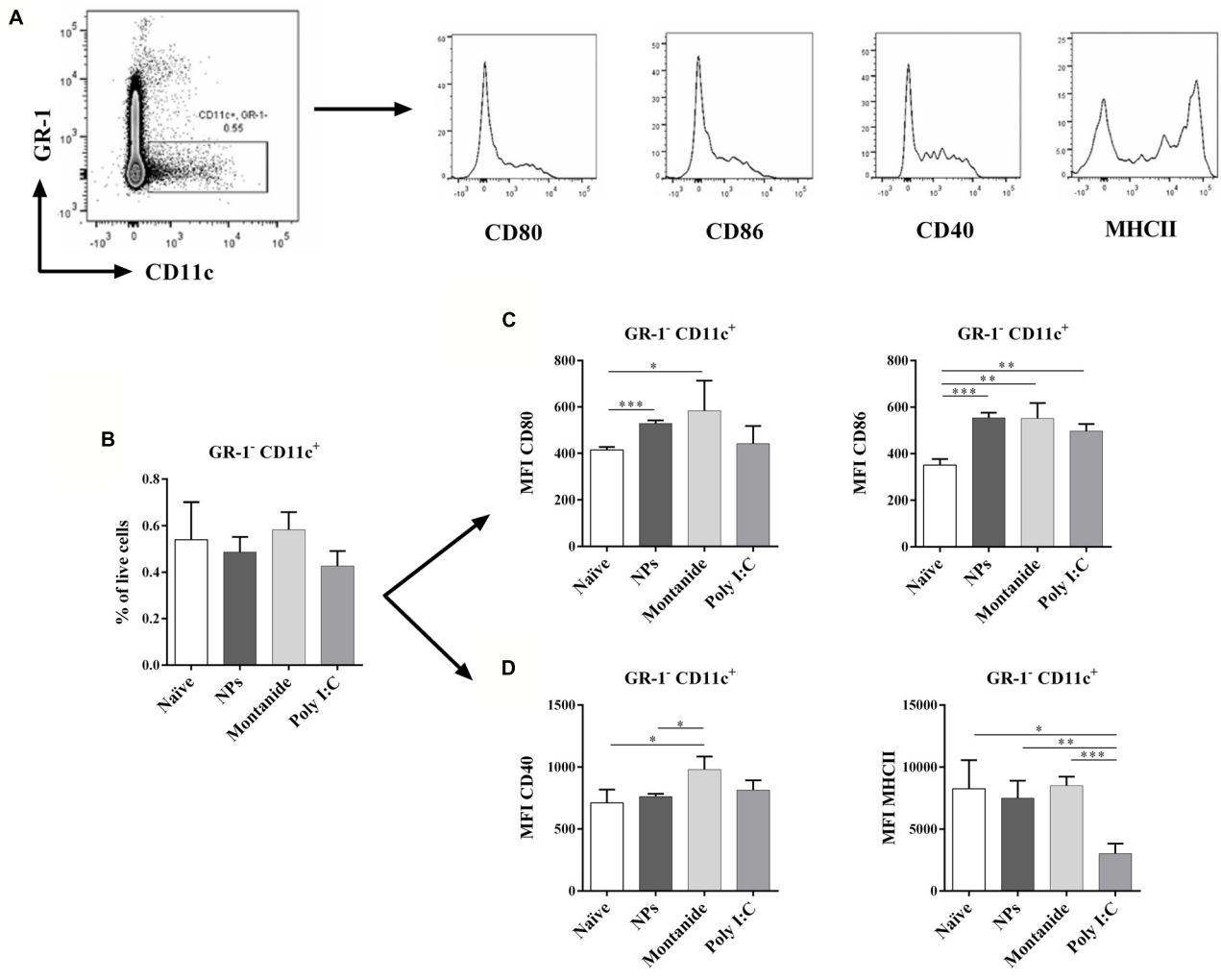

FIGURE 4 | Dendritic cell activation in dLNs after injection with PSNPs, Montanide, and Poly I:C. Mice (BALB/c) were injected once intradermally at the base of tail with the different adjuvants alone. 48 h after injection, mice were sacrificed, local (inguinal) dLNs were harvested and the levels of CD11c$^+$ DCs and various activation markers were assessed by flow cytometry. **(A)** gating strategy; **(B)** frequency of GR-1$^-$CD11c$^+$ cells; **(C)** Mean fluorescent intensity (MFI) of CD80 and CD86 on GR-1$^-$CD11c$^+$ cells; **(D)** MFI of CD40 and MHCII on GR-1$^-$CD11c$^+$ cells. Data presented as mean ± SD of MFI for each group of treatment ($n = 3$ mice/group). Statistical analysis was performed via *t*-tests, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

PSNPs AND MONTANIDE INDUCE AN ENVIRONMENT THAT ENCOURAGES STIMULATORY DCs, WHEREAS POLY I:C PROMOTES SUPPRESSIVE MDSCs 48 H POST INJECTION WITH THE ADJUVANTS ALONE

The balance between DCs and MDSCs in the priming lymph node would be predicted to influence the level of immunity subsequently induced by vaccines. We further assessed the MDSC and CD11c$^+$ DC populations in the dLN 48 h after injection (**Figure 5A**). Whilst PSNPs and Montanide maintained a normal MDSC to DC ratio in the dLN (**Figure 5B**), surprisingly, Poly I:C promoted a significantly higher ratio of MDSCs to DCs, compared to all other groups ($p < 0.01$, **Figure 5B**). Further analysis of the subsets within MDSCs, based on their level of Gr-1 expression, showed that Poly I:C significantly increased the ratio of MDSCs expressing intermediate levels of Gr-1 (monocytic or suppressive MDSC, moMDSC; Kong et al., 2013) over DC, moMDSC/CD11c, when compared to naïve, Montanide or PSNPs treated groups ($p < 0.01$, **Figure 5C**). There was also a significant increase in the ratio of MDSC expressing high levels of Gr-1 (granulocytic MDSC, gMDSC; Kong et al., 2013) to DC (gMDSCs/CD11c) for all treatments ($p < 0.01$ compared to naïve and PSNPs, and $p < 0.05$ compared to Montanide treatment, **Figure 5D**), however, the magnitude of this increase was not as high as the increased ratio of moMDSC/CD11c cells. Hence Poly I:C induced an environment abundant in suppressive (monocytic) phenotype MDSC in the dLN within a short time frame, whereas PSNPs and Montanide did not promote increases in such MDSCs.

POLY I:C AND MONTANIDE PROMOTE THE INDUCTION OF TNFR2$^+$ TREG CELLS

Inflammatory, tumor necrosis factor (TNF) inducing, adjuvants such as Montanide and Poly I:C, have the potential to stabilize FoxP3 expression on Treg that express the TNF receptor 2 (TNFR2; Chen and Oppenheim, 2011). TNFR2 has also previously been found to identify the most highly active and immunosuppressive Treg subset (Govindaraj et al., 2014). We speculated that pro-inflammatory adjuvants could therefore increase the Treg to T effector ratio in the dLN, and if this occurred during the T cell priming phase, it could potentially interfere with effective CD8 T cell induction. We further analyzed the Treg and T effector cells in the dLN (**Figure 6A**), and found that whilst there was no overall increase in total Treg to T effector cell ratio (CD25$^+$FoxP3$^+$ to CD25$^-$FoxP3$^-$ cells; **Figure 6B**), Poly I:C and Montanide significantly increased the frequency of TNFR2$^+$ Treg (FoxP3$^+$CD25$^+$TNFR2$^+$ cells) compared to TNFR2$^-$ Treg (FoxP3$^+$CD25$^+$TNFR2$^-$ cells) in the dLN 48 h post injection,

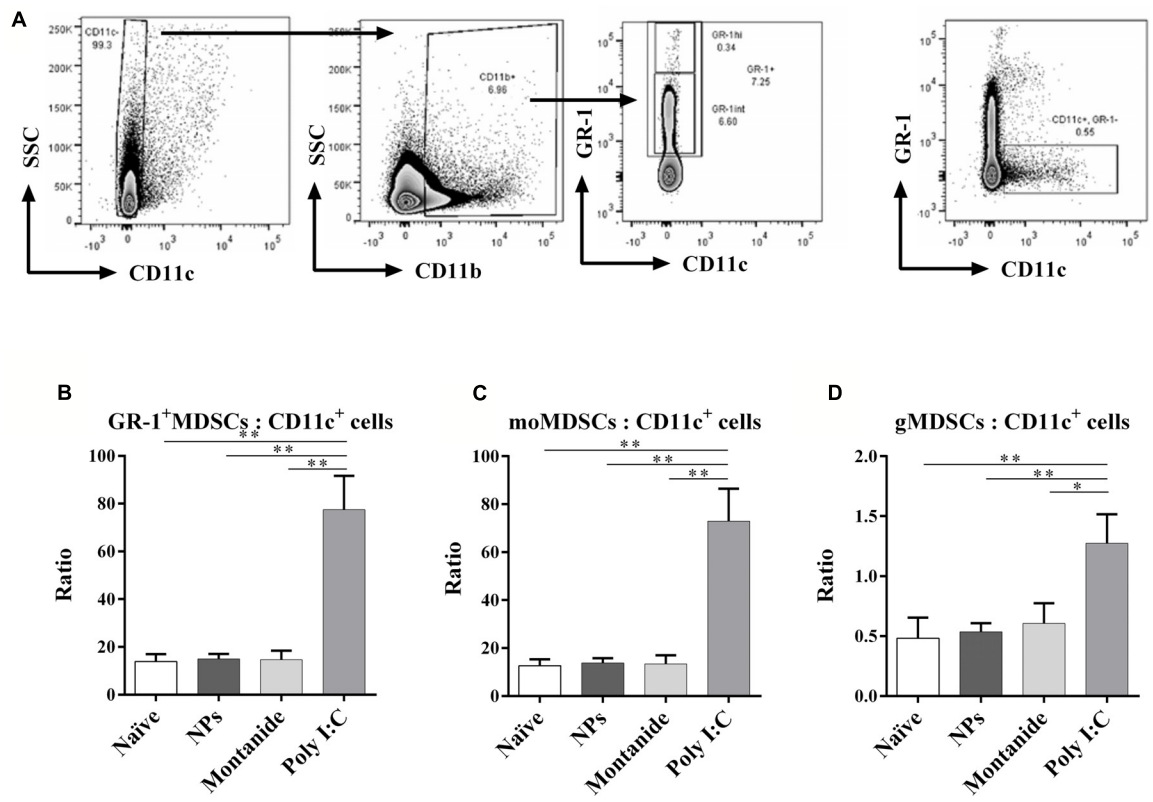

FIGURE 5 | Differential expression of GR-1$^+$ MDSCs and DCs in the local dLN. Mice (BALB/c) were injected once intradermally at the base of the tail with the different adjuvants alone. 48 h after injection, mice were sacrificed, local (inguinal) dLNs were harvested and levels of GR-1$^+$ MDSCs and DCs, and their ratios, were assessed by flow cytometry. **(A)** gating strategy; **(B)** ratio of GR-1$^+$ MDSCs: CD11c$^+$ cells; **(C)** ratio of moMDSCs: CD11c$^+$ cells; **(D)** ratio of gMDSCs: CD11c$^+$ cells. Data presented as mean ± SD of ratio for each group of treatment ($n = 3$ mice/group). Statistical analysis was performed via *t*-tests, $*p \leq 0.05$, $**p \leq 0.01$.

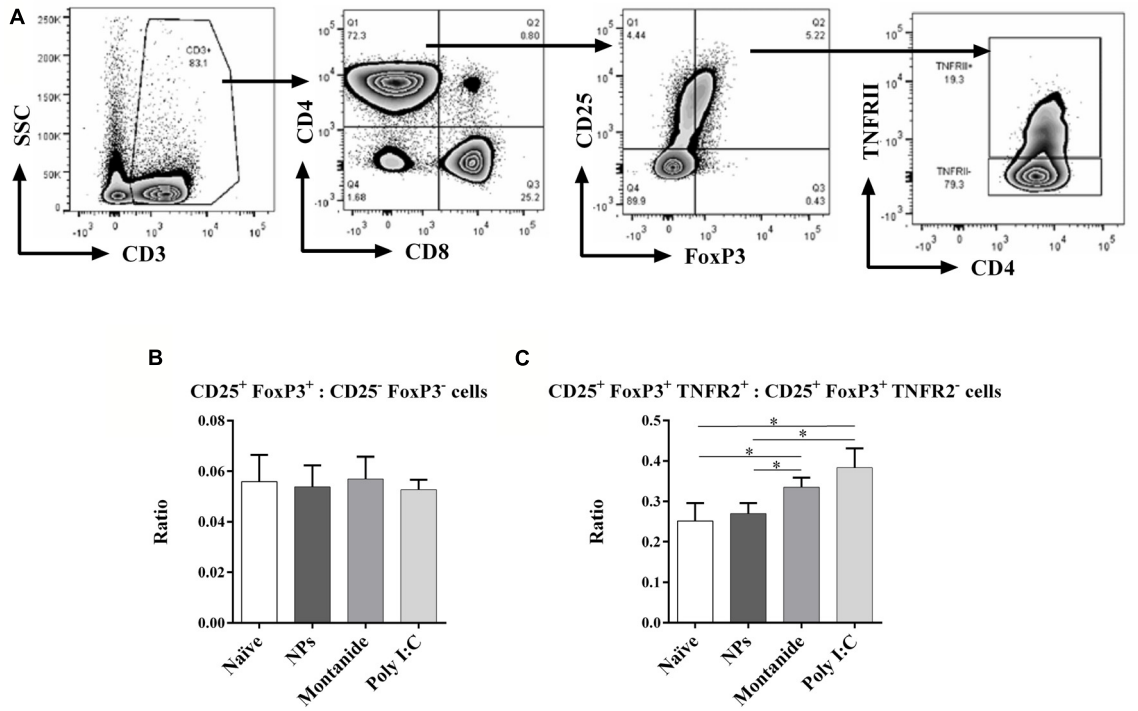

FIGURE 6 | Differential expression of CD4+ Treg and effector T cells in the local dLN. Mice (BALB/c) were injected once intradermally at the base of tail with the different adjuvants alone. 48 h after injection, mice were sacrificed, local (inguinal) dLNs were harvested and the levels of $CD4^+$ Treg and effector T cells, and their ratios, were assessed by flow cytometry. **(A)** gating strategy; **(B)** ratio of $FoxP3^+CD25^+$: $FoxP3^-CD25^-$ cells; **(C)** ratio of $FoxP3^+CD25^+TNFR2^+$: $FoxP3^+CD25^+TNFR2^-$ cells. Data presented as mean ± SD of ratio for each group of treatment ($n = 3$ mice/group). Statistical analysis was performed via *t*-tests, $^*p \leq 0.05$.

compared to the naïve and PSNPs groups ($p < 0.05$, **Figure 6C**). PSNPs maintained the balance of $TNFR2^+$ to $TNFR2^-$ Treg subpopulations.

DISCUSSION

The side-by-side comparison of three different adjuvant systems for the induction of highly responsive CD8 T cells to a minimal peptide epitope antigen from CSP of *P. berghei* demonstrated that: (1) Non-inflammatory and inflammatory vaccines can elicit similarly high levels of immune responses, (2) Non-inflammatory nanovaccines require the minimal CD8 T cell epitope peptide to be covalently attached to the nanoparticle carrier, suggesting peptide delivery *in vivo* is key for antigenic stimulation, (3) Vaccines can prime high levels of CD8 T cells by delivering the minimal CD8 T cell epitope, without helper CD4 epitopes, (4) Inflammatory, but not non-inflammatory, adjuvants result in the induction of $TNFR2^+$ Treg in dLNs during a timeframe consistent with the priming of an immune response, and (5) Together with the induction of enhanced numbers of suppressor moMDSC, such findings may explain the particularly poor capacity of Poly I:C to induce CD8 T cell immune responses.

Both "Montanide + KI" and "PSNPs-KI" formulations induced a similar magnitude of response after two immunizations, reaching the minimum threshold IFN-γ production levels determined to be required for sterile protection in the *P. berghei* challenge model (Plebanski et al., 1998). Given a threshold of 100 spots was required to start seeing sterile protection in about 74% of animals in previous studies (Plebanski et al., 1998), it is likely that the 200 spots achieved by the nanovaccines would also be protective, although it will be important to confirm this formally. The potentially protective IFN-γ levels produced in this study merit additional validation in further direct challenge studies. There may be additional advantages in using a non-inflammatory nanoparticle approach over Montanide. Montanide is a viscous combination of adjuvant with peptide, creating a depot at the injection site with the antigen, associated with some pain and local inflammation. As well as increasing compliance with vaccination, the use of a non-inflammatory adjuvant system that substantially drains to the lymph nodes, may, in the case of immunization of individuals in malaria endemic areas, help minimize the risk of triggering inflammatory feedback loops, such as those associated with cerebral malaria. Previous studies have shown nanoparticle based vaccines do not need to engage conventional inflammatory pathways to induce adaptive immunity (Karlson Tde et al., 2013; Xiang et al., 2013), and act by selectively targeting DCs, particularly $CD8^+$ DCs, directly in the local lymph nodes (Fifis et al., 2004a; Mottram et al., 2007; Xiang et al., 2013), as well as by promoting uptake by DC in the periphery followed by subsequent migration via the afferent lymphatics (Gamvrellis et al., 2013). The critical factor identified that promotes $CD8^+$ DCs targeting was found to be particle size (40–50 nm; Fifis et al., 2004a; Mottram et al., 2007). The fact that mixed-in nanoparticles in this study did not

act as conventional adjuvants, and hence the carrier activity of the nanoparticles was sufficient and necessary to induce high levels of immune responses, predicts that nanoparticle carriers of the correct size to target $CD8^+$ DCs *in vivo* (made of non-inflammatory materials) would also be capable of inducing high levels of immunity. Given the explosion in nanomaterials and delivery systems, this appears to be a promising and timely finding.

It was surprising to find that the "Poly I:C + KI" formulation was unable to induce similarly high CD8 T cell responses when compared side-by-side with the "Montanide + KI" and "PSNPs-KI" formulations. This could be mechanistically explained by the new finding that Poly I:C promotes dramatic increases in the ratio of MDSCs to DCs, including moMDSCs, in the LNs draining the injection site, within a timeframe capable of interfering with local CD8 T cell priming. Moreover, whereas the frequency of DCs remained the same in the dLN 48 h post injection with either PSNPs, Montanide or Poly I:C, there were significantly lower levels of MHCII expression on DCs treated with Poly I:C. Down-regulation of some activation markers on DCs has been associated in the literature with increases in suppressor MDSC frequencies and their subsequent apoptosis (Shen et al., 2014). MDSCs can suppress effector T cell responses directly, or by promoting the expansion of Tregs in the presence of IFN-γ (Kong et al., 2013).

Together our results suggest that non-inflammatory nanoparticles 40–50 nm or Montanide can be used to induce potent CD8 T cell responses, even when used with purely a minimal CD8 T cell peptide epitope. Generally, the results herein also suggest a new paradigm for highly immunogenic vaccines, which could instead of delivering pro-inflammatory danger signals, be designed to 'keep under the radar' to deliver antigen to cross-priming $CD8^+$ DCs whilst avoiding the expansion of some key immunosuppressive and inflammation reactive cell populations.

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Malaria and the liver: immunological hide-and-seek or subversion of immunity from within?

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During the pre-erythrocytic asymptomatic phase of malarial infection, sporozoites develop transiently inside less than 100 hepatocytes that subsequently release thousands of merozoites. Killing of these hepatocytes by cytotoxic T cells (CTLs) confers protection to subsequent malarial infection, suggesting that this bottleneck phase in the parasite life cycle can be targeted by vaccination. During natural transmission, although some CTLs are generated in the skin draining lymph nodes, they are unable to eliminate the parasite, suggesting that the liver is important for the sporozoite to escape immune surveillance. The contribution of the organ to this process is unclear. Based on the known ability of several hepatic antigen-presenting cells (APCs) to induce primary activation of CD8 T cells and tolerance, malarial antigens presented by both infected hepatocytes and/or hepatic cross-presenting APCs should result in tolerance. However, our latest model predicts that due to the low frequency of infected hepatocytes, some T cells recognizing sporozoite epitopes with high affinity should differentiate into CTLs. In this review, we discuss two possible models to explain why CTLs generated in the liver and skin draining lymph nodes are unable to eliminate the parasite: (1) sporozoites harness the tolerogenic property of the liver; (2) CTLs are not tolerized but fail to detect infected cells due to sparse infection of hepatocytes and the very short liver stage. We propose that while malaria sporozoites might use the ability of the liver to tolerize both naive and effector cells, they have also developed strategies to decrease the probability of encounter between CTLs and infected liver cells. Thus, we predict that to achieve protection, vaccination strategies should aim to boost intrahepatic activation and/or increase the chance of encounter between sporozoite-specific CTLs and infected hepatocytes.

Keywords: sporozoite, hepatocytes, LSEC, Kupffer cells, hepatic stellate cells, dendritic cells, tolerance, T cells

INTRODUCTION

Worldwide, infection by malaria-causing parasites kills nearly one million people every year (Murray et al., 2012), and is one of the most important causes of morbidity globally. Human disease is caused by the five species of the *Plasmodium* genus. Parasites are introduced by the bite of female *Anophelese* mosquitoes, which act as a primary host in the parasite life cycle. The remainder of the cycle continues in the human host. In endemic areas, individuals are repeatedly infected, and co-infections by different species are common. Natural infection does not generally confer protection and therefore the development of effective vaccines is critical.

As the mosquito probes for blood, the sporozoites contained in the mosquito salivary glands are predominantly released into the dermis. Most of the parasites reside in the skin for between 1 and 6 h, while 20% migrate via the lymph directly into the skin draining lymph nodes (LNs) (Sidjanski and Vanderberg, 1997; Amino et al., 2006) where they are thought to induce or modulate the subsequent anti-parasite immune response (Yamauchi et al., 2007; Guilbride et al., 2012). It is thought that most of the sporozoites fail to migrate to the LNs and are cleared at the site of inoculation, while a small proportion randomly finds its way to the nearest blood vessel (Sidjanski and Vanderberg, 1997). After crossing the endothelial barrier, the sporozoites enter the circulation to reach the liver (Mota et al., 2001; Ishino et al., 2004, 2005; Frevert et al., 2005). This organ is critical for the next phase of the parasite life cycle, the pre-erythrocytic stage.

The pre-erythrocytic asymptomatic cycle phase may represent the Achilles heel of the parasite, as it is during this bottleneck phase that infected hepatocytes could be efficiently targeted and eliminated by cytotoxic CD8 T cells (CTLs) (Lau et al., 2014). CD8 T cells and the cytokines IFN-γ and TNF-α have been reported as being critical for sterile protection against pre-erythrocytic parasites inside hepatocytes in both animal models and humans (Schofield et al., 1987; Weiss et al., 1988; Krzych et al., 2000; Overstreet et al., 2008; Good and Doolan, 2010; Obeid et al., 2013). Interestingly, the most promising and efficient

Abbreviations: APC, Antigen Presenting Cell; CTL, Cytotoxic T Cell; KC, Kupffer Cell; LSEC, Liver Sinusoidal Endothelial Cell; MHC, Major Histocompatibility Complex; TCR, T cell Receptor; CSP, Circumsporozoite Protein; ICAM-1, Intercellular Adhesion Molecule 1; LFA-1, Lymphocyte Function-Associated Antigen 1; LN, Lymph Node; PBL, Peripheral Blood Lymphocytes; HSC, Hepatic Stellate Cells; rAAV, Recombinant Adeno-Associated Virus; DC, Dendritic Cell.

vaccine candidates in human clinical trials are based on live genetically attenuated whole parasites that infect the liver but do not progress to the blood stage (Epstein and Richie, 2013). Thus, targeting antigens expressed during the pre-erythrocytic cycle phase holds great hope and promise for anti-malaria vaccination.

The liver is acknowledged as a site of primary T cell activation that promotes tolerance rather than effective priming (Bertolino et al., 2002; Benseler et al., 2007; Crispe, 2014). Although activation of protective effector T cells specific for one of the major immunodominant sporozoite-derived antigen has been shown to be restricted to skin draining LNs (Chakravarty et al., 2007; Obeid et al., 2013), it is not clear to what extent the liver contributes to the activation of T cells specific for malarial antigens expressed by infected hepatocytes, or whether this activation generates effector T cells. This question is particularly relevant to vaccination strategies that immunize recipients with irradiated sporozoites that infect the liver but arrest their development in hepatocytes without generating blood stage parasites. The high number of irradiated sporozoites used in this type of immunization is several-fold higher than during natural infection by mosquitos, and is necessary to generate sufficient numbers of T cells for protection.

The liver stage of the parasite is often referred to as a silent phase during which the parasite goes under the immunological radar. It is possible that during this phase, the parasite harnesses the ability of the liver to induce tolerance to avoid being eliminated, and thus establishes productive infection. Alternatively, it is possible that CTLs generated in LNs, and potentially liver, fail to detect the very few infected hepatocytes during the short period of the liver phase. This is particularly a limiting factor in rodent models of malaria as the pre-erythrocytic phase lasts for only 2 days. Both models could explain why this phase is asymptomatic, and apparently immunologically silent.

In this review, we will discuss the likelihood that a T cell response is elicited in the liver during the pre-erythrocytic phase, and the predicted outcome arising from such intrahepatic activation. Our arguments support a model in which sporozoites have developed strategies to induce tolerance but also reduce to a minimum the chances of encountering T cells by delaying efficient elimination of infected hepatocytes.

SITES OF T CELL PRIMING DURING THE PRE-ERYTHROCYTIC PHASE

Unless an individual has been previously infected with malaria parasites, their immune repertoire is predominantly naïve with regard to parasite-specific responses. Naïve T cells recirculation is restricted to the blood and lymph. Via these routes, these cells can also access secondary lymphoid tissues (spleen and LNs). In LNs, naive T cells scan for foreign antigens presented by dendritic cells (DCs) in the form of peptide-MHC (p:MHC) complexes. Primary T cell activation by DCs allows blast formation, cytokine secretion, proliferation, and differentiation into cells endowed with effector function and the capacity for trans-endothelial migration and entry into tissue parenchyma.

As the dermis is the site of inoculation and the first site exposed to sporozoites (**Figure 1**), it is not surprising that, as assessed by IFN-γ production, the first signs of T cell activation after mouse immunization with irradiated sporozoites (via mosquito bite or intradermal injection) were detected in the skin draining LNs at 48 h (Chakravarty et al., 2007). TCR transgenic T cells specific for the immunodominant antigen, the circumsporozoite protein (CSP), were activated only in these LNs, but effector T cells secreting IFN-γ were not detected in spleen, liver, or liver-draining LNs of the recipients at this time point (Chakravarty et al., 2007). Using a subcutaneous immunization protocol and $CD81^{-/-}$ mice that facilitate restriction of antigen presentation to the skin draining LN rather than the liver, it has been shown that T cell priming in the skin draining LN is sufficient to confer protection (Obeid et al., 2013). Depletion of $CD11c^{+}$ cells abrogated efficient CD8 T cell priming (Jung et al., 2002; Obeid et al., 2013), supporting the model that DCs carrying the sporozoite-derived antigen from the dermis migrated to the draining LNs, where they induced intranodal priming of CSP-specific T cells. Although there is strong evidence that the skin draining LNs prime T cells specific for early pre-erythrocytic proteins such as CSP, other sporozoite proteins are specifically expressed by hepatocytes during the subsequent liver stage. Furthermore, although CSP is an immunodominant antigen in Balb/c mice, it is not immunogenic for CTLs in C57Bl/6 mice, and in most humans naturally infected with malaria parasites (Gruner et al., 2007). Evidence that CSP is not the only protein that contributes to protection includes the development of protective immunity in mice tolerant for CSP that were immunized with irradiated sporozoites (Kumar et al., 2006), and the occurrence of CSP-independent sterile protection under chloroquine prophylaxis after immunization with irradiated sporozoites (Gruner et al., 2007; Mauduit et al., 2010). It is unlikely that the skin-draining LNs play any role in priming CD8 T cells against these mid to late pre-erythrocytic proteins expressed by hepatocytes.

A recent study (Lau et al., 2014) demonstrated that activated TCR transgenic CD8 T cells specific for an unidentified antigen expressed by both liver and blood stage parasites protected against subsequent *Plasmodium* infection, indicating that targeting antigens expressed by hepatocytes can lead to protective immunity. The compartment in which priming occurs is not clear. The liver draining LNs are the most likely site in which CD8 T cells can be primed following cross-presentation of hepatocyte-derived proteins by DCs. However, it is possible that proteins expressed by hepatocytes induce T cell activation and priming in the liver itself.

THE LIVER: A SITE OF PRIMARY ACTIVATION FOR CD8 T CELLS

The liver is now recognized as an alternative site (and the only non-lymphoid organ in the body) in which naive CD8 T cells can be activated independently of lymphoid tissues (Bertolino et al., 2001). First demonstrated for CD8 T cells, this remarkable hepatic immunological property has recently been extended to CD4 T cells (Tay et al., 2014b). Thus, in addition to the hepatic draining LNs, primary activation of T cells specific for mid to late pre-erythrocytic proteins could potentially occur in the liver itself. As proteins such as CSP are also expressed during the early phase of liver infection, intrahepatic tolerance might not only

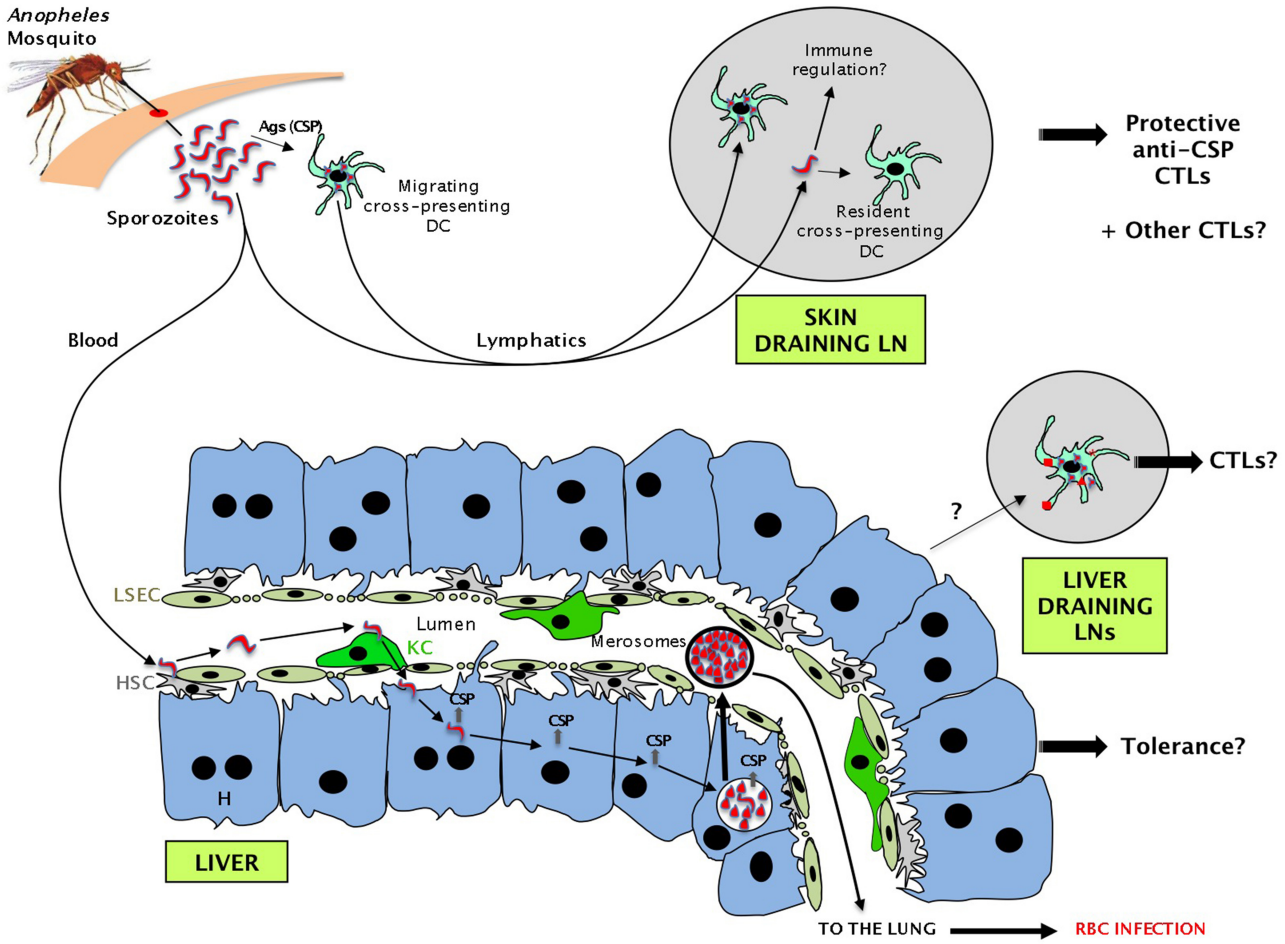

FIGURE 1 | Sequence of events during the pre-erythrocytic phase of malaria parasite infection leading to the presentation of sporozoite antigens to CD8 T cells in several compartments. Some sporozoites injected by the mosquito migrate via the lymph into the skin draining lymph nodes (LNs). Migratory DCs or resident DCs capturing sporozoite proteins activate naive CD8 T cells and promote the generation of CTLs. Other sporozoites migrate via the blood to the liver where they are retained by hepatic stellate cells (HSC) and glide. After binding to a Kupffer cell (KC), they cross the liver sinusoidal endothelial cells (LSEC) barrier, traverse through several hepatocytes (H), before entering one in which they establish a parasitophorous vacuole. After a short period (2 days in mice for *P. berghei*/*P. yoelii* or 7 days in humans for *P. falciparum*), each infected hepatocyte releases thousands of merozoites that will subsequently infect red blood cells. While traversing hepatocytes, sporozoites leave a trail of CSP and possibly other proteins. Antigens shed by infected hepatocytes could also potentially be presented in the liver draining LNs.

affect naive T cells undergoing primary activation in this organ, but also alter the fate of anti-CSP effector T cells primed in skin draining LNs.

The ability of the liver to support primary CD4 and CD8 T cell activation is likely enabled by its unusual architecture and distinct resident populations of antigen-presenting cells.

UNIQUE ARCHITECTURE OF THE LIVER

The liver receives up to 25% of the cardiac output. Thus, T cells pass through this organ multiple times during their lives, and have the opportunity to interact with hepatic cells located in the sinusoids. The structure of the liver sinusoids is key to understand its role in intrahepatic T cell retention. Liver sinusoids are narrow capillaries with an average diameter of 10 μm, in which blood flow is more intermittent and slower than in other capillaries. This unusual pattern of blood flow is contributed to by the narrow diameter of the sinusoids, the contraction of contractile smooth muscle sphincters in the walls of hepatic arterioles (Macsween and Scothorne, 1979; Macphee et al., 1995), and intermittent obstruction of the lumen by KCs (McCuskey and Reilly, 1993). This unique architecture favors interactions between leukocytes and hepatic cells, and intrahepatic recruitment of T cells recognizing their cognate antigen within this organ. Selectin-dependent leucocyte rolling normally observed in capillaries is not observed in liver sinusoids, and selectins are not required for the recruitment of leucocytes to this organ (Wong et al., 1997; Bowen et al., 2004). In contrast, TCR/p:MHC complex interactions and those between the adhesion molecules LFA-1 and ICAM-1 are important to the retention of lymphocytes, and these have been shown to play a critical role in the recruitment of CD8 T cells to the liver (Bertolino et al., 2005).

POTENTIAL APCs IN THE LIVER SINUSOIDS

Several hepatic cell types have been shown to be able to activate naive CD8 T cells *in vitro* (reviewed in Bertolino et al., 2002). It is now accepted that most liver APCs located in the sinusoids are able to induce primary activation *in vivo* as long as they can access circulating T cells and express or capture antigen.

Hepatocytes are located behind the endothelial cell layer, and are the principal cell type of the liver, comprising 75% of hepatic cells. These very large polyhedral, mono or bi-nucleated cells contain up to 16N DNA content, and synthesize and secrete a wide variety of biologically crucial molecules. They also metabolize and excrete many endogenous and xenobiotic substances. Steady state hepatocytes express MHC class I molecules (both classical and monomorphic CD1 molecules), and ICAM-1; inflammation also induces expression of MHC class II molecules, CD40L, and costimulatory molecules such as CD80 and CD86 (Bertolino et al., 1998; Holz et al., 2008). Since they normally lack MHC class II expression, resting hepatocytes may therefore only act as APCs for CD1d- and MHC class I-restricted T cells. Primary hepatocytes have also been demonstrated to drive efficient activation and proliferation of naïve TCR transgenic CD8 T cells *in vitro*, confirming their role as efficient APCs (Bertolino et al., 1998).

Despite the widespread view that hepatocytes are in a concealed location, express low levels of MHC class I, and therefore cannot be "seen" by T cells, available data suggests that, despite their sub-endothelial location, this cell population efficiently interacts with circulating CD8 T cells, and functions as effective APCs. *In vivo* interactions between hepatocytes and naïve CD8 T cells have been demonstrated or suggested in different animal models including transgenic mice (Bertolino et al., 2001; Bowen et al., 2004; Derkow et al., 2007), liver transplants (Klein and Crispe, 2006a; Tay et al., 2013), and mice treated with recombinant adeno-associated viral (rAAV) vectors (Wuensch et al., 2006, 2010; Tay et al., 2014a,b). Using electron microscopy, we have shown that circulating T cells make direct contact with hepatocytes through cytoplasmic extensions penetrating the endothelial fenestrations that perforate the LSECs (Warren et al., 2006). Furthermore, in contrast to early data, this study also demonstrated that interactions between T cells and liver parenchymal cells are maximized by the polarized expression of molecules required for primary T cell activation, MHC class I and ICAM-1, on the perisinusoidal cell membrane of hepatocytes (Warren et al., 2006).

Liver sinusoidal endothelial cells are efficient scavenger cells strategically located in the sinusoids, and able to clear low-density lipoprotein (LDL) and capture particulate antigens and immune complexes circulating via the blood and deliver them to hepatocytes (Sorensen et al., 2012). This uptake is mediated by mannose receptor, and has been shown to be more efficient than that mediated by KC, suggesting that LSEC specialize in this function. LSEC constitutively express both MHC class I and class II molecules, low levels of CD86, as well as adhesion molecules including ICAM-1, VCAM-1 and dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN). They have been shown to activate both naïve CD4 and CD8 T cells *in vitro* (Knolle et al., 1999) and *in vivo* (Limmer et al., 2000).

Kupffer cells (KC) are the major population of tissue macrophages in the body, and are relatively heterogeneous (Ikarashi et al., 2013). Although some cells with KC-like properties can be reconstituted from bone marrow after lethal irradiation, most KC are derived from the yolk sac early after birth (Jakubzick et al., 2013). Intravital spinning disk and multiphoton microscopy experiments have demonstrated that KC are able to efficiently capture pathogens circulating within the sinusoids, such as *Borrelia burgdorferi* (Lee et al., 2010), and contribute to the formation of hepatic granulomas in *Leishmania donovani* (Beattie et al., 2010) and *Mycobacterium bovis* BCG (Egen et al., 2008) infections. KCs express MHC class I and II molecules, ICAM-1, CD86, CD80 and function as APCs for naïve CD8 T cells and CD4 T cells *in vitro* (Bertolino et al., 2002; Ebrahimkhani et al., 2011). *In vivo*, there is now data showing that liver bone marrow-derived cells (which include both KC and DC) are able to efficiently retain adoptively transferred naïve TCR transgenic CD8 T cells in an antigen-specific manner. This retention leads to the rapid activation of the T cells within the liver (Bowen et al., 2002; Holz et al., 2012; Tay et al., 2014b).

Hepatic stellate cells (HSC), also known as Ito cells, are located between LSEC and hepatocytes. Following liver damage and regeneration, they secrete the extracellular matrix that is critical for tissue repair. However, this process is also responsible for the development of fibrosis. In a non-inflamed liver, these cells play a role in regulating blood flow via constriction to modulate the diameter of the sinusoids (Rockey, 2001). Cultured HSC express MHC Class I and II, CD1d, and low levels of CD80 and CD86 (Vinas et al., 2003; Winau et al., 2007). Contact between HSC and T cells has not been directly observed; however, it is likely that they occur via LSEC fenestrations as demonstrated for hepatocytes (Warren et al., 2006). The role of these cells in intrahepatic T cell activation is still controversial. However, a key study has demonstrated that HSCs are also able to activate naïve CD8 T cells within the liver (Winau et al., 2007). In contrast, a more recent study suggested that HSC are poor T cell stimulators and function more as regulatory bystanders by promoting Tregs and suppressing Th17 differentiation (Ichikawa et al., 2011). These variant results might be due to contaminants in HSC preparations or to phenotypic changes after culture (Vinas et al., 2003). Thus, further *in vivo* data is required to clearly demonstrate the role of HSC as APCs in the intact liver.

It is important to note that the liver also contains DCs and biliary epithelial cells. However, these cell types are located within the portal tracts, behind a non-fenestrated endothelial cell layer, and there is no experimental evidence suggesting that they are able to initiate intrahepatic primary T cell activation (Holz et al., 2010b). In addition, hepatic DCs are mostly immature, suggesting that, like most tissue DCs, these cells induce primary T cell activation in liver draining LNs following maturation and migration triggered by inflammation.

CROSS-PRESENTATION IN THE LIVER

Antigen cross-presentation is a specialized function restricted to a limited number of APCs. In lymphoid tissues, only some DC subsets can perform this function. In the liver, besides liver resident $CD8^+$ DCs, cross-presentation has been clearly

demonstrated for LSEC, but the role of other hepatic APCs remains controversial.

The role of LSEC in cross-presenting antigen *in vivo* has been demonstrated in a series of *in vitro* and *in vivo* studies by Knolle and colleagues, clearly establishing these cells as the main cross-presenting cells of the liver. Primary activation of CD8 T cells by LSEC generally promotes tolerance (Limmer et al., 2000) via several mechanisms, including decreased expression of CD80 and CD86 molecules required for efficient priming by secretion of the immunosuppressive molecules PGE2 and IL-10 (Knolle et al., 1998), up-regulation of the inhibitory ligand PD-L1 (Diehl et al., 2008), and interaction with DC leading to loss of IL-12, CD80 and CD86 expression (Schildberg et al., 2008). However, LSEC-activated CD8 T cells appear to survive *in vivo* for prolonged periods, and can be reactivated and rescued from deletion upon secondary encounter with DC (Bottcher et al., 2013).

The role of KC in cross-presenting antigen *in vivo* and the outcome of such activation are more controversial. *In vitro*, KC have been shown to inhibit T cell activation by secreting IL-10 (Knolle et al., 1995), but these cells also increase their expression of MHC class II and their APC function upon TLR3 ligation (Maemura et al., 2005; You et al., 2008). *In vivo*, CD8 T cell activation by KC leads to CD8 T cell apoptosis (Holz et al., 2012), suggesting that these APCs induce tolerance in the absence of inflammation. By comparing the ability of hepatocytes, LSEC, and KC to cross-present cell-associated antigen *in vitro*, Ebrahimkhani and colleagues have recently shown that LSEC are more efficient than KC in cross-presenting antigen (Ebrahimkhani et al., 2011).

Although one study also suggests that HSC can cross-present antigen (Winau et al., 2007) they have also been described as poor APCs and tolerogenic (Eksteen et al., 2009). HSC also appear to promote the expansion of $FoxP3^+$ regulatory T cells (Jiang et al., 2008; Ichikawa et al., 2011) due to their high expression levels of retinoic acid and TGF-β (Schwabe et al., 2006).

The role of hepatocytes in cross-presentation remains controversial: although some studies have shown that purified hepatocytes cross-present cell-associated antigen *in vitro* (Ebrahimkhani et al., 2011), other studies (Limmer et al., 2000; Bongfen et al., 2007; Cockburn et al., 2011) suggest the opposite.

INTERACTION OF SPOROZOITES WITH LIVER APCs

After entering via the skin, sporozoites migrate to the liver where they glide along the hepatic sinusoids (**Figure 1**). The various cell populations located in the hepatic sinusoids are critical for the malaria sporozoites to establish infection in the liver.

HSC proteoglycans protruding through the endothelial fenestration into the sinusoidal lumen have been shown to be critical to the initial retention of circulating sporozoites (Frevert et al., 1993; Pradel et al., 2002). Real time intravital microscopy experiments have revealed that following retention, some sporozoites glide along the sinusoid till they interact with KC (Frevert et al., 2005). These cells express chondroitin and heparan sulfate proteoglycans that promote sporozoite arrest (Pradel et al., 2002). The apical cell pole of the parasite then positions itself against KC and after a pause, the sporozoite traverses the KC, pushes slowly across the sinusoidal cell barrier, and reaches the space of Disse (**Figure 1**) (Frevert et al., 2005). Whether the sporozoite enters via LSEC fenestrations or through the gap separating two adjacent LSEC has not been resolved, but the results of a recent study suggest that 25% of sporozoites can cross LSEC via the paracellular route (Tavares et al., 2013). Once in the space of Disse, sporozoites seem to increase their velocity and migrate for many minutes through several hepatocytes, before they eventually localize to a final one (Frevert et al., 2005) in which they establish a parasitophorous vacuole (Mota et al., 2001). Hepatocytes are required for the parasite to change its form and multiply. After a period of 7 days in humans for *P. falciparum* or 2 days in mice for *P. berghei/P. yoelii*, each infected liver cell releases 10,000–30,000 merozoites that will subsequently infect red blood cells (**Figure 1**). This classically marks the end of the silent pre-erythrocytic phase, and the beginning of the symptomatic erythrocytic stage of the infection (Kappe et al., 2003; Frevert, 2004; Heussler et al., 2006).

The gateway model, based on observations made using real time intravital microscopy, suggests that sporozoite migration from the sinusoidal lumen into the space of Disse require KC (Baer et al., 2007). Consistent with the critical role of KC, *P. yoelii* infection of mutant CSF-1-deficient *op/op* mice, that possess 75% less KC (and other macrophages), leads to an 80% decrease in the number of infected hepatocytes (Baer et al., 2007). It is not entirely clear why the parasite would require KC to cross the endothelium, and take the risk of triggering innate immunity, instead of directly crossing the endothelium. Interestingly, avian and reptilian *Plasmodium* species invade and replicate in KC and other macrophages instead of infecting hepatocytes. It has been postulated that during evolution in mammalian hosts the parasite has retained the ability to bind to KC, but gained the property of infecting hepatocytes instead of KC (Baer et al., 2007). Electron micrographs often show KC resting in the lumen of the sinusoids, anchored between two adjacent LSEC. For this reason, it has been proposed that KC act as a portal for sporozoites to cross the endothelial barrier and reach the parenchyma (Baer et al., 2007).

A more recent study using intravital laser spinning-disk confocal microscopy has however demonstrated that, although the majority (78%) of all sporozoite crossing events associated with cell-traversal activity involve KC, 22% involve direct cell traversal of the LSEC without involving a KC (Tavares et al., 2013). KC are thus not an absolutely mandatory gateway for sporozoite crossing of the hepatic sinusoidal barrier. Interestingly, this study also provided evidence that live sporozoites deficient in cell traversal activity were trapped and degraded in KC, while only ∼10% of control sporozoites (probably those that have exhausted their CTL capacity or alternatively those that are already dead before they arrive in the liver) displayed similar lasting interactions with KC. Although live sporozoites endowed with cell traversal ability are not trapped and killed by KC, they have been reported to inactivate and eventually kill the traversed macrophage resident cell (Klotz and Frevert, 2008). The authors of this study speculated that the main purpose of sporozoite cell traversal in the liver is to avoid parasite clearance (Tavares et al., 2013).

ROLE OF THE LIVER IN PRIMARY T CELL ACTIVATION DURING THE PRE-ERYTHROCYTIC PHASE

Due to the very low number of hepatocytes infected during natural transmission, and the short duration of the liver phase (between 2 and 7 days depending on the host species and the malaria parasite strain) (Overstreet et al., 2008), the role of the liver in priming CD8 T cells during natural transmission has been difficult to address. Some studies have suggested that the low dose of immunogen transmitted via one mosquito inoculum is not sufficient to induce a clonal burst sufficient to generate an effective T cell response (Hafalla et al., 2002). In addition, *Plasmodium* blood stage infection has been shown to inhibit the maturation and the capacity of DCs to initiate immune responses and suppress protective CD8 T cell responses against the liver stage *in vivo* (Ocana-Morgner et al., 2003). Although it does not necessarily extrapolate to natural *Plasmodium* transmission, immunization with high numbers of irradiated or genetically attenuated sporozoites that are capable of infecting hepatocytes, but do not develop in the liver, excludes any effect of blood stage parasites. This type of immunization allows a better understanding of the liver contribution to CD8 T cell priming and is critical in optimizing vaccine strategies.

Experiments performed using CSP-specific transgenic T cells adoptively transferred into mice immunized with irradiated or non-irradiated sporozoites argued against any significant effective CD8 T cell activation in the liver and liver draining LNs (Chakravarty et al., 2007). However, although this study excluded any contribution of the liver in priming protective anti-CSP CD8 T cells after *Plasmodium* infection, it did not rule out a role for the liver in T cell activation during malaria parasite infection for 2 main reasons. Firstly, T cell activation in this study was assessed by IFN-γ production at 48h instead of other functionally independent markers of T cell activation at earlier time points. Although a hallmark of efficient priming and effector CD8 T cell function, IFN-γ is poorly expressed by hepatocyte-activated CD8 T cells (Holz et al., 2008, 2012). In addition, the majority of T cells activated in the liver undergo rapid deletion, leading to tolerance (Holz et al., 2010a). Secondly, this study focused on CSP, a protein expressed by sporozoites before they invade hepatocytes. Expression of this protein decreases after invasion, while other proteins more specific for the liver stage of the parasite start to be expressed.

The role of the liver in priming T cells specific for mid to late pre-erythrocytic proteins therefore remains to be addressed. Genetically attenuated sporozoites, designed to have arrested development at a late liver stage, induced larger and broader CD8 T cell responses compared to radiation attenuated sporozoite or early-arresting genetically attenuated parasite immunizations (Butler et al., 2011). They also promoted superior protection in inbred and outbred mice (Butler et al., 2011), providing further support for the idea that bona-fide liver stage antigens can prime protective immune responses.

A recent study by Michael Bevan and colleagues illustrates why this question is important for anti-malaria parasite vaccination. By using high-throughput screening and irradiated or attenuated sporozoites, these investigators identified a unique CTL response against an epitope derived from the parasite ribosomal L3 antigen (Murphy et al., 2013), a protein expressed during the liver-stage and in erythrocytes. Surprisingly, these CTLs did not follow the general pattern of anti-CSP CTL responses, as they could be expanded by heterologous prime-boost regimens, but not by multiple sporozoite immunizations. These findings illustrate that CTL responses specific for liver-stage proteins might require different immunization strategies than those specific for antigens expressed by the incoming parasite. Although this study did not clarify the mechanisms responsible for these distinct CTL patterns or whether other liver-stage proteins generate similar patterns, it is tempting to speculate that they are caused by different priming conditions in the liver and LNs.

Several studies have shown that intravenous administration of attenuated sporozoites is superior to other routes in conferring protection (Spitalny and Nussenzw, 1972; Richards, 1977; Chatterjee et al., 1999; Douradinha et al., 2007; Epstein et al., 2011) As sporozoites traveling via the intravenous route reach the liver, these experiments support a major contribution of this organ in priming effector cells. A recent study reporting the results of a clinical trial of an anti-malaria parasite vaccination on 80 volunteers, confirmed that intravenous administration of purified irradiated sporozoites promoted protection, while inoculation of the vaccine via the skin was suboptimally immunogenic and protective (Epstein et al., 2011). A high frequency of sporozoite-specific IFN-γ–producing CD8 T cells in the liver was shown to confer protection in mice (Epstein et al., 2011). Although it does not demonstrate the direct role of hepatic cells in priming effector T cells during natural transmission, this study suggests that targeting the liver route of activation might promote protection.

Presentation of liver-stage sporozoite proteins to antigen-specific CD8 T cells can occur either directly by infected hepatocytes (direct MHC Class I presentation pathway), or indirectly by APCs that capture extracellular sporozoite-derived proteins synthesized by hepatocytes and present them in the context of MHC I molecules (cross-presentation pathway). These two pathways are detailed below.

ROLE OF INFECTED HEPATOCYTES IN PRIMARY T CELL ACTIVATION IN MALARIA

Although sporozoites interact with many liver cell types, experiments using bone marrow chimeric mice have demonstrated that radio-resistant cells (most likely to be infected hepatocytes) are likely to be the main cells presenting antigens derived from irradiated sporozoites to effector CD8 T cells in the liver (Chakravarty et al., 2007). Furthermore, it has been shown that while DCs cross-present CSP via endosomes, presentation of CSP by hepatocytes requires expression of the protein in the cytosol rather than in endosomes (Cockburn et al., 2011). CSP presentation by hepatocytes was independent of cross-presentation (Cockburn et al., 2011). Collectively, these results provide strong evidence that hepatocytes are the main liver cells infected by irradiated sporozoites, and able to present cytosolic sporozoite antigens to T cells via the direct MHC class I presentation pathway.

Some evidence also suggests that replication-competent malaria parasite-infected hepatocytes contribute to CD8 T cell priming: (i) elimination of hepatic stage parasites abrogates

protection generated by administration of irradiated sporozoites (Scheller and Azad, 1995); and (ii) protection induced by live sporozoites in mice treated with chloroquine was abrogated by primaquine treatment that eliminates the parasite from hepatocytes (Belnoue et al., 2004).

Most studies investigating the early immune events in malaria have used mice immunized with a large dose of irradiated sporozoites, and have focused on the abundant CSP that covers the surface of sporozoites. This protein has multiple functions in several stages of the parasite life cycle. It is thought to be a master regulator of the development of the pre-erythrocytic stages (Singh et al., 2007), and inhibits KC immune defense (Usynin et al., 2007). In infected hepatocytes, CSP traverses the membrane of the parasitophorous vesicle, enters the cytoplasm, and translocates to the nucleus where it downregulates expression of the transcription activation factor NF-κB (Singh et al., 2007). This inhibits host inflammatory immune functions, and promotes the development of the liver stage parasite (Singh et al., 2007). Paradoxically, by entering the cytoplasm, CSP exposes itself to proteasomal degradation and processing by the MHC Class I presentation pathway. CSP has been shown to be an immunodominant protective T cell antigen (Kumar et al., 2006). Mouse primary hepatocytes have also been shown to be able to process *Plasmodium berghei* CSP after live sporozoite infection, and present CSP-derived peptides to specific CD8 T cells *in vitro* in a proteasome-dependent manner (Bongfen et al., 2007). A recent study (Ma et al., 2013) performed using human primary hepatocytes has also confirmed that the direct MHC class I presentation pathway is not markedly affected by live *P. berghei* sporozoites: mRNA expression levels of all major components of the MHC class I processing and presentation machinery were not significantly affected by parasite replication at 24 or 48 h post-infection, while the levels of MHC class I expression were decreased only by 10–20% at the surface of infected host cells as compared to either uninfected or untreated controls. Furthermore, ectopic expression of CSP did not interfere with basal MHC class I expression, or IFN-γ and TNF-α-induced upregulation of MHC class I expression (Ma et al., 2013).

These results therefore imply that CSP translocation to the cytoplasm increases the risk of recognition and elimination of infected hepatocytes following presentation of CSP-peptides by the classical MHC class I presentation pathway. More recently, LISP-2, a protein specifically expressed by mid to late stage parasites, has also been shown to be exported to the cytosol from the parasitophorous vacuole (Orito et al., 2013). If this protein is immunogenic, it also have the potential to be an immune target.

It is thought that the important function of CSP in the parasite development justifies why the parasite exposes itself to the immune system in such a way. An alternative view is that primary activation by hepatocytes leads to tolerance, and therefore this is a low risk strategy for the parasite. Expression of CSP and other liver-stage specific proteins on liver cells might also be critical to tolerize effector CD8 T cells primed in skin-draining LNs.

OUTCOME OF PRIMARY CD8 T CELL ACTIVATION BY HEPATOCYTES

Hepatocytes are not "professional" APCs as they do not express known costimulatory molecules such as CD80 and CD86. Although these cells are able to induce primary activation and proliferation of CD8 T cells, it is expected that this activation would not be as efficient as priming induced by mature DCs. Our initial *in vitro* work demonstrated that naive CD8 T cells co-cultured with antigen-expressing hepatocytes were activated, proliferated, and started to acquire cytotoxic activity, but died prematurely by apoptosis (Bertolino et al., 1998, 1999), suggesting that hepatocytes promoted T cell deletion and tolerance.

To investigate the fate of CD8 T cells activated by antigen-expressing hepatocytes *in vivo*, we have used transgenic models and rAAV vectors to restrict antigen or relevant p:MHC expression to these cells *in vivo*. Hepatocyte-activated T cells adoptively transferred into mice expressing the cognate p:MHC complex on all hepatocytes were deleted within the first 4 days post-activation, without developing significant cytotoxic activity (Bertolino et al., 2001; Bowen et al., 2004; Holz et al., 2008; Tay et al., 2014a), suggesting that this activation induced tolerance. In these studies, most CD8 T cells were rapidly deleted within the first few hours post-activation as a result of invading hepatocytes and subsequent degradation inside lysosomal compartments (Benseler et al., 2011). Invasion of a cell into another cell is known as "emperipolesis" (Humble et al., 1956; Overholtzer and Brugge, 2008), and is observed in liver sections in autoimmune and viral hepatitis (Dienes, 1989). To distinguish this process of CD8 T cell deletion within hepatocytes from emperipolesis without destruction of the invading cell, we have termed this process "suicidal emperipolesis" (Benseler et al., 2011).

Some T cells surviving suicidal emperipolesis proliferate, but subsequently die as a result of primary activation in the absence of costimulatory molecules. This "death by neglect" has been shown to be associated with low expression of cytokines and high expression of the pro-apoptotic molecule Bim (Holz et al., 2008).

Although hepatocytes generally induce T cell tolerance *in vivo*, recent studies have suggested that this is not always the case. In some experimental systems, CD8 T cells activated by hepatocytes survived and became full effector cells (Wuensch et al., 2006; Klein and Crispe, 2006b; Derkow et al., 2007). Our results suggest that generation of CTLs by hepatocytes requires high TCR affinity interactions and low frequencies of antigen-expressing hepatocytes; in the absence of these requirements, CD8 T cell tolerance ensues in response to hepatocyte expressed antigen (Tay et al., 2014a).

In malaria parasite infection, the frequency of sporozoite-infected hepatocytes is low. Our recent studies therefore predict that infected hepatocytes should be able to prime at least some high affinity sporozoite antigen-specific T cells and generate full effector cells that contribute to the anti-malaria CTL pool.

ROLE OF CROSS-PRESENTING APCs IN THE ANTI-MALARIA RESPONSE

Unless the parasite has developed strategies to inhibit this process, it is very likely that following entry into hepatocytes, some antigens are shed, captured, and cross-presented by other cell types in lymphoid tissues and/or the liver (**Figure 1**).

Although liver DCs are not accessible to naive T cells and do not therefore contribute to intrahepatic activation, it is likely that these APCs play a major role in cross-presenting antigens

in the liver draining LNs. In studies of transgenic models and using the rAAV system mentioned above, our results suggest that cross-presentation of antigens expressed by hepatocytes leads to efficient T cell priming, resulting in full effector function (Tay et al., 2014a). We have postulated that antigen presentation in lymphoid tissues is the main pathway facilitating the generation of CTLs directed against liver pathogens (Bowen et al., 2004, 2005).

Until recently, the exact location of the liver-draining LNs in mice had not been identified (Barbier et al., 2012). Although likely, the contribution of these lymph nodes to CTL generation during natural malaria parasite infection has not been clearly demonstrated. However, a recent study (Lau et al., 2014) in which mice were intravenously injected with high numbers of irradiated sporozoites demonstrated preferential malarial-antigen specific CD8 T cell activation and proliferation in the 3 hepatic draining LNs (Barbier et al., 2012). The number of intrahepatic $CD11c^+$ cells has also been reported to increase in mice injected with irradiated *P. yoelii* sporozoites or from live parasites inoculated by infected mosquitoes (Leiriao et al., 2005b). These potential APCs have been shown to contain intracellular hepatocyte proteins, suggesting that debris of apoptotic hepatocytes were taken up by liver DCs (or recruited circulating DCs or monocytes) (Leiriao et al., 2005b). Whether these cells migrate to the draining LNs and cross-present sporozoite antigens to T cells has not been investigated in this study. Another study (Jobe et al., 2009) used irradiated sporozoites to demonstrate that conventional $CD8^+$ DC able to induce the differentiation of naïve CD8 T cells into $CD44^{hi}CD45RB^{lo}CD62L^{lo}$ effector memory T cells accumulated in the livers of sporozoite-immunized mice, suggesting that this liver DC subset is involved in the induction of liver-stage antigen-specific CD8 T cells.

It is important to emphasize that most of these studies have used high numbers (5×10^4 to 1×10^6) of irradiated parasites injected intravenously, or mice bitten by 50 mosquitoes, that do not reflect natural transmission. Thus, although DCs might capture and cross-present sporozoite proteins from infected hepatocytes in the liver and/or draining LN when high numbers of sporozoites are administered, the role of DCs in cross-presenting sporozoite antigens derived from hepatocytes in the liver draining LNs during natural transmission requires further investigation.

The function and fate of anti-malaria T cells activated by potential cross-presenting hepatic APCs is relatively unknown. Experiments performed using radiation-induced bone marrow chimeras (Chakravarty et al., 2007) excluded any role for bone marrow–derived hematopoietic-lineage liver cells, such as blood-derived DCs or monocyte-derived macrophages, in the presentation of irradiated and live sporozoite-derived antigens to effector CD8 T cells, suggesting that these APCs did not present antigens to T cells. It is important to note that, despite the conclusions of the study, these experiments did not exclude a role for KC, as a large proportion of these liver-resident macrophages are radio-resistant (Kennedy and Abkowitz, 1997; Klein et al., 2007). In addition, the role of radio-resistant LSEC and stellate cells has not been clarified. Thus, in the absence of further experimental evidence, any discussion on this topic remains speculative.

It is clear that the parasite avoids being detected by CTLs primed in the skin draining LNs by changing expression of proteins after settling in hepatocytes. Once in the liver, it is likely that it uses other strategies to avoid being eliminated by CD8 T cells specific for liver stage proteins.

COULD SPOROZOITES HARNESS THE TOLEROGENIC PROPERTY OF THE LIVER TO SUBVERT INTRAHEPATIC IMMUNITY AND PREVENT ELIMINATION OF INFECTED CELLS?

The first model is that malaria sporozoites prevent the generation of efficient CTLs that would kill infected cells by inducing tolerance ("*subversion from within*" model, see **Figure 2**). This possibility is more relevant to infection by irradiated sporozoites that do not induce long lasting responses and could be tolerized.

Many studies in the literature suggest that the liver can tolerize effector T cells. In transplantation, an hepatic allograft can reverse rejection of previously transplanted organs from the same donor (Benseler et al., 2007), even when the liver was transplanted up to 6 days after the initial transplant, indicating that the liver allograft is able to induce tolerance in the recently activated T cell compartment. More recently, we have shown that expression of an allo-MHC class I molecule in most hepatocytes prevented the rejection of skin grafts expressing this allo-MHC molecule despite prior sensitization of recipient mice (Cunningham et al., 2013). Finally, analyses performed in transgenic mouse models indicated that TCR transgenic T cells activated in the LNs of recipient mice accumulated in the antigen-expressing liver, killed some hepatocytes, but never induced chronic liver damage, again suggesting that these effector T cells were silenced (Holz et al., 2010b, 2012).

How does the liver tolerize effector T cells? The original "graveyard" model proposed by Crispe and colleagues (Huang et al., 1994; Mehal et al., 1999) suggested that the liver was a disposal site for T cells activated in lymphoid tissues. This was consistent with several studies showing efficient intrahepatic retention of activated CD8 and CD4 T cells. The same group later proposed that the liver actively killed effector T cells recognizing their antigen within the liver. However, as this theory could not account for the presence of effective immune responses in the liver under some circumstances, and did not allow for the presence of high numbers of effector/memory T cells in this organ (Belz et al., 1998; Keating et al., 2007), this concept was subsequently abandoned (Crispe et al., 2006; Bertolino et al., 2007).

We have recently revisited this question by showing that the frequency of antigen-expressing hepatocytes is critical in determining tolerogenic outcome. When at least 25% of hepatocytes expressed antigen, effector T cells primed in lymphoid tissues were eventually silenced, while below this threshold cytotoxic T cells survived after clearing antigen-expressing hepatocytes (Tay et al., 2014a). This might explain the outcome of tolerance in transplantation and transgenic systems, where there is ubiquitous expression of antigen within the liver, and detection of memory T cells in some viral models (Belz et al., 1998; Keating et al., 2007).

The mechanisms by which the liver silences effector CD8 T cells in the presence of high dose antigen is not entirely clear, and molecules such as Galectin-1, TNFR, and FasL on hepatic cells have been suggested to play a key role in this process (reviewed in

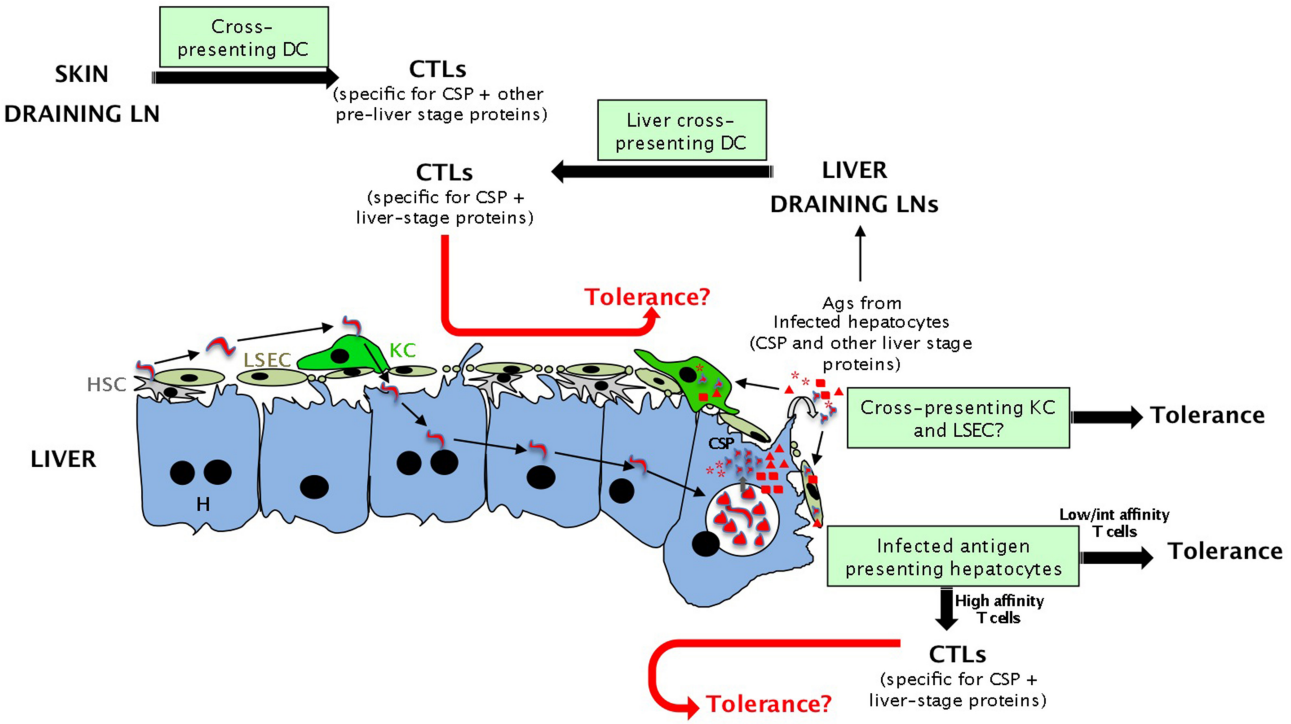

FIGURE 2 | Potential antigen presenting cells during the pre-erythrocytic phase of malaria parasite infection eliciting tolerance or effective immune responses (CTLs). Presentation of sporozoite antigens to CD8 T cells in the liver could occur either by hepatocytes or cross-presenting hepatic cells (LSEC and maybe KC). Although activation by all these cells should lead to tolerance, T cells recognizing sporozoite antigens expressed by infected hepatocytes with high affinity might differentiate into full effector cells. Malarial antigens can also be presented in skin and liver draining LNs by cross-presenting DCs. This activation should lead to efficient priming and CTLs. The fate of effector cells generated in the liver and lymphoid tissues and re-encountering tolerogenic APCs in the liver (red arrows) is unknown.

Holz et al., 2010b, 2012). Our results suggest that most effector T cells surviving the early phases of activation expressed high levels of the pro-apoptotic molecule Bim, and died by apoptosis. T cells that survived this phase displayed poor CTL function, associated with an exhausted phenotype, including expression of high levels of the inhibitory molecule PD-1, as well as reduced TCR and CD3 expression (Holz et al., 2010b, 2012; Tay et al., 2014a), indicative of prolonged antigen exposure and reduced T cell activity (Ferber et al., 1994). It is also possible that suicidal emperipolesis plays an important role in the clearance of effector T cells recognizing their cognate antigen within the liver. This question is currently under investigation in our laboratory.

The extremely low frequency of infected hepatocytes during the pre-erythrocytic stage (even when a high number of irradiated sporozoites is used for immunization) is far from the high antigen dose promoting silencing of effector cells. Based on this argument, we predict that the few infected hepatocytes and potential cross-presenting hepatic APCs should be killed by CTLs, rather than inducing tolerance.

DO SPOROZOITES AVOID BEING DETECTED BY CTLs?

The alternative model to explain how malaria sporozoites use the liver to avoid being eliminated by the immune system is to hide in this large organ to minimize as much as possible the probability of encountering CTLs *("hide-and-seek" model,* see **Figure 3**). This model would apply to both live and irradiated sporozoites. From the parasite perspective, it might be efficiently achieved by infecting the minimum number of hepatocytes and by spending the shorter possible time in the host cell.

Using quantitative PCR assays, it has been estimated that a mosquito bite inoculates between 0 and 1297 live sporozoites into the skin, with a mean number of 123 and a median number of 18 (Medica and Sinnis, 2005). As some of these parasites remain in the skin or migrate to the LNs, only a few will make it to the liver (Sidjanski and Vanderberg, 1997; Jin et al., 2007). As a result, an extremely low number of hepatocytes become infected by live sporozoites. It is estimated that in mice, 1 in a million hepatocyte is infected while in humans the frequency is even lower (one in a billion) (Van Braeckel-Budimir and Harty, 2014).

It is not clear whether T cells specific for the liver-stage malaria parasite efficiently find the very few infected hepatocytes during the short period of time they reside in the liver. It is likely that they do not. This is particularly true for naive T cells for primary activation, but it would also be difficult for effector T cells to find their targets (secondary activation). As the liver is made of hundreds of millions of hepatocytes, this is akin to finding a needle in a haystack. Thus, the low number of infected hepatocytes combined with the short amount of time sporozoites reside in the cell might be a strategy used by the parasite to avoid both T cell priming in the liver and escape immune surveillance by

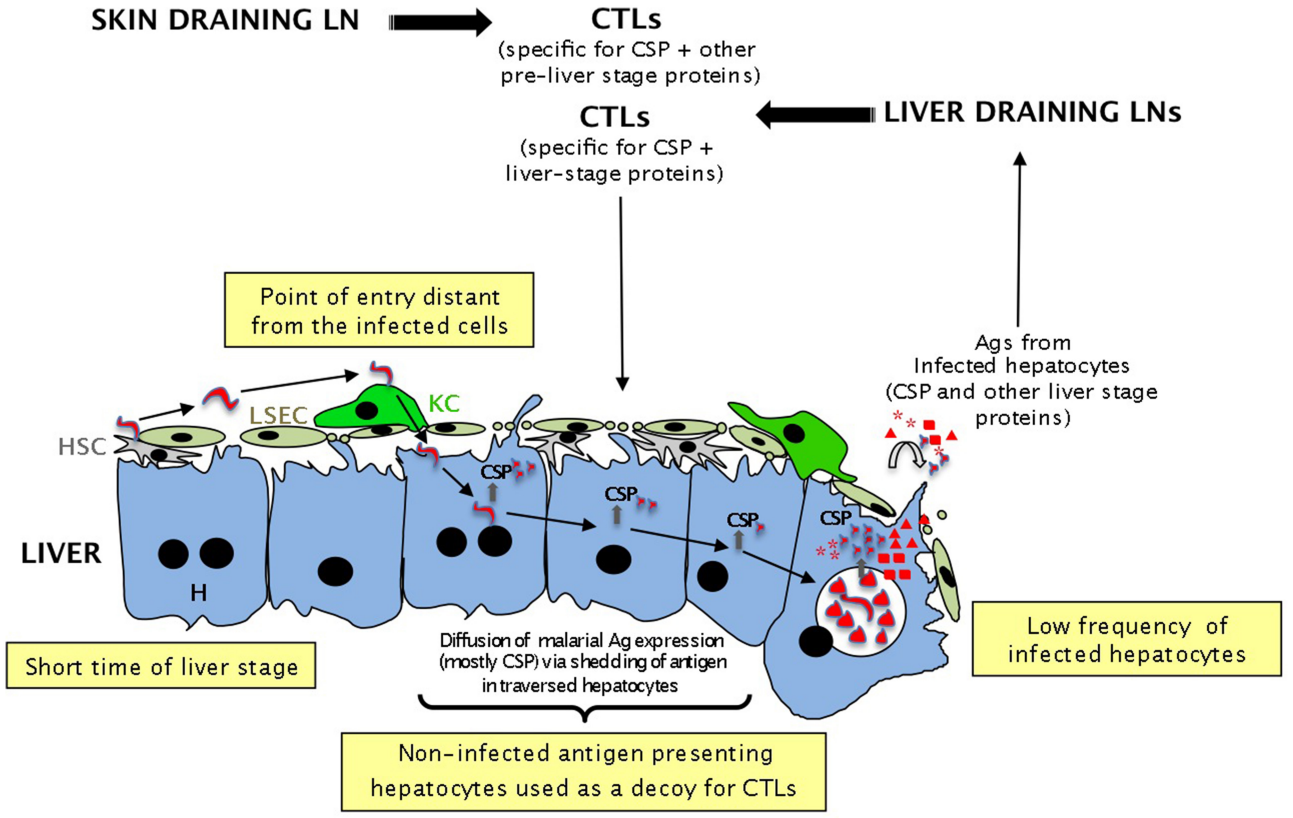

FIGURE 3 | Different potential strategies used by sporozoites to evade immune surveillance by CTLs according to the "hide and seek" model. To avoid being eliminated by CTLs generated in the LNs or intrahepatically, malaria sporozoites might have developed several strategies: (i) the very low frequency of sporozoites lowers the probability of encountering CTLs; (ii) the short time spent in the liver (2–7 days) gives CTLs a very short time to kill all infected cells; (iii) infection of hepatocytes at a distance from the entry point might divert the CTLs to the wrong area; (iv) the trail of CSP left in non-infected hepatocytes during cell traversal by sporozoites might be used as a decoy to "buy" some precious time for the sporozoites.

CTLs. This might explain the apparent lack of effective immune response during the pre-erythrocytic stage and the lack of long lasting protection induced by both live and irradiated sporozoites. Consistent with this model, high numbers (10^5) of irradiated sporozoites injected intravenously did not elicit a very significant activation and proliferation of naive TCR transgenic CD8 T cells in the liver during the first 2 days in comparison to the spleen and liver draining LNs, although these T cells recognized an antigen expressed by hepatocytes (Lau et al., 2014). Effector cells generated in lymphoid tissues recirculated, however, to the liver at day 3 (Lau et al., 2014). These findings suggest that, in vaccination protocols using high numbers of irradiated sporozoites, most of the primary T cell activation occurs in lymphoid tissues rather than the liver. Whether this also occurs during natural transmission (lower physiological numbers of live sporozoites inoculated by mosquitos) requires, however, further demonstration. Regardless of whether they are generated in LNs or spleen, to achieve protective immunity CTLs would need to detect and kill all infected hepatocytes during the very short period of the pre-erythrocytic phase (2–7 days). One can predict that the success of this killing is likely to be critically dependent on the total number of effectors.

Results obtained using mouse and human immunization protocols using both attenuated or live sporozoites suggest that this assumption is correct. These protocols can only achieve protection by injecting 1000-fold or more sporozoites than occurs in natural transmission and/or by boosting the number of CSP-specific CD8 T cells (Overstreet et al., 2008). Immunization experiments in mice have suggested that long-term protection requires a remarkably strong CD8 T-cell response, representing a substantial fraction of the total CD8 T-cell pool (Schofield et al., 1987; Weiss et al., 1988; Romero et al., 1989; Schmidt et al., 2008). Optimal CD8 T cell priming was associated with persistence of CSP in both lymphoid tissues and livers of mice, and was mediated by DCs or macrophages (Cockburn et al., 2010). By investigating the CD8 T-cell immune response in Balb/c mice immunized with DC loaded with the immunodominant CSP, followed by a boost provided by *Listeria monocytogenes* expressing CSP, Harty and colleagues have recently shown that stable long term protection upon live sporozoite challenge required CSP-specific CD8 T-cell frequencies exceeding a threshold of 1% of total PBLs (Schmidt et al., 2008). This threshold depends on the mouse MHC strain and *Plasmodium* species (Van Braeckel-Budimir and Harty, 2014). However, it significantly exceeds

frequencies of antigen-specific CD8 T cells required for plausible protection against various viral and bacterial infections.

In addition to minimizing the probability of being recognized by T cells whilst within host hepatocytes, it is tempting to speculate that the sporozoite has developed several strategies to gain precious time and further decrease this probability. For rodent malaria parasites (*P. berghei* and *P. yoelii)*, the timing of T cell activation might be sufficient to avoid immune recognition by CTLs activated in LNs: by the time CTLs leave the LNs and circulate to the liver (2–3 days), merozoites would have been generated and the liver phase would be over. The pre-erythrocytic phase of *P. falciparum* in humans is longer (7–9 days) and other strategies might be used by the pathogen to evade or delay immune detection.

One of these potential strategies is to use a decoy to divert the T cells to non-infected cells. When live sporozoites traverse through several hepatocytes before establishing the parasitophorous vacuole in a selected host cell (Frevert et al., 2005), they disrupt the plasma membrane of several other hepatocytes (Mota et al., 2001). During this process, they leave a trail of CSP (and possibly other proteins) in the cytosol of affected cells (**Figures 1**, **3**). It is not clear whether hepatocytes recover from this cell traversal. Studies using high number of live parasites have detected many necrotic hepatocytes and increases in serum ALT (Frevert et al., 2005), suggesting that sporozoites cause hepatocellular injury. Whether this is an experimental artifact due to the high number of parasites, or whether it occurs during natural malaria transmission needs to be clarified. However, if some hepatocytes quickly repair their perforated membranes, it is likely that the CSP contained in the cytosol of non-infected cells will be processed by the MHC class I presentation pathway and form p:MHC complexes. Consistent with this, by using live *P. berghei* sporozoites deficient for the SPECT protein that is essential for cell traversal, Bongfen et al. (2007) have demonstrated that both infected and traversed primary hepatocytes process and present CSP to CD8 T cells. The processing and presentation pathway involved the proteasome, antigen transport through a post-endoplasmic reticulum compartment, and aspartic proteases (Bongfen et al., 2007).

Non-infected hepatocytes presenting CSP might be used by the sporozoite as a decoy strategy to divert CD8 T cells toward non-infected hepatocytes. This tactic might delay the time for T cell detection, and increase the chance of survival of infected hepatocytes. Interestingly, some reports suggested that infected hepatocytes become resistant to apoptosis (Leiriao et al., 2005a; Van De Sand et al., 2005), conferring an additional selective advantage to infected cells.

The reason why the sporozoite needs to traverse several hepatocytes before settling in one cell located at a distant site from the point of entry is also intriguing and could be an additional strategy developed by the parasite to further reduce the risk of being detected by T cells (**Figure 3**). Upon KC traversal, the abundant CSP on the sporozoite surface has been shown to induce cyclic AMP (cAMP) that inhibits the assembly of the NADPH oxidase (Usynin et al., 2007). This blocks the generation of reactive oxygen species, a potent macrophage defense mechanism required to destroy parasites (Usynin et al., 2007). Although this prevents the parasite from being eliminated by the macrophage defense system while it crosses the endothelium, it is possible that the traversed KC releases cytokines and chemokines able to attract immune cells. This might explain why in addition to blocking the respiratory burst, viable sporozoites also generate an anti-inflammatory cytokine secretion profile in KC (Klotz and Frevert, 2008). KC exposed to infectious sporozoites down-modulated MHC class I, secreted no IL-12p40, and had reduced antigen presenting function (Steers et al., 2005), suggesting that the sporozites minimize the potential signals that KC might release to attract immune cells. Interestingly, in contrast to KC from naive mice exposed to infectious sporozoites, KC from irradiated sporozoite-immune-challenged mice up-regulated class I and costimulatory molecules and produced elevated IL-12p40 relative to naive KC. These KC also exhibited augmented APC activity (Steers et al., 2005).

Settling in the first hepatocyte at the initial entry point would be highly risky for the parasite, as CD8 T cells would rapidly find and kill the infected cell located close to the signaling KC. Traveling to a distant site might buy precious time for the parasite while it differentiates at a distant location.

In summary, malaria sporozoites seem to have developed several strategies to avoid killing of infected hepatocytes by sporozoite-specific CTLs. These include changing expression of proteins within the infected hepatocyte, infecting an organ where primary activation of CD8 T cells is biased toward the induction of tolerance, and developing several tactics to decrease the probability that infected hepatocytes encounter antigen-specific T cells. These arguments support the view that the predominant limitation for malaria-specific T cells is finding/eliminating all infected hepatocytes during the short period of the hepatic cycle phase, rather than overcoming tolerance in the liver.

Thus, although both malaria sporozoites and hepatotropic viruses target the liver, these pathogens seem to use totally different strategies to escape immune recognition. Instead of infecting many hepatocytes in a short period of time and exploiting the tolerogenic properties of the liver, malaria sporozoites infect very few cells, and have developed additional strategies to decrease the probability of being detected by CTLs. This model predicts that to be effective, anti-malaria vaccination protocols should aim to boost the number of CTLs to increase their probability of finding and killing all infected hepatocytes.

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Corrigendum: Malaria and the liver: immunological hide-and-seek or subversion of immunity from within?

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A corrigendum on

Malaria and the liver: immunological hide-and-seek or subversion of immunity from within?
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In the original article on page 4, there is an error in the following sentence:

"[...] Liver sinusoidal endothelial cells are efficient scavenger cells strategically located in the sinusoids and able to clear low-density lipoprotein (LDL) and capture particulate antigens and immune complexes circulating via the blood and deliver them to hepatocytes (Sorensen et al., 2012). This uptake is mediated by mannose receptor, and has been shown to be more efficient than that mediated by KC, suggesting that LSEC specialize in this function. [...]"

The correct sentence is:

"[...] Liver sinusoidal endothelial cells are efficient scavenger cells strategically located in the sinusoids, and able to clear oxidised or acetylated low-density lipoprotein (LDL) and capture small particles (<200 nm) circulating via the blood (Sorensen et al., 2012). This uptake is mediated by several receptors and has been shown to be more efficient than that mediated by KC, suggesting that LSEC specialize in this function. [...]"

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Antigen-driven focal inflammatory death of malaria liver stages

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Multiple immunizations using live irradiated sporozoites, the infectious plasmodial stage delivered into the host skin during a mosquito bite, can elicit sterile immunity to malaria. $CD8^+$ T cells seem to play an essential role in this protective immunity, since their depletion consistently abolishes sterilizing protection in several experimental models. So far, only a few parasite antigens are known to induce $CD8^+$ T cell-dependent protection, but none of them can reach the levels of protection afforded by live attenuated parasites. Systematic attempts to identify novel antigens associated with this efficient cellular protection were so far unsuccessful. In addition, the precise mechanisms involved in the recognition and elimination of parasitized hepatocytes *in vivo* by $CD8^+$ T cells still remain obscure. Recently, it has been shown that specific effector $CD8^+$ T cells, after recognition of parasitized hepatocytes, recruit specific and non-specific activated $CD8^+$ T cells to the site of infection, resulting in the formation of cellular clusters around and in the further elimination of intracellular parasites. The significance of this finding is discussed in the perspective of a general mechanism of antigen-dependent focalized inflammation and its consequences for the elimination of malaria liver stages.

Keywords: $CD8^+$ T cells, inflammatory infiltrate, cooperative behavior, *in vivo* imaging, *Plasmodium*

THE PRE-ERYTHROCYTIC PHASE OF MALARIA INFECTION

Malaria is a mosquito borne disease caused by *Plasmodium spp.* parasites. Despite advances in control and prevention measures, malaria still kills > 600,000 people annually and no effective licensed vaccine is available so far (www.who.int/malaria/en). The disease is a consequence of repeated cycles of parasite invasion and replication inside red blood cells (RBC). However, before infecting the blood and causing the disease, the parasite must pass through a silent and asymptomatic pre-erythrocytic (PE) phase. In mammals, the PE phase starts with the inoculation of sporozoites into the extravascular regions of the host skin during a mosquito bite. Some of these highly motile stages get access to the blood circulation and home to the liver, where they traverse several hepatic cells before invading and developing, as liver stages, inside hepatocytes. One infected hepatocyte generates thousands of RBC-infective stages in 2 to ∼10 days depending on the species. Finally, the PE phase finishes with the release of these invasive stages into the blood circulation (Ménard et al., 2013).

In contrast to the symptomatic erythrocytic phase of infection, which can reach the magnitude of $\sim 10^{12}$ circulating infected RBCs in hyperparasitemic adults (World Health Organization [WHO], 1990), the asymptomatic PE stages represent the smallest parasite burden (1∼1000 sporozoites and liver stages) inside the mammalian host (Medica and Sinnis, 2005). Consequently, these stages are considered as ideal targets for vaccine intervention, since early elimination of this minute population of extracellular sporozoites and intracellular liver stages could strategically block infection before pathogenesis and transmission of parasites to mosquitoes. Most importantly, immunizations using live sporozoites, which are blocked during hepatic development as consequence of irradiation (Nussenzweig et al., 1967), genetic modification (Mueller et al., 2005) or drugs (Friesen and Matuschewski, 2011), confer sterile protection against sporozoite re-infection in several experimental models, as well as in humans (Seder et al., 2013).

THE PUZZLE OF LIVER STAGE KILLING BY $CD8^+$T CELLS

Although there is evidence that antibodies and $CD4^+$ T cells contribute to the protection induced by live irradiated sporozoites (Schofield et al., 1987; Tsuji et al., 1990; Rodrigues et al., 1993; Doolan and Hoffman, 2000), $CD8^+$ T cells seem to be the major players of this sterilizing immunity since in almost all tested rodent (Schofield et al., 1987; Doolan and Hoffman, 2000; Schmidt et al., 2010) and primate (Weiss and Jiang, 2012) models, sterile protection is abolished when $CD8^+$ T cells are depleted before sporozoite challenge. Accordingly, the transfer of parasite-specific $CD8^+$ T cells can also protect mice from sporozoite infection (Romero et al., 1989; Weiss et al., 1992).

This protective cellular response is associated with a high number of specific $CD8^+$ T cells circulating in the peripheral blood of protected mice, ranging from ∼5 to 60% of total circulating $CD8^+$ T cells (Van Braeckel-Budimir and Harty, 2014). Similarly, adoptive transfer of $\sim 10^7$ activated specific $CD8^+$ T cells, which totalize ∼26 to 60% of $CD8^+$ T cells circulating in the blood, is

required to sterilize the infection in the liver, while the transfer of 10^6 $CD8^+$ T cells, which represents ∼3% of $CD8^+$ T cells circulating in the blood, is not enough to completely protect mice against sporozoite challenge (Kimura et al., 2013). In addition, interactions between $CD8^+$ T cells and infected hepatocytes could not be observed after adoptive transfer of 10^6 primed $CD8^+$ T cells to infected mice (Cabrera et al., 2013), suggesting that the recognition of infected hepatocytes is dependent on high numbers of specific $CD8^+$ T cells. In humans, however, the levels of $CD8^+$ T cells correlated with protection seem to be much lower than in rodents (Ewer et al., 2013). Protective activity is dependent not only on the quantity, but also on the quality of $CD8^+$ T cells. For example, high expression levels of cell adhesion molecules such as CD44 and VLA-4 are correlated with the *in vivo* anti-parasite activity of $CD8^+$ T cells, but not with their *in vitro* cytotoxic activity (Rodrigues et al., 1992). These molecules have been implicated in homotypic and heterotypic adhesion of lymphocytes, in the trafficking of T cells to inflamed site (Nandi et al., 2004), and could play an important role in the clustering of T cells at the site of infection (next sections).

Very little is known about the nature of protective parasite epitopes presented on the surface of infected hepatocytes by class I major histocompatibility complex molecules (MHC I). In rodent models, protective MHC I-restricted epitopes were described in the circumsporozoite protein (CSP, Romero et al., 1989; Weiss et al., 1992; Rodrigues et al., 1994; Schmidt et al., 2008) and in the thrombospondin-related anonymous protein (TRAP, Hafalla et al., 2013). Both CSP and TRAP are membrane proteins expressed mainly in sporozoites. In humans, immunization using adjuvanted, truncated CSP induced only partial protection without a detectable specific $CD8^+$ T cell response (Moorthy and Ballou, 2009). Immunization using TRAP delivered by viral vectors, which elicit robust T-cell responses, induced also a partial protection which was correlated with the frequency of monofunctional interferon gamma (IFNγ)-producing $CD8^+$ T cells (Ewer et al., 2013). Intriguingly, several attempts to identify new epitopes targeted by $CD8^+$ T cells among thousands of profiled peptides, mini-genes or genes covering hundreds of parasite proteins, have revealed only a few new PE candidate antigens, but none of them induced protection against sporozoite infection (Mishra et al., 2011; Murphy et al., 2013; Hafalla et al., 2013). Whether the difficulty to unravel new protective epitopes is a consequence of the method of screening, which relies on the secretion of IFNγ by $CD8^+$ T cells detected by ELISPOT, or of immunization, which may not reach the critical $CD8^+$ T cell threshold necessary for protection, remains to be determined.

Given the scarcity of protective CD8 epitopes, another convenient way to study $CD8^+$ T cell responses against infected hepatocytes is to use the epitope of ovalbumin (OVA) fused to endogenous and exogenous antigens expressed in malaria PE stages (Cockburn et al., 2011; Kimura et al., 2013; Montagna et al., 2014). This strategy showed that membrane, cytoplasmic and tubulovesicular network exported proteins can harbor epitopes that are presented on the surface of infected hepatocytes, leading to the parasite elimination by MHC I–restricted ($H\text{-}2K^b$), OVA-specific $CD8^+$ T cells (OT-I cells). Although antigen-presentation is dependent on the transporter associated with antigen processing (TAP; Cockburn et al., 2011; Kimura et al., 2013), which translocates peptides from the cytosol into the endoplasmic reticulum where they are loaded onto MHC I molecules, the mechanism by which these membrane, cytoplasmic and exported parasite antigens cross the parasitophorous vacuole membrane and reach the host cell cytoplasm is still controversial and unclear (Singh et al., 2007; Cockburn et al., 2011; Montagna et al., 2014).

The mechanisms by which $CD8^+$ T cells eliminate liver stages are also puzzling. Systemic depletion of IFNγ consistently abolishes the sterile protection induced by irradiated sporozoites in rodent models (Schofield et al., 1987; Doolan and Hoffman, 2000). However, activated $IFN\gamma^{-/-}$ $CD8^+$ T cells harboring transgenic T-cell receptors (TCRs), which can recognize specific cognate epitopes presented on the surface of infected hepatocytes, are still capable of controlling infection as their IFNγ-proficient counterparts (Chakravarty et al., 2008; Kimura et al., 2013). The same is observed for FasL and/or perforin knockout $CD8^+$ T cells (Morrot and Zavala, 2004; Kimura et al., 2013). Finally, sterile protection is only lost in a proportion of animals injected with double IFNγ and perforin knockout $CD8^+$ T cells (Kimura et al., 2013). Altogether these loss-of-function studies depict a scenario where the elimination of liver stages by specific $CD8^+$ T cells seems to be a complex process involving several and redundant effector molecules and probably other IFNγ-producing immune cells.

$CD8^+$ T CELL–DEPENDENT INFLAMMATORY FOCI AND THE DEATH OF LIVER STAGES

While molecular mechanisms underlying the elimination of liver stages by $CD8^+$ T cells are still unclear, the cellular events leading to the death of these hepatic parasites are only slowly being revealed. In 1989, multiple inflammatory foci containing numerous $CD11b^+$, $CD8^+$, and at lesser extent $CD4^+$ cells were first observed in the liver of BALB/c mice immunized with *P. berghei* (Pb) irradiated sporozoites, 43 h after challenge with normal sporozoites. Although no parasites could be observed in association with these infiltrates, the formation of these cellular foci was dependent on the presence of $CD8^+$ T cells (Hoffman et al., 1989).

Several years later, DNA *in situ* hybridization revealed remnants of *Pb* parasites in close association with cellular infiltrates in the liver of rats immunized with irradiated sporozoites and challenged with normal sporozoites (Scheller et al., 1997). The number of infiltrates increased after 24 h post-challenge and coincided with the decrease in the number of liver stages in the immunized rats. $CD4^+$ and $CD8^+$ T cells were also identified in these infiltrates. The number of $CD4^+$ cells was maintained constant at 31 h and 44 h post-infection, while the number of $CD8^+$ T cells was four and sixfold superior to those of $CD4^+$ T cells at these respective time points.

Notably, systemic inhibition of nitric oxide (NO) production by aminoguanidine reduced the number of inflammatory foci after 24 h post-infection with a clear diminution of the number of $CD8^+$ lymphocytes in the focal infiltrates (Scheller et al., 1997). This treatment also abolished the elimination of liver stages in the immunized animals indicating that the production of NO is critical for the accumulation of $CD8^+$ T cells, which

are necessary for the parasite elimination. However, the source of inducible NO synthase in the liver of immunized rats challenged with sporozoites seems to be restricted to infected hepatocytes (Klotz et al., 1995). Since NO produced by infected hepatocytes is involved in the elimination of parasites *in vitro* in the absence of T cells (Nussler et al., 1993; Mellouk et al., 1994), it is not possible to distinguish if aminoguanidine abolished elimination of liver stages in the immunized animal by the direct inhibition of NO anti-plasmodial activity, by the inhibition of inflammatory responses that induce the accumulation of $CD8^+$ T cells around the infected hepatocytes, or both. Studies using specific $CD8^+$ T lymphocytes or liver cells that are deficient in the inducible synthesis of NO could precise the role of this molecule in the killing of liver stages.

ANTIGEN-DRIVEN INFLAMMATORY FOCI

Recently, *in vivo* imaging revealed $CD8^+$ T cell infiltrates in close association with infected hepatocytes in BALB/c mice immunized with irradiated *P. yoelii* (Py) sporozoites (Cockburn et al., 2013). The use of fluorescent $CD8^+$ T cells harboring transgenic TCRs, specific for epitopes expressed by the parasite and presented by MHC I molecules on the surface of infected hepatocytes, also showed that cluster formation mediated by $CD8^+$ T cells is antigen specific. Activated $CD8^+$ T cells, which recognize specifically a *Py*CSP-epitope, clustered around and eliminated *Py* infected hepatocytes. Conversely activated OT-I cells did not cluster or eliminate *Py* and *Pb* infected hepatocytes, but readily clustered around and eliminated hepatocytes infected with *Pb* expressing the CD8 epitope of OVA (Cockburn et al., 2013; Kimura et al., 2013). The frequency distribution of *Py*CSP-specific $CD8^+$ T cells around infected hepatocytes fitted a mathematical model where the recruitment of T cells was density-dependent, indicating that activated $CD8^+$ T cells were attracted to a cluster. The close apposition of $CD8^+$ T cells with infected hepatocytes suggests that this first step of specific recognition and recruitment is initiated by the direct contact between these two cells (**Figure 1A**). Hepatocytes traversed by sporozoites are also thought to present antigens to $CD8^+$ T cells. Although traversed hepatocytes do not alter the elimination of infected cells by $CD8^+$ T cells *in vitro* (Bongfen et al., 2007), the role of liver cells traversed by sporozoites (Mota et al., 2001; Tavares et al., 2013a,b) located adjacent to infected hepatocytes cannot be formally excluded in the process of initiation and formation of these cellular inflammatory foci (**Figure 1B**). This capacity of $CD8^+$ T cells to recognize a cognate antigen and recruit other $CD8^+$ T cells to the site of infection seems to be a general and novel function of $CD8^+$ T cells, as reported recently in a model of mouse reproductive tract infection by the lymphocytic choriomeningitis virus (Schenkel et al., 2013).

Surprisingly, when activated *Py*CSP-specific and non-specific $CD8^+$ T cells were transferred together in mice infected with *Py* sporozoites, non-specific $CD8^+$ T cells, which usually did not cluster around *Py* infected hepatocytes, modified their behavior and were recruited together with *Py*CSP-specific $CD8^+$ T cells to the site of infection (Cockburn et al., 2013). In a tumor model, activated OT-I cells were also responsible for the deep infiltration and accumulation of activated non-specific T cells in OVA-expressing EL4 tumors (Boissonnas et al., 2007), indicating

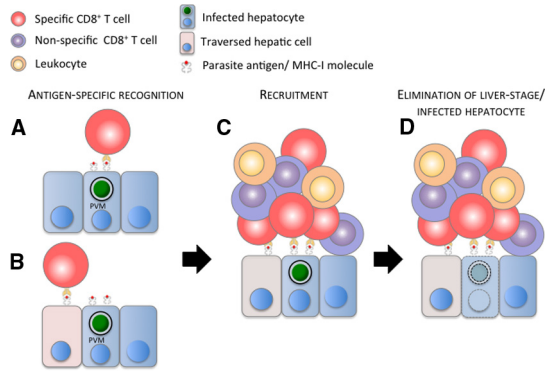

FIGURE 1 | Model of the elimination of malaria liver stage by a $CD8^+$ T cell-dependent inflammatory cell cluster. (A) Parasite antigens cross the parasitophorous vacuole membrane (PVM), are processed and presented on the surface of the infected hepatocyte by MHC I molecules, and/or **(B)** are released in the cytoplasm and presented on the surface of hepatic cells (hepatocytes, Kupffer cells, endothelial cells—figuratively represented by the pink cells) traversed by sporozoites. **(A,B)** Parasite-specific, activated $CD8^+$ T cell (red cell) recognizes its cognate antigen and **(C)** recruits other specific and non-specific (purple cells) $CD8^+$ T cells, and presumably other leukocytes (orange cells) to the site of infection. **(D)** These immune cells cluster around the infected hepatocyte, leading to the elimination of the malaria liver stage and presumably of the infected hepatocyte. For clarity and due to the lack of information about its position during cluster formation, the sinusoidal barrier is not represented in the model.

that $CD8^+$ T cells not only exert a direct cytotoxic activity on target cells, but can also orchestrate a focal inflammatory response by the recognition of a specific antigen and further recruitment of specific and non-specific T cells to the site of infection or tumor growth. This inflammatory role is in agreement with the recent observation that $CD8^+$ T cells express a burst of inflammatory cytokines immediately after cognate antigen stimulation recruiting diverse cellular types involved in inflammatory reactions (Sung et al., 2013).

MULTIPLICITY OF PARASITE DEATH PHENOTYPES

Dynamic imaging revealed how clusters of activated $CD8^+$ T cells eliminate liver stages *in vivo* (Cockburn et al., 2013; Kimura et al., 2013). The utilization of a vitality index (VI) based on the fluorescence intensity of GFP-expressing liver stages permitted the monitoring of parasite viability/death over hours of observation after the transfer of activated and specific $CD8^+$ T cells. Strikingly, killing of liver stages was a lasting process, which on average occurred after several hours of association between $CD8^+$ T cells and infected hepatocytes. In addition, the kinetics of the VI of fluorescent parasites allowed the discrimination of at least three distinct death phenotypes (DPs) associated with the presence of activated $CD8^+$ T cells. An abrupt decrease in the parasite VI followed by the diffusion of a weak GFP signal through the host cell cytoplasm characterized the first DP. The second DP was characterized by a slow decrease in the VI over hours of interaction. The blebbing of the infected hepatocyte with the release of parasite material into the sinusoids characterized the third DP. All these distinct DPs suggest again that parasite elimination could rely on multiple and redundant mechanisms of killing.

A better understanding of these killing mechanisms might be achieved using functional *in vivo* imaging. This strategy is a double approach that combines the quantitative imaging of mutant cells with the imaging of non-mutant cells using specific fluorescent reporters to investigate function (Tavares et al., 2013a). For example, the correlation between the ablation of a cytotoxic effector molecule in protective $CD8^+$ T cells and the loss of a given DP could indicate a causal relation between these two variables. Complementary, the use of fluorescent markers that report the level of expression of cytotoxic molecules (Croxford and Buch, 2011; Mouchacca et al., 2013) could be used to discriminate different populations of protective $CD8^+$ T cells and their relationship with the DPs could be assessed by *in vivo* imaging. The latter approach could also reveal what is the activation phenotype of $CD8^+$ T cells associated with efficient homing to the liver, recognition of infected hepatocytes, clustering formation and killing of liver stages.

CLUSTERING OF CELLS FACILITATES ELIMINATION OF LIVER STAGES

Qualitatively a few $CD8^+$ T cells are sufficient to rapidly eliminate a liver stage *in vitro* (Trimnell et al., 2009), however, the killing process *in vivo* seems to be much longer and complex, involving multiple cells (Cockburn et al., 2013). Whether these differences are due to intrinsic characteristics of $CD8^+$ T cells used in these studies or are a consequence of limitations imposed by an *in vitro* system is not yet determined. The liver possesses an immune-privileged environment, which confers a relatively high resistance against $CD8^+$ T cell responses (Pircher et al., 2006). This fact could explain the long time that these cells require to eliminate liver stages *in vivo*. Cell clustering triggered by antigen-specific $CD8^+$ T cells might possibly facilitate the process of parasite killing by augmenting the local concentration of protective cellular and molecular effectors. To test this hypothesis, cluster formation was inhibited by targeting chemokine signaling. Chemokines act on T cells via G-Protein Coupled Receptors (GPCRs) signaling. Pertussis toxin (PTx) was used to inhibit GPCR signaling through the ADP-ribosylation and uncoupling of G-Proteins (Cotton and Claing, 2009). Activated *Py*CSP-specific $CD8^+$ T cells treated with PTx had an impaired capacity to cluster around and eliminate infected hepatocytes, while maintaining their capacity to kill target cells and secrete cytokines *in vitro*, as well as, to home to the liver *in vivo* (Cockburn et al., 2013). Although this pharmacological approach indicates that the clustering of cells triggered by *Py*CSP-specific $CD8^+$ T cells around infected hepatocytes facilitates the elimination of malaria liver stages, the identification of the chemokine(s) involved in the recruitment of cells to a cluster is still missing. The discovery of these molecules will permit a precise assessment, e.g., using knockout animals, of the role of cell clustering in the elimination of malaria liver stages.

CONCLUDING REMARKS AND PERSPECTIVES

Altogether these data suggest that $CD8^+$ T cell-dependent elimination of liver stages is a complex and cooperative process involving multiple cells and effector molecules. An elevated number of $CD8^+$ T cells with a proper binding phenotype seem to be required for finding and eliminating the scarce population of infected hepatocytes in the liver. Following antigen-specific recognition of the site of infection by activated $CD8^+$ T cells, both specific and non-specific $CD8^+$ T cells and likely other immune cells are recruited to the site of infection. Finally infected hepatocytes are cleared by these antigen-driven inflammatory foci, displaying a multiplicity of DPs. This mechanism of killing places $CD8^+$ T cells as an essential linker between a specific adaptive immune response, which starts with the recognition of an infected cell, and a non-specific immune response, characterized by the recruitment of inflammatory cells to the site of infection and ensuing elimination of liver stages (**Figure 1**). This multi-cellular cooperative model of killing can also incorporate the relative importance of other cellular types, like $CD4^+$ T cells and other IFNγ-producing cells, in the foci composition and protection. It may also explain the general dependence on IFNγ for sterile immunity but the dispensable role of this cytokine for protection induced by parasite-specific $IFN\gamma^{-/-}$ $CD8^+$ T cells.

Important topics related to the formation, composition and killing efficacy of these $CD8^+$ T cell-dependent inflammatory foci are still unclear and need further investigation. One of these topics is the identification of novel protective CD8 epitopes presented on the surface of hepatocytes (Crabb et al., 2012) and the verification of their concerted action with other known protective epitopes, such as those described in CSP and TRAP. This approach could reveal a possible synergistic, additive, neutral or antagonistic effect on cluster formation and protection. Another important subject to address is the contribution of $CD8^+$ T cells, which cannot recognize infected hepatocytes, but are specifically activated in the lymph node that drains the bite site, for example, by mosquito saliva or sporozoite antigens (Chakravarty et al., 2007), in foci formation and elimination of liver stages. Once it is determined that the recruitment of these activated $CD8^+$ T cells to infected hepatocytes can improve the killing of liver stages, a vaccination strategy using protective epitopes, presented by infected hepatocytes, and inflammatory epitopes, which might improve killing by focal inflammation, could be envisaged and tested. Similarly the role of non-specific innate immune cells in cluster formation and killing activity is also an open question. It is well documented that inflammatory infiltrates are associated with the natural resistance of naïve rodents to hepatic infection (Khan and Vanderberg, 1991), but their role in immunized animals is not known. In addition, depletion studies using multiple doses of anti-asialo GM1 antibodies partially abrogated protection induced by irradiated sporozoites in several mouse strains (Doolan and Hoffman, 2000). This treatment is known to efficiently deplete NK cells and basophils, and despite the expression of asialo GM1 on subpopulations of NKT, $CD8^+$ T and γδ T cells, no significant depletion was observed after a single dose of anti-asialo GM1 antibodies in C57BL/6 mice (Nishikado et al., 2011). This suggests that NK cells, basophils and other innate immune cells could also participate in the focal inflammation orchestrated by specific $CD8^+$ T cells. Finally the unraveling of the molecular determinants of highly protective $CD8^+$ T cells is crucial for defining the type of effector cells a vaccine should generate. All this information could be

very useful for the design of an immunization strategy aiming at the efficient elimination of malaria liver stages mediated by $CD8^+$ T cells.

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A sufficient role of MHC class I molecules on hepatocytes in anti-plasmodial activity of CD8$^+$ T cells *in vivo*

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Although CD8$^+$ T cells are shown to mediate the protective immunity against the liver stages of malaria parasites in mice, whether the direct presentation of malaria antigen by major histocompatibility complex (MHC) class I molecules expressed on the liver of infected host is required for anti-plasmodial activity of CD8$^+$ T cells is still unknown. Presently, there is only one CD8$^+$ epitope, SYVPSAEQI, derived from the circumsporozoite protein of *Plasmodium yoelii* (PyCS), that mediates anti-malarial protection and is presented in the context of a K^d molecule. Therefore, to investigate the mode of anti-plasmodial activity of CD8+ T cells, we have previously generated C57BL/6 transgenic (Tg) mice, in which a K^d molecule is expressed only on hepatocyte (Alb-K^d) or dendritic cell (DC; CD11c-K^d), by using albumin promoter or CD11c promoter, respectively. We have also generated MHC-I-K^d Tg mice, which express the K^d molecule under the MHC class I (MHC-I) promoter, as a positive control. From splenocytes collected from CD11c-K^d Tg mice immunized with a synthetic peptide, SYVPSAEQI, which corresponds to the CD8$^+$ T-cell epitope of PyCS, emulsified in incomplete Freund's adjuvant , a PyCS-specific CD8$^+$ T-cell line was generated. This PyCS-specific CD8$^+$T-cell line was then adoptively transferred into a cohort of either MHC-K^d Tg or Alb-K^d Tg mice listed above, as well as wild-type C57BL/6 mice. Then both transferred and non-transferred mice were challenged with live malaria parasites. We found that the adoptive transfer of a PyCS-specific CD8$^+$ T-cell line resulted in a significant inhibition of the parasite burden in the liver of Alb-K^d Tg, as well as MHC-I-K^d Tg mice, but not of C57BL/6 mice. These results indicate that the K^d molecule expressed by hepatocytes is sufficient in mediating the anti-plasmodial activity of PyCS-specific CD8$^+$ T cells *in vivo*.

Keywords: malaria, CD8$^+$ T cell, liver, MHC class I, transgenic mouse

INTRODUCTION

Malaria is a severe disease that ranks among the most prevalent infections in tropical areas throughout the world. Approximately 250–300 million people become infected yearly with relatively high rates of morbidity and mortality. The WHO estimates that every year nearly one million children die of malaria in Africa alone (World Health Organization [WHO], 2008). The widespread occurrence and the increasing incidence of malaria in many countries, caused by drug resistant parasites and insecticide resistant vectors (*Anopheles mosquitoes*), underscore the need for developing new methods of controlling this disease, which includes more effective vaccines.

A number of mouse studies to date using *Plasmodium yoelii* and *P. berghei* parasites for challenge have shown that protective immunity against pre-erythrocytic stages is mediated in part by T cells, particularly CD8$^+$ T cells (Weiss et al., 1988, 1992; Rodrigues et al., 1991, 1997; Tsuji et al., 1998; Sano et al., 2001; Schmidt et al., 2008). Then, the essential role of major histocompatibility complex class I (MHC-I) molecules in mediating CD8$^+$ T-cell-dependent anti-malarial immunity was shown by the study in which adoptive transfer of malaria-immune splenocytes into β2 microglobulin (β2 m)-deficient mice failed to confer protection (White et al., 1996). Using transgenic (Tg) mice that express a T-cell receptor (TCR), based on the TCR sequence of CD8$^+$ T cells that recognize an immunodominant T-cell epitope of the PyCS protein, SYVPSAEQI, a recent study showed that the Tg T cells do not require bone marrow-derived antigen-presenting cells (APCs) for protection upon adoptive transfer; instead, they recognize antigen on parenchymal cells, presumably parasitized hepatocytes (Chakravarty et al., 2007). These studies all together strongly suggest that MHC-I molecules expressed by malaria-infected hepatocytes play a key role in mediating the anti-plasmodial activity of CD8$^+$ T cells *in vivo*. However, it is still unknown to which extent MHC-I molecules expressed by hepatocytes mediate the anti-plasmodial activity of CD8$^+$ T cells *in vivo*.

The immunodominant T-cell epitope, SYVPSAEQI, described above is presented by H-2K^d (K^d) molecules to CD8$^+$ T cells and is known to be the only epitope that can induce protective CD8$^+$ T cells against malaria. Therefore, to provide answers concerning the mechanisms underlying the anti-plasmodial activity of CD8$^+$ T cells, we have taken an approach in which Tg C57BL/6 mice, expressing H-2K^d molecules only on the surface of hepatocytes (Alb-K^d), DCs (CD11c-K^d), or all nucleated cells (MHC-I-K^d), have been generated (Huang et al., 2013) and used in the current studies.

MATERIALS AND METHODS

PARASITES AND ANIMALS

Female *Anopheles stephensi* mosquitoes infected with *P. yoelii* 17XNL strain were purchased from the New York University insectary. *P. yoelii* sporozoites were isolated from the salivary glands of infected *A. stephensi* mosquitoes 14 days after the mosquitoes had received an infectious blood meal (Huang et al., 2014). Six- to eight-week-old C57BL6 mice were purchased from Taconic (Germantown, NY, USA). Three tissues specific H-2K^d Tg mice models, Alb-K^d, CD11c-K^d, and major histocompatibility complex-I-K^d (MHC-I-K^d) Tg mice models, were established in our lab, in which H-2K^d were expressed under the control of albumin, CD11c, and MHC I promoters, respectively, in C57BL/6 mice (Huang et al., 2013, 2014). All mice were maintained under standard conditions in The Laboratory Animal Research Center of The Rockefeller University. Furthermore, all animal experiments were carried out in strict accordance with the Policy on Humane Care and Use of Laboratory Animals of the United States Public Health Service. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at The Rockefeller University (Assurance # A3081-01).

PEPTIDE, TETRAMER, AND CULTURE MEDIUM

A peptide, SYVPSAEQI, which corresponds to the CD8$^+$ T cell epitope of the CS protein of *P. yoelii* (PyCS), was synthesized by Peptide 2.0 Inc. (Chantilly, VA, USA). An allophycocyanin (APC)-labeled H-2K^d/SYVPSAEQI-tetramer was generated and provided to us by the NIH Tetramer Core Facility at Emory University. As culture medium we used DMEM-high glucose (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Thermo Scientific, Waltham, MA, USA) and 1% supernatant derived from phorbol myristate acetate (Fisher Scientific, Pittsburgh, PA, USA) activated EL-4 cells (Huang et al., 2013).

GENERATION OF A PyCS-SPECIFIC CD8+ T-CELL LINE

CD11c-K^d Tg mice were immunized twice with a 2-week interval and intra-peritoneally with10 μg of the synthetic peptide, SYVPSAEQI, emulsified in incomplete Freund's adjuvant (IFA; Sigma-Aldrich, St. Louis, MO, USA). Ten days after the last immunization, splenocytes collected from immunized CD11c-K^d mice were stimulated with radiation-attenuated (3000-rad) splenocytes isolated from naive MHC-I-K^d mice pulsed with SYVPSAEQI peptide. We repeated the stimulation for a few times every 10 days, as described Rodrigues et al. (1991), and 10 days after the last stimulation, the frequency of PyCS-specific CD8$^+$ T cells among the T-cell line was determined by either Tetramer staining or an ELISpot assay.

TETRAMER STAINING AND ELISPOT ASSAY

The specificity and frequency of a PyCS-specific CD8$^+$ T-cell line was determined by a flow cytometric analysis after staining the T-cell line with antibodies against various CD molecules and an APC-labeled H-2K^d/SYVPSAEQI-tetramer. All the antibodies used in this study were purchased from BioLegend (San Diego, CA, USA). Briefly, cells were first incubated with unlabeled anti-CD16/CD32 antibody (Clone 16) for 10 min at 4°C. Then the cells were incubated with FITC-labeled anti CD3 antibody (17A2), Pacific Blue-labeled anti-CD8 alpha antibody (clone 53–6.7), and APC-labeled H-2K^d/SYVPSAEQI-tetramer at room temperature for 30 min. A flow cytometric analysis was performed using an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA). The frequency of SYVPSAEQI-specific, IFN-γ–secreting T cells was determined by an ELISpot assay, as previously described Huang et al. (2013, 2014). Briefly, 1000 cells of the T cell line were co-cultured with 5×10^5 EL-4-K^d cells (Huang et al., 2013) loaded with SYVPSAEQI in a well of an ELISpot plate. Twenty-four hours later, the relative number of IFN-γ secreting cells was determined by counting the number of spots that corresponds to IFN-γ-secreting cells using a stereomicroscope. The number of IFN-γ secreting cells after co-culturing with EL-4-K^d cells without SYVPSAEQI peptide was used as negative control.

ADOPTIVE TRANSFER AND PARASITE CHALLENGE

Ten million cells of PyCS-specific CD8$^+$ T-cell line were adoptively transferred intravenously (i.v.) into each mouse (Rodrigues et al., 1991). Twenty-four hours later, 1×10^4 viable *P. yoelii* sporozoites were inoculated to each transferred, as well as non-transferred mice by tail vein injection (Rodrigues et al., 1991).

ASSESSMENT OF PARASITE BURDEN IN THE LIVER

Parasite burden in the liver was determined 42 h after sporozoite challenge by measuring the amount of parasite-specific 18S rRNA in the liver of challenged mice, using a real-time quantitative RT-PCR with the 7500 Fast Real-Time PCR System (Life Technologies, Grand Island, NY, USA; Huang et al., 2014). Parasite burden was described as a ratio of the absolute copy number of parasite-specific 18S rRNA to that of mouse GAPDH.

STATISTICAL ANALYSES

Statistical analyses were done using GraphPad Prism (version 5.03; GraphPad Software Inc., La Jolla, CA, USA). All data were expressed as the mean ± SD of three mice. Statistical analyses of experimental and control data were evaluated by one-way ANOVA and Student's *t*-test. A value of $p<0.05$ was considered statistically significant.

RESULTS

FREQUENCY OF PyCS-SPECIFIC CD8+ T CELLS EXPANDED *IN VITRO* FROM ISOLATED SPLENOCYTES OF CD11c-K^d MICE IMMUNIZED WITH SYVPSAEQI PEPTIDE

CD11c-K^d Tg mice were immunized twice with SYVPSAEQI peptide emulsified in IFA with a 2-week interval. Then splenocytes from peptide-immunized CD11c-K^d Tg mice were collected for the expansion of a PyCS-specific CD8$^+$ T-cell line *in vitro*, which was achieved by stimulating the cells for a few times with irradiated MHC-I-K^d Tg mouse-derived splenocytes pulsed with the SYVPSAEQI peptide in a 10–14-day interval, as similarly performed previously (Rodrigues et al., 1991). After successful expansion of a PyCS-specific CD8$^+$ T-cell line, the specificity and frequency of the PyCS-specific CD8$^+$ T-cell line were determined by either APC-labeled H-2K^d/SYVPSAEQI-tetramer staining or IFN-γ ELISpot assay, as shown in **Figure 1**. The tetramer staining results depicted

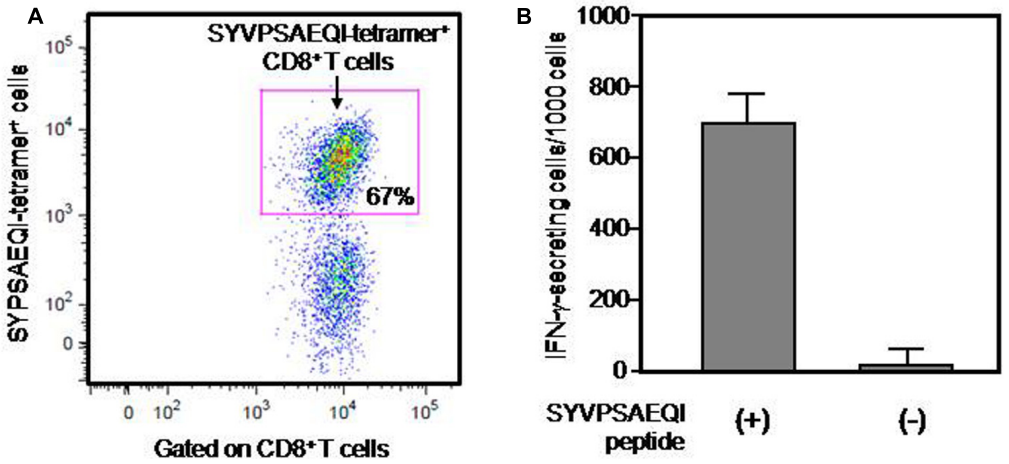

FIGURE 1 | Frequency of a PyCS-specific CD8$^+$ T-cell line, as determined by either a tetramer staining or an ELISpot assay. Frequency of PyCS-specific CD8$^+$ T cells among the T-cell line was determined by either a tetramer staining **(A)** or an IFN-γELISpot assay **(B)**. In **(A)**, CD3$^+$ T cells gated with anti-CD8 antibody were stained with APC-labeled H-2K^d/SYVPSAEQI-tetramer. The number shows the percentage of CD8$^+$ T cells that are positive with H-2K^d/SYVPSAEQI-tetramer. In **(B)**, 1000 cells of the T-cell line were co-cultured with 5 × 10^5 EL-4-K^d cells loaded with SYVPSAEQI in a well of an ELISpot plate, and 24 h later, the relative number of IFN-γ-secreting cells was determined by an ELISpot assay. The number of IFN-γ-secreting cells after co-culturing with EL-4-K^d cells without SYVPSAEQI peptide loading was used as a negative control.

that more than two third of the cells of a PyCS-specific CD8$^+$ T-cell line could be identified as H-2K^d/SYVPSAEQI tetramer CD8$^+$ T cells (**Figure 1A**). By assessing the ELISpot assay, more than 700 cells out of 1,000 cells of the T cell line were found to get activated by the SYVPSAEQI peptide and secrete IFN-γ, whereas in the absence of the SYVPSAEQI peptide, the cells did not display any responses, indicating that there was no non-specific or auto-reactive T cells present in the T cell line (**Figure 1B**). This indicates that a majority (>65–70%) of the population in the T cell line are specific to the SYVPSAEQI epitope.

INHIBITION OF *P. yoelii* HEPATIC STAGE DEVELOPMENT IN MHC-K^d MICE AND Alb-K^d MICE AFTER ADOPTIVELY TRANSFERRING PyCS-SPECIFIC CD8$^+$ T CELLS

Now that we generated a PyCS-specific CD8$^+$ T-cell line having more than two third of the cells specific to SYVPSAEQI peptide,

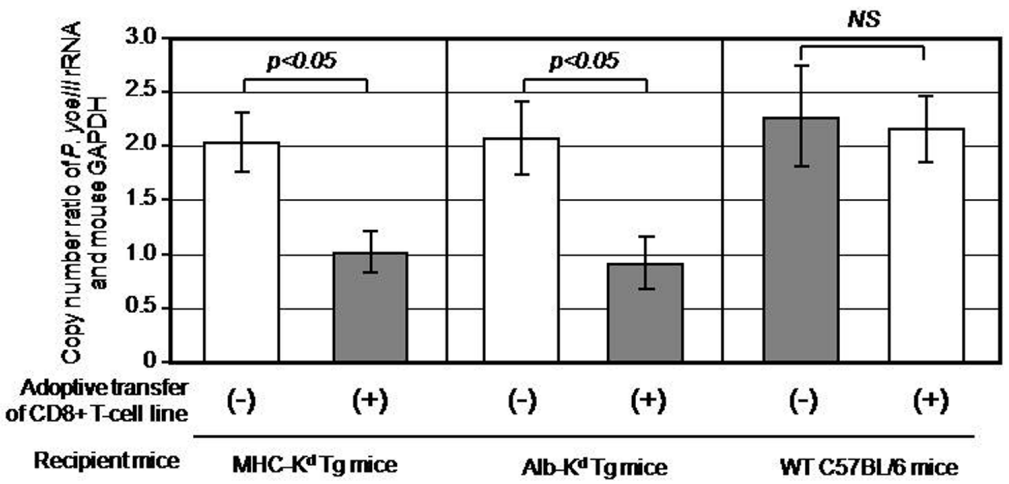

FIGURE 2 | Inhibition of *Plasmodium yoeli* liver stage development in MHC-K^d mice and Alb-K^d mice upon adoptive transfer of PyCS-specific CD8$^+$ T cells, as determined by a real-time qRT-PCR. Each mouse of MHC-K^d Tg mice, Alb-K^d Tg mice, and wild-type C57BL/6J mice (three mice per group) received 1 × 10^7 cells of a PyCS-specific CD8$^+$ T-cell line intravenously. Twenty-four hours later, the transferred, as well as non-transferred Tg mice and wild-type C57BL/6 mice, were challenged by 1 × 10^4 viable *P. yoelii* sporozoites. After 42 h, the parasite burden in the liver was determined by measuring the relative copy number of parasite-specific 18S rRNA to that of mouse GAPDH using a real-time qRT-PCR. The mice that do not receive PyCS-specific CD8$^+$ T-cell transfer served as negative controls. Error bars represent means ± SEM ($n = 3$). A value of $p < 0.05$ was considered statistically significant, whereas N.S. means "not significant."

we sought to address one of the key questions regarding the manner in which CD8$^+$ T cells recognize and eliminate the hepatic stage of malaria *in vivo*. More specifically, we aimed to determine the role of MHC-I molecules expressed on hepatocytes in mediating CD8$^+$ T cells' recognition of malaria-infected hepatocytes and their anti-plasmodial activity *in vivo*. For this purpose, we adoptively transferred 1×10^7 cells of PyCS-specific CD8$^+$ T-cell line to three groups (three mice each): MHC-I-K^d Tg mice (as a positive control), Alb-K^d Tg mice and wild-type C57BL/6 mice (as a negative control). Respective Tg mice that do not receive the PyCS-specific CD8$^+$ T-cell transfer were used as a negative control for each group of transferred Tg mice. All experimental mice were then challenged with 1×10^4 live *P. yoelii* sporozoites. Forty-two hours after the challenge, the livers were collected from challenged mice, and the liver parasite burden was determined by measuring the parasite-specific rRNA by real-time PCR and quantified by a ratio of the absolute copy number of parasite-specific 18S rRNA to that of mouse GAPDH. After the *P. yoelii* sporozoites challenge, the PyCS-specific CD8$^+$ T-cell line inhibited almost 50% of the parasite burden in the liver of MHC-I-K^d Tg mice, but not in C57BL/6 mice (**Figure 2**). Most importantly, a PyCS-specific CD8$^+$ T-cell line transferred to Alb-K^d Tg mice could inhibit (55%) the liver stage development as potently as those transferred to MHC-I-K^d Tg mice. These results indicate that K^d molecules expressed on hepatocytes are sufficient in mediating the anti-parasitic effect of PyCS-specific CD8$^+$ T cells *in vivo*.

DISCUSSION

The role of MHC-I molecules in mediating CD8$^+$ T cell-dependent immunity against the liver stages of rodent malaria parasites has been shown by a few studies as described in the introduction section (White et al., 1996; Chakravarty et al., 2007). These studies have clearly led to a conclusion that CD8$^+$ T cells are unable to confer protection in the absence of MHC-I molecules (White et al., 1996) and that CD8$^+$ T cells do not require bone marrow-derived APCs for protection (Chakravarty et al., 2007). However, whether CD8$^+$ T cells need to recognize MHC-I expressed on hepatocytes in order to exert anti-plasmodial effector activity is yet unknown. Therefore, for the purpose of addressing this key question, we have generated B6 Tg mice that express K^d molecule only on hepatocyte (Alb-K^d; Huang et al., 2013) in addition to those that express K^d molecules in all nucleated cells (MHC-I-K^d; Huang et al., 2013) and performed a set of adoptive transfer experiments, in which malaria-specific CD8$^+$ T cell-line was transferred to Alb-K^d Tg mice, MHC-I-K^d Tg mice (as a positive control), and B6 mice (as a negative control).

We first sought to establish a PyCS-specific CD8$^+$ T-cell line from MHC-K^d Tg mice, which is a rather straightforward approach. However, such T cell-line bears K^d molecule, which should cause allogeneic reaction to wild-type C57BL/6 mice (K^d negative) upon being adoptively transferred. Therefore, we decided to generate a PyCS-specific CD8$^+$ T-cell line from CD11c-Kd Tg mice, in which DCs express K^d molecules, and can present SYVPSAEQI peptide to CD8$^+$ T cells that lack K^d expression.

In the present study, we found that a PyCS-specific CD8$^+$ T-cell line adoptively transferred to Alb-K^d Tg mice inhibited more than 50% of the liver stage development, which is equal to the degree of inhibition by the CD8$^+$ T cell-line found in MHC-I-K^d Tg mice. These results indicate that K^d molecules expressed on hepatocytes play a major role in mediating the effector phase of anti-malaria CD8$^+$ T-cell response *in vivo*.

As we previously shown, we have also generated C57BL/6 Tg mice that express K^d only on DCs (CD11c-K^d Tg) and macrophages (hCD68-K^d Tg; Huang et al., 2013) for the purpose of determining the role of cells other than hepatocytes, like Kupffer cells and DCs, in triggering PyCS-specific CD8$^+$ T cells. However, the difficulty of expanding and generating a large number of PyCS-specific CD8$^+$ T cells from CD11c-K^d Tg or hCD68-K^d Tg mice, thus far, has hampered us from determining the role of DCs and Kupffer cells in mediating anti-plasmodial activity of the CD8$^+$ T cells. In order to generate a PyCS-specific CD8$^+$ T-cell line from these K^d Tg mice, we had tried different prime–boost immunization regimens, i.e., a combination of immunizations with a recombinant adenovirus expressing PyCS, a whole irradiated PySpz in addition to SYVPSAEQI peptide, unsuccessfully. It was also documented in our previous research that CD11c-K^d Tg or CD68-K^d Tg mice elicited much fewer number of PyCS-specific CD8 T cells *in vivo* than that of MHC-I-K^d mice after immunization with either SYVPSAEQI peptide or recombinant adenovirus expressing PyCS (Huang et al., 2013). The maximum number of PyCS-specific CD8$^+$ T cells we could obtain from a group of three immunized K^d Tg mice was 1×10^7 total cells after stimulating them 3–4 times *in vitro* with radiation-attenuated, APCs derived from MHC-I K^d Tg mice. The difficulty of expanding a PyCS-specific CD8$^+$ T-cell line *in vitro* may be due to the lack of K^d expression by CD8$^+$ T cells themselves for presenting the peptide to each other. In any case, we are currently attempting to breed and increase to a large number of CD11c-K^d Tg or hCD68-K^d Tg mice, so that we will be able to expand PyCS-specific CD8$^+$ T cells *in vivo* before direct cell sorting in the future.

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No more monkeying around: primate malaria model systems are key to understanding *Plasmodium vivax* liver-stage biology, hypnozoites, and relapses

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Plasmodium vivax is a human malaria parasite responsible for significant morbidity worldwide and potentially death. This parasite possesses formidable liver-stage biology that involves the formation of dormant parasites known as hypnozoites. Hypnozoites are capable of activating weeks, months, or years after a primary blood-stage infection causing relapsing bouts of illness. Elimination of this dormant parasitic reservoir will be critical for global malaria eradication. Although hypnozoites were first discovered in 1982, few advancements have been made to understand their composition and biology. Until recently, *in vitro* models did not exist to study these forms and studying them from human *ex vivo* samples was virtually impossible. Today, non-human primate (NHP) models and modern systems biology approaches are poised as tools to enable the in-depth study of *P. vivax* liver-stage biology, including hypnozoites and relapses. NHP liver-stage model systems for *P. vivax* and the related simian malaria species *P. cynomolgi* are discussed along with perspectives regarding metabolite biomarker discovery, putative roles of extracellular vesicles, and relapse immunobiology.

Keywords: malaria, *Plasmodium*, *P. vivax*, *P. cynomolgi*, liver, hypnozoite, dormancy, non-human primate animal models

Malaria is responsible for significant morbidity, mortality, and socioeconomic hardships in about 100 countries (World Health Organization, 2014). The causative agents of the disease are parasitic protists of the genus *Plasmodium*, which have a complex life-cycle involving a vertebrate and invertebrate host. After infecting susceptible mammals, the parasite undergoes obligate, clinically silent development in the liver prior to entering the blood and causing the clinical symptoms and pathology associated with malaria. Neutralizing the parasites in the liver has been a goal to prevent blood-stage infection and, therefore, clinical disease and transmission. Indeed, targeting liver stage forms (LSFs) has been a strong theme in current anti-malarial drug and vaccine efforts (Abdulla et al., 2011; Duffy et al., 2012). Moreover, preventing relapse infections is especially important in light of research demonstrating that the majority of *Plasmodium vivax* malaria episodes are due to relapses, which result from the activation of dormant forms in the liver, and not from new, mosquito-borne infections (Betuela et al., 2012; White et al., 2014).

Naturally, human clinical studies relating to the biology of LSFs and host–pathogen interactions in the liver are prohibitive, and regardless, influenced by uncontrollable variables; e.g., diet and medications. Unlike human studies, experimental NHP model systems are well suited for studying LSFs and relapse biology. Future malaria research with non-human primates (NHPs) on these topics will undoubtedly include large-scale 'omics,' advanced immune profiling, mathematical modeling, computational biology, and the integration of clinical and 'omics' datasets (Galinski et al., 2013, 2014; Voit, 2013).

While research using NHPs will inevitably remain limited worldwide within a few capable biomedical research centers, such investigations with the public release of datasets will enable many more investigators to participate in associated areas of research and development. Moreover, collaborations with investigators at these centers can lead to new research directions including much needed translational studies to improve diagnostics and clinical care and to develop and test new anti-malarial interventions and vaccine candidates. From our perspective, these factors make NHP model systems critical to advancing the world toward malaria eradication.

NHP-MALARIA MODELS OVERVIEW

Non-human primate model systems have been instrumental in malaria research for decades whether for furthering basic understanding of *Plasmodium* biology, malaria pathogenesis, or preclinical investigations pertinent to developing new interventions (Coatney et al., 1971; Collins, 1974; Galinski and Barnwell, 2012; Beignon et al., 2014). Notably, they were critical for the discovery of dormant forms of the parasite in the liver, known as hypnozoites. These forms were first discovered in rhesus macaques (*Macaca mulatta*) experimentally infected with *P. cynomolgi* (Krotoski et al., 1982b), and then in chimpanzees infected with *P. vivax* (Krotoski et al., 1982a). Research from the field has confirmed that hypnozoites can stay dormant for weeks, months, or years after a primary infection and then activate and result in relapses, with new cycles of blood-stage parasitemias and illness (White and Imwong, 2012). Recently, NHPs were critical for the development of an *in vitro*, primary hepatocyte culture system that

supported the cultivation of *P. cynomolgi* LSFs for approximately 40 days and provided the first tangible evidence that hypnozoites existed and were capable of activating and multiplying to generate merozoites (Barnwell and Galinski, 2014; Dembele et al., 2014).

Various NHP-simian and human malaria parasite combinations can be used to study *Plasmodium* biology. Many strains of the parasites that infect NHPs, including four validated relapsing species, are available and can be used to address scientific questions relating to LSF biology (**Table 1**). Different strains of *P. cynomolgi* and *P. vivax* that possess distinctive relapse patterns can be utilized to study the consequences of frequent versus infrequent relapses on the host immune system. A suitable mouse model is not currently available to study such phenomena. Indeed, humanized mice containing human hepatocytes have been demonstrated to support *P. falciparum* liver-stage growth (Vaughan et al., 2012; Kaushansky et al., 2014). These models also appear to have some utility for *P. vivax* because they appear to support the development of hypnozoites (Mikolajczak et al., 2013). However, these mice lack intact immune systems and, thus, are deficient when addressing immunobiological questions, whether for *P. falciparum* or other primate malaria species (Kaushansky et al., 2014).

VIVAX MALARIA – NHP MODELS

While NHP models have been used occasionally to supplement fundamental *P. falciparum* research findings from culture systems and for pre-clinical studies, *P. vivax* research over the last few decades would have been virtually impossible without NHP models; i.e., *Aotus* and *Saimiri* monkeys (Galinski and Barnwell, 2012). Unlike *P. falciparum*, a long-term *in vitro* culture system for *P. vivax* does not currently exist due to the need for a regular supply of reticulocytes (Noulin et al., 2013), and thus, we believe yet to be defined culture media components that better mimic the host environment are also needed (unpublished data). In the meantime, NHP models have been critical for generating *P. vivax* material for in-depth analyses (Anderson et al., 2015) and NHP experimental studies continue to complement and expand upon blood-stage analyses that are now possible with small clinical samples attained from human infections (Russell et al., 2012; Galinski et al., 2014).

To investigate hypnozoites and relapses, *Aotus* or *Saimiri* species can be infected with NHP-adapted *P. vivax* strains via mosquito inoculation or syringe injection of sporozoites into a blood vessel (**Table 1**; Galinski and Barnwell, 2012; Galinski et al., 2013). Similar to human infections, relapsing, recrudescing, or chronic infection profiles can be observed in these models provided the animals are splenectomized to interfere with an overly robust removal of infected erythrocytes (**Figure 1**). In contrast to relapses, recrudescences are the result of untreated or persistent blood-stage infections that become sub-patent, below the level of detection by microscopy, followed by an eventual return to patency; such recurring parasitemias are distinct from relapse parasitemias that are due to the activation of hypnozoites and release of a new brood of merozoites from the liver.

Blood-stage parasitemias, which begin to develop within 8–10 days, can be curatively treated without destroying the hypnozoites. PCR testing can confirm the absence of blood-stage parasites, and thus, any subsequent blood-stage infections can be confirmed as relapses and not recrudescences. This experimental strategy is currently the only reliable means to study vivax relapses, with the caveats that these animals are small (typically about 1 kg), parasitemia is typically low or moderate (1–2%), and only small blood volumes can be taken (6 ml/kg/month) based on Institutional Animal Care and Use Committee (IACUC) guidelines. One strain in particular (named the Brazil VII strain) is being developed at the Centers for Disease Control and Prevention (CDC) for studying relapses as it shows multiple relapse patterns over a period of several months similar to that observed previously in humans with "tropical strains" (**Table 1**).

SIMIAN PARASITE – NHP MODELS FOR VIVAX MALARIA

Simian malaria parasite-NHP models are powerful systems to investigate LSF biology, hypnozoites, and relapses compared to the small New World NHPs. Simian malaria parasites productively infect Old World monkeys, including rhesus macaques (*M. mulatta*) and long-tailed macaques (*M. fascicularis*), which possess similar genetic composition and physiology to humans (Gardner and Luciw, 2008; Messaoudi et al., 2011; Zimin et al., 2014). The macaques are much larger than New World monkeys, which allows for greater blood or bone marrow draws (up to a maximum of 10 ml/kg/month) for isolation of parasite material and host cells for immunobiological studies. Additionally, more reagents exist for experimentation with these NHP species. Furthermore, large amounts of liver-material can be collected via biopsies or whole livers to isolate LSFs for downstream experiments.

Plasmodium cynomolgi is a "sister species" of *P. vivax*. These closely related parasites share similar biology such as the formation of hypnozoites and caveolae vesicle complexes in infected erythrocytes (Aikawa et al., 1975; Akinyi et al., 2012; Tachibana et al., 2012). Tens of millions of *P. cynomolgi* sporozoites can be generated in a specified, experimental time-frame compared to *P. vivax* sporozoites which are more difficult to generate (Rosenberg and Rungsiwongse, 1991; unpublished data). They can then be used to experimentally infect macaques and lead to productive blood-stage parasitemias (Collins et al., 1999). Furthermore, the same experimental approach used in Small New World Monkey infections with *P. vivax* can be employed to study relapses (see Vivax Malaria – NHP Models). Two other simian malaria species that could be useful for studying hypnozoites or relapse mechanisms are *P. fieldi* and, especially, *P. simiovale* (**Table 1**).

As with *P. vivax*, many *P. cynomolgi* isolates exist that have their own infection and relapse characteristics (**Table 1**). The number and frequency of relapses in rhesus infected with different strains of *P. cynomolgi* can be predicted with greater accuracy than typically possible with New World monkey-adapted *P. vivax* isolates although *P. vivax* Brazil VII has so far been uniquely dependable in this regard (unpublished data). In rhesus, variables such as the sporozoite inoculum can be altered to produce consistent infection patterns even though inter-individual variability between animals as well as inherent properties of the strain being used in the study can influence infection kinetics (Schmidt, 1986).

Plasmodium cynomolgi can be genetically manipulated more easily than *P. vivax*, though *P. vivax* transfection has been accomplished at the CDC and Emory, where transient transfection

Table 1 | *Plasmodium* species and strains with critical characteristics for studying liver-stages.

Characteristics of each species and strain						
***Plasmodium* species (strains)**	**Isolated from**	**Principal NHP host(s)**	**Primary cycle (days)**	**Relapses (yes/no)**	**Time between relapses**	**Reference**
Plasmodium falciparum (Salvador I or Santa Lucia)	Human	*Aotus griseimembra, A. vociferans, A. nancymaae*	5.5 – 6.5	No	NA	Fairley (1947), Shortt et al. (1949), Collins et al. (1977)
P. malariae (Uganda I)	Human	*Aotus* sp. and *Saimiri* sp.	14 – 15	No	NA	Chin et al. (1965), Lupascu et al. (1967), Collins et al. (1975)
P. vivax (Brazil VII)	Human	*Aotus* sp. and *Saimiri* sp.	7 – 8	Yes	Early and frequent (1 to 2 months)	Fairley (1947), Collins and Barnwell (unpublished)
P. vivax (Chesson)	Human	*Aotus* sp. and *Saimiri* sp.	7 – 8	Yes	Early and frequent (1 month)	Fairley (1947), Ungureanu et al. (1976), Collins et al. (1980)
P. vivax (Salvador I)	Human	*Aotus* and *Saimiri* sp.	7 – 8	Yes	Infrequent (2 to 4 months)	Fairley (1947), Contacos et al. (1972), Collins et al. (1973)
P. vivax (North Korea)	Human	*Aotus* and *Saimiri* sp.	7 – 8	Yes	Late (6 to 12 months)	Fairley (1947), Shute et al. (1976)
P. cynomolgi (B, M, Berok, Ceylon)	*Macaca fascicularis* and *M. sinica*	*M. mulatta*	8 – 10	Yes	Early and frequent (1 to 2 months)	Garnham (1966), Coatney et al. (1971)
P. simiovale	*M. sinica*	*M. mulatta*	>12 (uncertain)	Yes	Varied: frequent (2 to 4 weeks) to infrequent (few to many over 2 years)	Coatney et al. (1971), Collins and Contacos (1974)
P. fieldi	*M. nemestrina* and *M. fascicularis*	*M. mulatta*	>12 (uncertain)	Yes	Varied: frequent (2 to 4 weeks) to infrequent (few to many over 1 year)	Held et al. (1967), Coatney et al. (1971)
P. knowlesi	*M. fascicularis* and *M. nemestrina*	*M. mulatta* and *M. fascicularis*	5	No	NA	Garnham et al. (1957), Coatney et al. (1971)
P. coatneyi	*M. fascicularis*	*M. mulatta*	10	No	NA	Coatney et al. (1971)

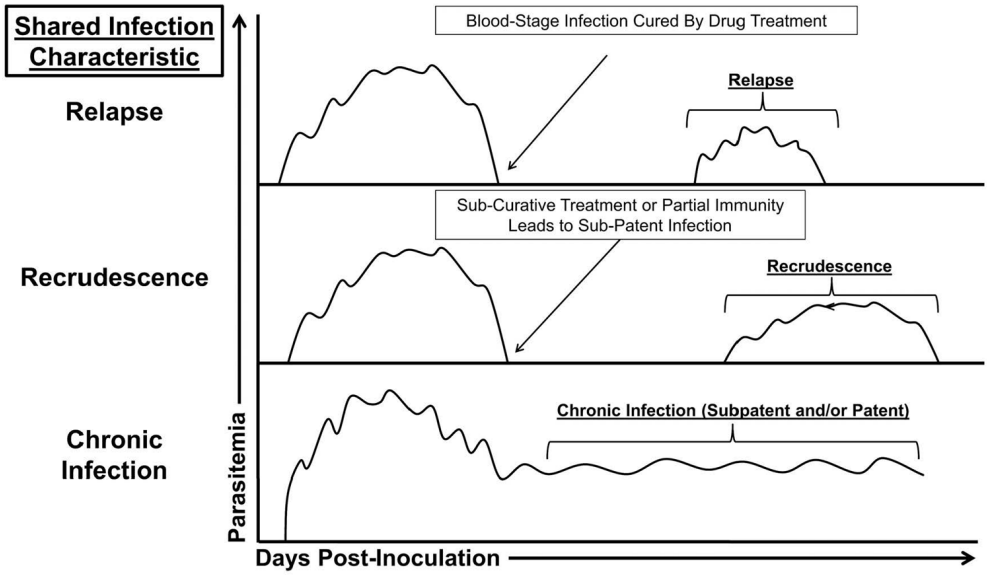

FIGURE 1 | Shared infection characteristics of human and simian malaria parasites.

of trophozoites was demonstrated in *Saimiri boliviensis* (Pfahler et al., 2006). In recent years, we have developed *P. cynomolgi* parasites with integrated transgenes, including a *red fluorescent protein* (*rfp*) gene (Akinyi et al., 2012; unpublished data). Transient RFP- and green fluorescent protein-expressing *P. cynomolgi* parasites have also been reported and used to purify *P. cynomolgi* LSFs from NHP primary hepatocyte cultures using fluorescence-activated cell sorting (Voorberg-van der Wel et al., 2013). Technical hurdles such as achieving better yields of purified parasites for downstream experiments still remain. Nonetheless, these are monumental breakthroughs given the challenges working with these parasites.

PRIMARY NHP HEPATOCYTE CULTURES

NHP primary hepatocyte cell cultures are valuable for exploratory studies and may prove to be crucial for validating *in vivo* experiments as well as mechanistic studies requiring gene knockdowns, drug treatment, etc. Critical advancements have been made toward optimizing *in vitro* studies with *Plasmodium* LSFs, particularly with *P. cynomolgi* and rhesus monkey primary hepatocytes, even though infection rates, and thus, parasite yields still remain low (Voorberg-van der Wel et al., 2013). The most critical advancement has been the establishment of a long-term culture system using primary NHP hepatocytes that can support *P. cynomolgi* hypnozoites and other LSFs for up to 40 days; importantly, this system allows for hypnozoites to activate and develop into schizonts capable of releasing merozoites (Barnwell and Galinski, 2014; Dembele et al., 2014). This breakthrough ramps up research in this area and provides a workable system for validating *in vivo* findings.

SYSTEMS BIOLOGY APPROACHES

Systems biology approaches have been recently pioneered to study infectious diseases and vaccine efficacy (Li et al., 2014; Petrizzo et al., 2014; Zak et al., 2014). Systems biology foregoes traditional reductionist approaches and focuses on a biological system in its entirety. Typically, multiple 'omics technologies are employed to generate large datasets and methods are developed to integrate those datasets. Advanced mathematics and statistics are required to generate computational models of specific biological processes, such as hematopoiesis, immune responses, and infectious disease pathogenesis. Indeed, these strategies are being utilized by the Malaria Host–Pathogen Interaction Center (MaHPIC) using Old World and New World monkey models to study malaria and bring a wealth of data and novel results to the research community via online resources. We believe that systems biology approaches will likewise have utility for studying *Plasmodium* LSFs and the mechanisms behind relapses.

Systems biology methods can be attempted to study LSFs using transgenic, fluorescent LSFs isolated by fluorescence-activated cell sorting from *ex vivo* liver tissue. We and others (Voorberg-van der Wel et al., 2013) hope to demonstrate that adequate, purified *ex vivo* parasite material for downstream experimentation can be attained using such strategies. Performing transcriptomic, proteomic, and metabolomic analyses on the *ex vivo* material holds potential for identifying biochemical and molecular pathways important in hypnozoite biology. A hypnozoite proteome may help elucidate new vaccine candidates whereas transcriptome and metabolome data could give insight into other potential biochemical and molecular pathways that could become drug targets. Indeed, headway is being made in understanding hypnozoite biology with the recent demonstration that epigenetic programming could be responsible for latency (Dembele et al., 2014). Notably, however, modifying epigenetic changes *in vivo* may have detrimental side effects to the host organism, and thus, this may not be a feasible treatment strategy in humans.

We are well aware of the scientific and physiological challenges, but also optimistic that highly sensitive systems biology approaches that include metabolomics can help identify biomarkers that could predict the presence of hypnozoites and serve as diagnostic tools. Metabolomics is a relatively new, yet powerful, scientific discipline that is gaining traction in the fight against malaria (Olszewski et al., 2009; Kafsack and Llinás, 2010; Lakshmanan et al., 2011; Salinas et al., 2014). It is exciting to consider how high-resolution mass spectrometry could potentially identify a metabolic biomarker(s) (predictably of host origin) in the serum, plasma, urine, or saliva that is indicative of the presence of LSFs and the need for treatment. Potential biomarkers are best sought from an *in vivo* infection where host–parasite interactions can be investigated in the context of the normal physiology of the parasite and the host. Experimental NHP models can be informative in this regard. Diets and other variables can be controlled, and infected samples can be collected daily, or even multiple times daily if useful, over the course of a designated infection period through multiple relapse episodes without the immediate, ethical need for treatment.

EXTRACELLULAR VESICLES: POTENTIAL DIAGNOSTIC AND THERAPEUTIC TARGETS

Extracellular vesicles (EVs) are a heterogeneous population of small vesicles found in virtually all bodily fluids including serum, plasma, urine, saliva, etc., and are categorized into subtypes based on their physical properties such as density, size, and shape as well as their biogenesis (Kowal et al., 2014; Robbins and Morelli, 2014). These vesicles are produced by all multi- and unicellular organisms examined to date, and different types of EVs contain specific protein and RNA cargo dependent upon the cell-type the EV originated from (Villarroya-Beltri et al., 2014).

The roles of liver-derived EVs on liver physiology have been reviewed elsewhere (Imani Fooladi and Mahmoodzadeh Hosseini, 2014). Notably, multiple studies have implicated liver-derived EVs in the life-cycle of liver pathogens. For example, exosomes, a specific EV subtype that originates from a multivesicular body within the cell, derived from liver non-parenchymal cells were demonstrated to transfer resistance against hepatitis B virus infection to hepatocytes via an IFN-α mediated mechanism (Li et al., 2013). Despite these implications of EVs in the liver, putative roles of EVs have not been reported with regards to *Plasmodium* liver-stage biology, but warrant attention. Indeed, EVs were recently shown to be released from *Plasmodium* infected erythrocytes and implicated in a "density-dependent sensing mechanism" that influences *P. falciparum* gametocytogenesis (Mantel et al., 2013; Regev-Rudzki et al., 2013). These studies were the first to raise the possibility for a natural role of EVs during the life-cycle and provide a firm rationale that such mechanisms may exist for LSFs.

Extracellular vesicles could predictably serve as a communication mechanism between LSFs and influence hypnozoite activation, and NHP, primary hepatocyte cultures can be used to assess if such mechanisms exist. Cultures can be infected with sporozoites, and EVs derived from uninfected and infected hepatocytes can be isolated from the culture medium (Momen-Heravi et al., 2013). The EVs can then be placed on LSF cultures harboring different forms of the parasites, including hypnozoites, to directly test if EVs isolated at different points of the LSF cycle (e.g., when hypnozoites are activating) result in specific biological outcomes. For example, one may predict that EVs isolated from cultures with activated hypnozoites may signal other hypnozoites to exit dormancy and multiply. These experiments are not perfect, however, and will be unable to delineate if the effect is caused by host and/or parasite-derived EVs, but it is a starting point to explore mechanisms and specific EV components that could potentially be used to force hypnozoite activation.

If such a strategy of 'waking up' hypnozoites is feasible, akin to the novel ideas suggested by others based on drug interventions to target putative epigenetic control mechanisms of dormancy (Dembele et al., 2014), only the blood-stages would have to be treated, making treatment more straightforward with multiple safe options compared to primaquine with its contraindications (White et al., 2014). Indeed, as members of the MaHPIC, we have been developing EV research strategies utilizing NHPs infected with simian malaria parasites. EVs are challenging to purify, but we have managed to purify them from small volumes of plasma, observe them by electron microscopy, and confirm their identity through other biochemical and physical means (unpublished data).

Hepatocyte-derived EVs in the circulation could also comprise possible biomarkers of hypnozoites similar to biochemical biomarkers identified by metabolomics (Deng et al., 2009; Vlassov et al., 2012). Hepatocytes and other tissue-resident cells in the liver release EVs that contain specific protein and RNA cargo that become altered during liver injury (Farid et al., 2012). Similarly, we hypothesize that the microRNA and protein content of liver-derived EVs will be altered when hepatocytes are infected with *Plasmodium*. If this proves to be the case, EVs could be potentially isolated from serum, plasma, saliva, and/or urine and specific diagnostic tests developed to use EVs as diagnostic markers of hypnozoites. Although it may seem unlikely that EVs from *Plasmodium*-infected hepatocytes could serve as biomarkers because of the scarcity of infected hepatocytes (a relative few in an entire liver), EVs are currently being explored to develop sensitive methods to detect cancer in bodily fluids (Rak, 2013). If EV-based diagnostic approaches are feasible and promising for cancers where few malignant cells exist, such approaches are worth exploring for developing technologies to detect hypnozoites.

RELAPSE IMMUNOBIOLOGY

The immunobiology behind relapses, recrudescences, and chronic infections (**Figure 1**) during malaria is poorly understood although "immune exhaustion" during chronic infections has been investigated recently (Wykes et al., 2014). This neglected area of research needs attention because each of these distinctive infection profiles could have unique effects on the host immune response; e.g., to alter the host's memory pool.

The team of immunologists at the MaHPIC has been using NHP models to understand the effects of relapses on the host immune system. Indeed, the consortium has determined that relapses cause continuous expansion of the circulating memory B-cell pool using the *P. cynomolgi*-rhesus model system (unpublished data). Currently, the team is performing follow-up experiments to

better understand this phenomenon and its immunological relevance using sampling strategies that are not possible in humans. Blood is being collected before, during, and after relapses to monitor the alterations in the B-cell compartment by flow cytometry, with a special interest in memory B cells. The identities of predominant B-cell clones based on immunoglobulin sequences are being examined using Ig-Seq technologies (Georgiou et al., 2014). The first goal is to determine the clonal diversity of the B-cell recall response against relapsing or challenge parasites. The second goal is to assess which B-cell clones respond during consecutive blood-stage infections. If consecutive blood-stage infections are selecting for a particular subset of B-cells or, contrastingly, inducing proliferation of different B-cells, the host's memory B-cell pool could be significantly altered. We predict that alterations of the B-cell compartment could translate into poor recall responses because memory B-cells, critical for detecting, expanding, and producing antibodies to eliminate the parasites, might be eliminated from the memory B-cell niche by other B-cells that predominantly proliferate. If this proves to be the case, the impact must be considered in light of developing a vaccine that relies on neutralizing antibodies mediated by memory B-cells.

CONCLUSION

Non-human primate models of malaria have enabled major contributions toward understanding liver-stage biology, hypnozoites, and relapses, and will continue to provide the means to investigate this enigmatic part of the *Plasmodium* life-cycle. Primaquine is the only FDA-approved drug against hypnozoites despite its contraindications. Additionally, excessive use of this drug can support the rise of primaquine resistant parasites (John et al., 2012; Price, 2014). A biomarker test would help restrict treatment to only those individuals in need, and be useful in malaria elimination campaigns where it is preferable to only treat infected individuals instead of everyone to ensure elimination of the parasite reservoir. New knowledge, techniques, and possible diagnostics, vaccines, and medications that may result from studying LSFs using NHP models will inevitably be key to malaria eradication efforts.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing, figure and table, and reviewed and approved the finalized article.

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Liver-stage specific response among endemic populations: diet and immunity

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Developing effective anti-malarial vaccine has been a challenge for long. Various factors including complex life cycle of parasite and lack of knowledge of stage specific critical antigens are some of the reasons. Moreover, inadequate understanding of the immune responses vis-à-vis sterile protection induced naturally by *Plasmodia* infection has further compounded the problem. It has been shown that people living in endemic areas take years to develop protective immunity to blood stage infection. But hardly anyone believes that immunity to liver-stage infection could be developed. Various experimental model studies using attenuated parasite suggest that liver-stage immunity might exist among endemic populations. This could be induced because of the attenuation of parasite in liver by various compounds present in the diet of endemic populations.

Keywords: *Plasmodia*, liver-stage immunity, natural habit, sterile protection, chloroquine and chemoprophylaxis

INTRODUCTION

Malaria along with HIV and TB poses great challenge to human health. More than 200 million people are at high risk and millions are dying (particularly, children) every year across the globe (1). Although anti-malarial drugs have helped bring down the severity and mortality of malaria in endemic regions, emergence of drug resistant parasite poses a great challenge to the human health prompting the urgent need for vaccine(s). Developing effective vaccines, however, has been challenging because of the complex life cycle of *Plasmodia*, which starts with the asymptomatic liver stage followed by the symptomatic blood stage infection (**Figure 1**). Various studies have shown the generation of protective immunity against the blood stage infection after repeated exposure to the parasite (2), but it is questionable against the liver stage (3, 4). However, experiments, using radiation or genetically attenuated sporozoite (RAS or GAS) that fails to complete their developmental cycle in liver, demonstrate the induction of sterile immunity in rodents and humans (2, 5–7). Even chemoprophylaxis and sporozoite (CPS) immunization that kills the parasite at early stage in RBCs (restricting its development to the liver) has shown similar results (2, 5, 7, 8). Although humoral and cell mediated responses are required to develop protection, $CD8^+$ T cell response, generated by attenuated parasite, seems to play a critical role in providing protracted protection at liver stage (2, 3, 5–8). These findings prompted us to think that there is possibility of inducing liver-stage specific immune responses in humans by the parasite that might be attenuated or restricted to liver during natural infection.

People living in different subcontinents with high endemicity for malaria have been shown to have varying degree of susceptibility to infection (**Table 1**) (1). It becomes difficult to protect the host if the parasite enters the blood stage without being interrupted at liver stage as parasite load in blood could be uncontrollably high. Therefore, restricting the parasite to the liver could generate the protective immunity against malaria indicating the differential susceptibility of endemic people to challenge. Food habits have been shown to have a major impact on the health and modulation of immune response. For example, in India, people consume many herbs/spices as a part of their daily diet, which has been shown to have an anti-malarial activity, as explained later. This article has made an attempt to explain how parasites could be attenuated or restricted "naturally" to either liver stage or blood stage by the diet of people living in malaria endemic areas, potentially helping generate liver-stage specific immune responses.

CHALLENGES IN MALARIA VACCINE DEVELOPMENT

Sterile protection to malaria even in endemic populations is not yet fully understood. Even vaccination has failed to induce the desired protection because of involvement of many complex factors (9–16). However, researchers have designed strategies that could provide sterile protection. Both blood and liver vaccination strategies are under different phases of clinical trials (17). The majority of the efforts to understand the immune response against *Plasmodia* infection in humans are directed against blood stage infection. But none of the blood stage vaccine candidates that have been tried so far demonstrated appreciable efficacy (18, 19). The same is also true for the liver-stage subunit vaccine candidates (20). Moreover, unlike the rationale of choosing blood stage vaccine candidates, the antigens for liver-stage vaccines have not been selected based on the understanding of protective immune response in humans against liver-stage infection, a probable reason for not having the right liver stage antigenic target. RAS, GAS, and CPS have shown appreciable efficacy (2, 5–8). Despite promising results, these approaches might not be feasible to adopt for mass vaccination. Considering the world population at risk of malaria, the feasibility of making billions of doses and maintaining the quality are very challenging. Second, GAS has been shown to revert to infectious parasite (21, 22) posing a threat to people expected to take vaccine for prophylaxis. Although CPS immunization is an

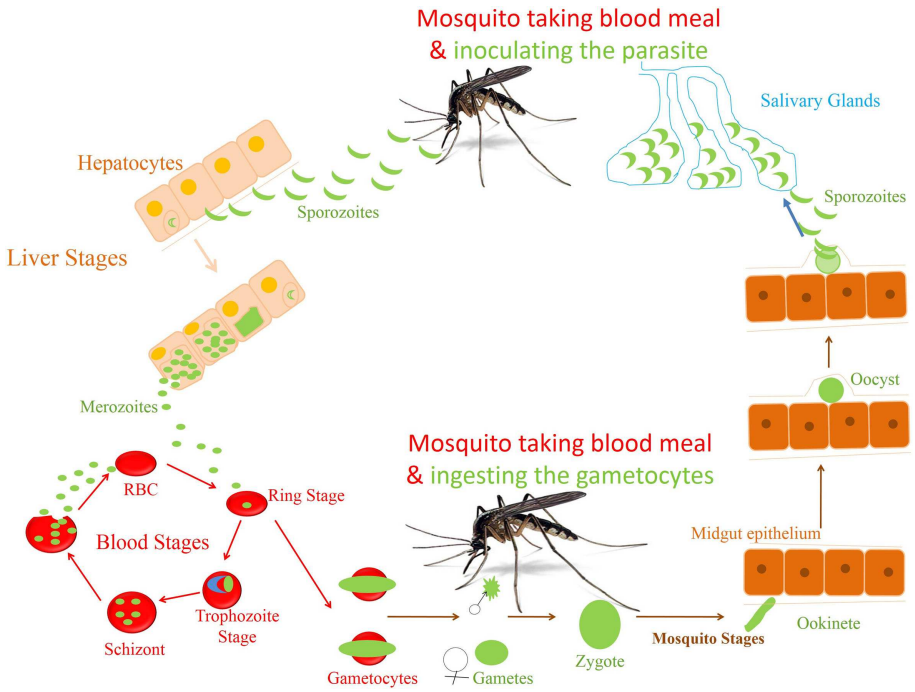


FIGURE 1 | Life cycle of *Plasmodium falciparum*. The life cycle of parasite *P. falciparum* starts in human with inoculation of parasite through mosquito. From the site of injection, sporozoite (SPZ) reaches to the liver and infect hepatocytes. The SPZ multiplies and produces thousands of blood stage infective merozoites. These merozoites enter the blood stream and infect the RBCs to start the erythrocytic cycle of parasite. In RBC, they go through different stages of development before they release the merozoites to infect new RBCs. A small percentage of asexual parasite transforms into sexual form, i.e., gametocytes, which finally develop into sporozoites in the mosquitoes.

attractive vaccination approach, drug resistant parasites and side effects of drug possess challenges. Therefore, approaches like a subunit vaccine will be good alternative. Inducing sterile protection by subunit vaccines requires identification of the right antigenic targets. From the experience of LSA-1 as vaccine candidate, it is critical to have new targets that would induce humoral and T cell responses. Unless we understand the nature of immune response vis-à-vis protection that exists in endemic population, it will be difficult to identify the targets. Because of the lack of knowledge of liver-stage specific immune responses among endemic populations, it is strongly believed that protective immunity to liver stage does not exist; hence efforts to make a liver-stage vaccine have not been prioritized, a reason in our opinion, for not having an effective vaccine against malaria even decades after the trial of RAS vaccination.

EXISTENCE OF NATURAL IMMUNITY TO LIVER STAGE

Many factors including genetic diversity, environmental conditions, and mosquito species do contribute to the differential susceptibility to infection (23–25). While this is true for most of the people living in different parts of the world, we strongly feel that the gain of such differential protection could have a direct correlation with the ability of endemic populations to generate liver-stage specific immune responses. Even though there is blood stage specific protective immunity in people in malaria endemic areas (2), often they come down with the infection (3). It indicates that protective immunity against the blood stage might not be sufficient, and therefore, liver-stage immunity is required to eliminate the parasite. Experiments suggest that achieving protective immunity requires both $CD8^+$ T cells and antibody response (26) because $CD8^+$ T cells are essential for liver-stage parasite while humoral response is key to control blood stage (27). Although fewer studies have been done in endemic populations, there is clear indication that immune responses to liver stage leading to protection could be achievable. In support of this, the studies conducted by the research groups of Marc Connelly and Adrian Hill in Africa have shown that protection, although among a minute endemic population, correlates with immune response to liver-stage antigen (LSA) (23, 28, 29). As discussed before, immunization with attenuated or drug restricted *Plasmodium falciparum* sporozoite is very effective in both animal models and in humans. Results indicate that protection is achievable either by vaccinating the host or exposing the host to the parasite attenuated or restricted naturally.

ENDEMICITY VS. INFECTIVITY AMONG ENDEMIC POPULATIONS

Our analysis of WHO data shows that people of certain subcontinents are less susceptible to malaria than others having similar risk of high endemicity (**Table 1**). People living in Indian subcontinents have been showing lower incidence of malaria compared to other countries having endemicity either lower or higher than India. India is having 0.48% malaria incidence, which is seven times lower than that of Madagascar, i.e., 3.50%. Malarial endemicity of India is 22% while that of Madagascar is 30% (**Table 1**). Various states

Table 1 | List of countries with their Union Territory population, malaria endemic population, malaria confirmed cases, percent population at high risk, and percent incidence of malaria in 2011.

Country	(A) UN population	(B) Population at high risk	(C) Population at high risk (%) (B × 100)/A	(D) Malaria confirmed cases	(E) Incidence of malaria (%) (D × 100)/B
China	**1,347,565,324**	**191,908**	**0.014**	**4498**	**2.34**
Brazil	**196,655,014**	**4,523,065**	**2.3**	**267,045**	**5.9**
Nepal	30, 485, 798	1, 127, 975	3.7	71, 752	6.36
South Africa	50, 459, 978	2, 018, 399	4	9866	0.49
Thailand	69, 518, 555	5, 561, 484	8	24, 897	0.45
India	**1,241,491,960**	**273,128,231**	**22**	**1,310,367**	**0.48**
Madagascar	**21,315,135**	**6,394,541**	**30**	**224,498**	**3.51**
Myanmar	48, 336, 763	17, 884, 602	37	567, 452	3.17
Timor-Leste	1, 153, 834	888, 452	76.99	36, 064	4.06
Mali	15, 839, 538	14, 255, 584	89.99	1, 293, 547	9.07
Papua New Guinea	7, 013, 829	6, 592, 999	94	1, 025, 082	15.55
Vanuatu	245, 619	243, 163	99	5764	2.37
Solomon Islands	552, 267	546, 744	99	80, 859	14.79
Nigeria	162, 470, 737	162, 470, 737	100	3, 392, 234	2.09
Angola	19, 618, 432	19, 618, 432	100	2, 534, 549	12.92
Ghana	24, 965, 816	24, 965, 816	100	3, 240, 791	12.98
Zambia	13, 474, 959	13, 474, 959	100	4, 607, 908	34.19

of India, e.g., Arunachal Pradesh, Meghalaya, Mizoram, Tripura, Jharkhand, and Odisha are having high risk (72–100%) of infection (**Table 2**) (30), but the rate of malaria infection is about 0.72%. In contrast, China and Brazil where only 0.014–2.3% population at high risk but incidence of malaria is 2.3–5.9%, which is threefold to eightfold higher than that of India (**Table 1**), based on the data of 2011. Further analysis of data from 2008 to 2012 suggests that the incidence of malaria remain consistently low even if the high risk population has not decreased significantly as compared to countries like China and Brazil (**Figure 2**). It is possible that people living in specific regions of high endemic zones of African countries or Brazil might have the similar trends of malaria incidence. It is known that immune responses against blood stage infection are the major factor in providing protective response. However, it is possible that the low incidence of Malaria among endemic populations like in India could be due to the contribution of the liver-stage immune responses generated among them. This might be because of their unique diets playing an important role in attenuating the parasite, a critical factor in generating liver-stage specific response.

PROMOTING LIVER-STAGE IMMUNE RESPONSES BY NATURALLY ATTENUATED PARASITE

Based on our understanding from various reports, it is possible that many compounds derived from the herbs/spices could attenuate the parasite provoking immune responses to malaria. People, especially those living in the continents of Asia and Africa consume herbs and spices as part of their daily diet that have many medicinal effects; e.g., turmeric, garlic, and black pepper are consumed daily by Indians in almost all food items (31–34). The compounds from various sources listed in **Table 3** are shown to act on blood or liver stage parasite. It is possible that some of those compounds help induce liver-stage specific immune responses.

Quinine, arabinogalactan, curcumin, piperine, ellagic acid, quercetin, alkaloids, flavonoids, cinnamic acid derivatives, and allicin to name a few compounds are known to have anti-malarial activity, present in the diet. Many anti-malarial drugs target heme polymerization, essential for parasite to complete its life cycle. Quercetine (apples, oranges, lemons, onions, nuts, garlic, and neem leaves) is known to act on the blood stage parasite by preventing heme polymerization through sequestration of free hemin by forming quercetin–hemin complex (e.g., against *P. falciparum* 3D7) (35). It has also shown to inhibit parasite growth in a dose dependent manner by interfering in the permeability pathways. Quinine (grapefruit, lime, pomegranate, and parsley) also prevents heme polymerization as well as binds to the DNA of parasite at schizont stage and blocks its reproduction (36, 37). Ellagic acid (strawberry, pomegranates and the best source, red raspberry seeds/red Raspberries) has been shown to act on mature trophozoite and young schizont of blood stage parasite while inhibiting hemozoin formation (38, 39, 63). It has also been shown that ellagic acid potentiates the activity of anti-malarial drugs like chloroquine, mefloquine, artesunate, and atovaquone. According to a curative test, the ED_{50} of ellagic acid for *Plasmodium vinckeipetteri* was around 1 mg/kg/day by the intraperitoneal route. Under the same conditions, artesunate, the most effective semi-synthetic derivative of artemisinin, shows an inferior ED_{50} of 5 mg/kg/day. Several studies treating mice intra-peritoneally with ellagic acid before parasite inoculation showed high-level reduction (between 79 and 93%) of parasitemia by day 6 post-infection suggesting a prophylactic effect of ellagic acid (38, 39, 63).

Arabinogalactan (tomatoes, carrots, pears, coconut, leek, onion, spinach, broccoli, avocado, eggplant, mango, apples, apricot, banana, radish, turmeric, echinacea tea, and marshmallow root) enhances monocytes production and also activates macrophages that play an important role in phagocytosis of the parasite, thus

Table 2 | List of states of India with their population, malaria endemic population, malaria confirmed cases, percent population at high risk, and percent incidence of malaria.

State	(A) Population	(B) Population at high risk	(C) Population at high risk (%), (B × 100)/A	(D) Malaria confirmed cases	(E) Incidence of malaria (%), (D × 100)/B
Mizoram	1, 091, 014	1, 091, 014	100	8861	0.81
Tripura	3, 671, 032	3, 671, 032	100	14, 417	0.39
Arunachal Pradesh	1, 382, 611	1, 257, 586	91	13, 950	1.11
Jharkhand	32, 966, 238	28, 791, 697	87	160, 653	0.56
Odisha	41, 947, 358	36, 494, 063	87	308, 968	0.85
Meghalaya	2, 964, 007	2, 139, 948	72	25, 143	1.18
India	1, 210, 569, 573	26, 63, 25, 306	22	1, 310, 656	0.49

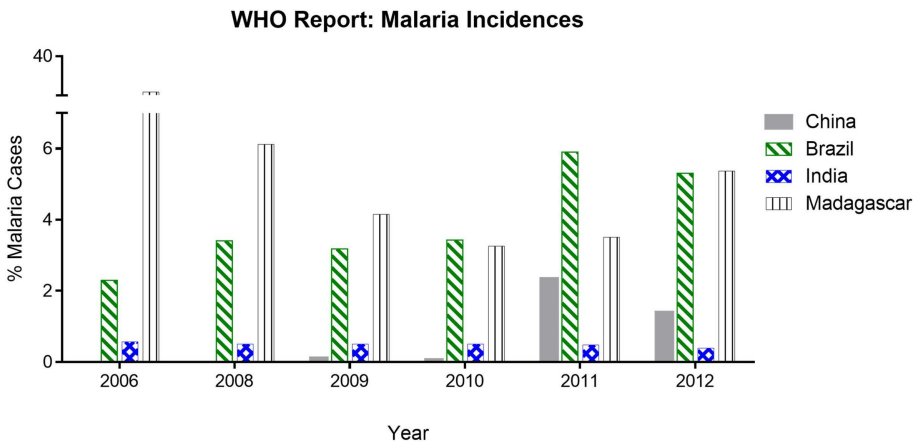


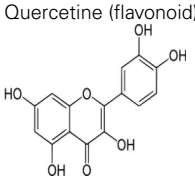
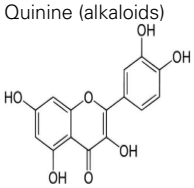
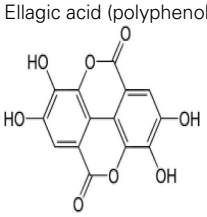
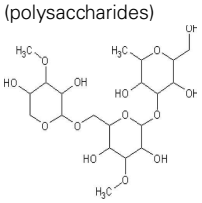
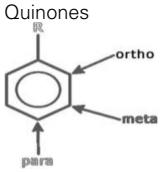
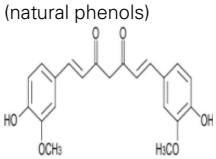
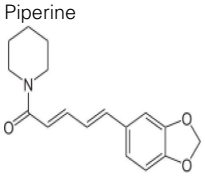
FIGURE 2 | Malaria incidences based on WHO report analysis. The data show the % of malaria cases (in China, Brazil, India, and Madagascar) during 2006–2012 reported in World Malaria Report. India and Madagascar have comparative high endemicity but varying infectivity whereas China and Brazil having low endemicity but proportionally higher infectivity.

lowering the parasite load, and presenting parasite antigens to help potentiate the liver-stage specific T cell responses (40, 41). Quinones (basil oil) inhibit the parasite development at schizont stage by blocking its mitochondrial electron transport and respiratory chain without affecting the host (42, 43).

Curcumin (turmeric) acts by damaging the parasite DNA and altering the histone acetylation that accounts for the parasiticidal activity on blood stage parasite (43–48). It has been shown that curcumin inhibits chloroquine-resistant *P. falciparum* growth in a dose dependent manner with an IC_{50} of 5 μM (46). Furthermore, oral administration of curcumin to mice infected with *Plasmodium berghei* reduces blood parasitemia by 80–90% and enhances the survival. Padmanaban et al. evaluated the *in vivo* efficacy of the curcumin–artemisinin combination (46). The results indicated that a, J3-arteether or curcumin monotherapy at the indicated doses prolongs the survival of *P. berghei*-infected mice but does not confer complete protection. However, combining curcumin with a, J3-arteether treatment reduces the infectivity further resulting 100% protection. Consumption of black pepper, source of piperine, is very common even among non-endemic populations. Piperine has been shown to enhance the bioavailability of curcumin by 2000-fold (33). The amount of curcumin consumed by endemic population (e.g., India) appears to support the fact that it would be enough to attenuate the parasite in the liver (34, 49–51). Cinnamic acid (cinnamon) derivatives have been shown to act at the young (ring) and the mature (trophozoite) stages of parasite development by inhibition of lactate transport or of mitochondrial respiration required for energy generation (52, 53, 64).

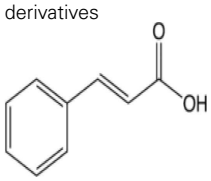
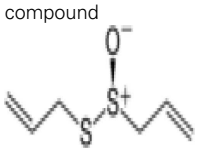
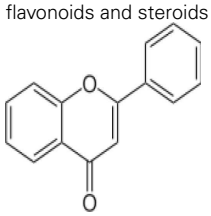
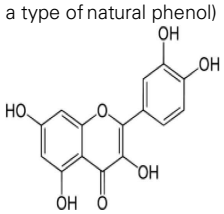
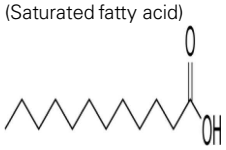
Allicin (garlic cloves) has been shown to prevent the parasite invasion in hepatocytes, reduce parasite load in blood, and enhance survival. It has been shown that the circumsporozoite protein (CSP) of Plasmodium sporozoites is proteolytically processed by a parasite-derived cysteine protease, and this processing event is associated with sporozoite invasion of host cells (54). It is found that 10, 25, or 50 μm allicin inhibit CSP cleavage, which is comparable to that observed with 10 μm E-64, a cysteine protease inhibitor that inhibits CSP processing and prevents invasion of host cells *in vitro* and *in vivo*. Mice injected with allicin showed reduced parasitemia compared to controls. Furthermore, treatment of sporozoite with allicin before injecting into mice completely prevented malaria infection suggesting allicin might directly attenuate the parasite (55). The protective effect of allicin seems to be influenced by improved host immune responses. It has been demonstrated in a rodent malaria model of *Plasmodium yoelli* (17XL) infection that allicin treatment enhances the production of pro-inflammatory mediators like IFN-γ, TNF, IL-12p70, and NO.

Table 3 | The compounds derived from various sources with their anti-malarial activity, mode of action and estimated concentration.

Fruits or herbs	Compound present	Estimated concentration	Mode of action (anti-malarial activity)	Reference
Apples, oranges, lemons, onions, nuts, garlic, neem leaves	Quercetine (flavonoid) 	32 mg/100 g of red onion; Daily intake 12.9 g/day)	Inhibition of heme polymerization by chelating free available hemin for polymerization	(35)
Grapefruit, lime, pomegranate, parsley	Quinine (alkaloids) 	~100 mg total alkaloids, including quinine in a cup of traditional quinine bark tea	Blocks malaria from reproducing by binding to the parasite's DNA Inhibition of hemozoin bio-crystallization, which facilitates the aggregation of cytotoxic heme. Free cytotoxic heme accumulates in the parasites, causing their deaths.	(36, 37)
Strawberry, pomegranates and the best source, red raspberry seeds/red raspberries	Ellagic acid (polyphenol) 	50.06 mg/10 gm of strawberry	Inhibition of β-hematin (hemozoin) formation Act on trophozoite and early schizont forms of the parasites. This erythrocytic stage of the malaria life cycle is the most metabolically active phase, with protein, RNA, and DNA synthesis taking place.	(38, 39)
Tomatoes, carrots, pears, coconut, leek, onion, spinach, broccoli, avocado, eggplant, mango, apples, apricot, banana, radish, turmeric, echinacea tea, marshmallow root	Arabinogalactan (polysaccharides) 	15–25% in larch	Macrophage activator Support the monocyte production	(40, 41)
Basil oil	Quinones 	N/A	Inhibition of parasite mitochondrial electron transport chain and respiratory chain without affecting the host mitochondrial system	(42, 43)
Turmeric	Curcumin (curcuminoid) (natural phenols) 	3.14% by weight in pure turmeric powder; Alleppey turmeric: 4–7% curcumin; Madras type: 2% curcumin	Anti-oxidant activity Curcumin induced generation of ROS may lead to histone hypoacetylation and DNA damage that account for the parasiticidal effect of curcumin	(34, 44–51)
Black pepper	Piperine 	5–10%	Enhances the bioavailability of curcumin by 2000-fold	(33)

(Continued)

Table 3 | Continued

Fruits or herbs	Compound present	Estimated concentration	Mode of action (anti-malarial activity)	Reference
Cinnamon	Cinnamic acid derivatives 	0.96–2.91%; 0.87 mg/g	Inhibit the transport of monocarboxylate across erythrocyte and mitochondrial membranes Inhibit parasite growth and they are equally effective at the young (ring) and the mature (trophozoite) stages of parasite development	(52, 53)
Garlic cloves	Allicin, organosulfur compound 	1–3% (2.8–7.7 mg/g found in Romanian red)	Inhibits circumsporozoite protein processing and prevents sporozoite invasion of host cells *in vitro*. *In vivo* mice injected with allicin had decreased *Plasmodium* infections compared to controls When sporozoites were treated with allicin before injection into mice, malaria infection was completely prevented Immunomodulatory activities (preferentially enhances pro-inflammatory immune responses)	(54–56)
Fenugreek	In leaves: alkaloids, saponin, tannin like phenolic compounds, flavonoids and steroids 	Fenugreek contains 35% alkaloids and 4.8% saponin	Hemozoin inhibitors The alkaloidal, ethanol, and butanol extract of fenugreek has been documented to possess anti-plasmodial activity against *in vitro* culture of chloroquine sensitive and resistant *Plasmodium falciparum* Presence of flavonoids and polyphenols has been found to be responsible for powerful anti-oxidant activity Fenugreek seeds also have capacity to increase the immunity power and to fight against the parasites	(57, 58)
Peanuts, grapes, grape juice, berries, e.g., blueberries and black berries	Resveratrol (stilbenoid, a type of natural phenol) 	0.01–0.26 mg in peanuts	Treatment of parasite-infected red blood cells with resveratrol significantly reduces their ability to adhere to the body's cells lining small blood vessels. That reduction in binding to blood vessels is predicted to greatly lessen the probability of developing severe clinical manifestations of malaria, according to the study.	(59)
Ginger	N/A	N/A	Nausea and vomiting are also common symptoms of malaria, which may explain the widespread use of ginger as one component of traditional remedies for malaria It stimulates production of the main anti-oxidant enzyme glutathione peroxidase, this detoxification-related enzyme improves the liver function and binds toxins. The compounds of ginger inhibit the malaria parasite.	(60)
Cold-pressed coconut oil, fresh and dried coconut, coconut milk, bitter melon	Lauric acid (Saturated fatty acid)  ferulic acid and *p*-coumaric acid	Pure coconut oil contains about 50% lauric acid	When lauric acid is converted into monolaurin, a monoglyceride compound, which exhibits antiviral, antimicrobial, anti-protozoal, and anti-fungal properties. It acts by disrupting the lipid membranes in organisms like fungus, bacteria, and viruses, thus destroying them Coconut has anti-oxidant compounds	(61, 62)

The numbers of CD4+ T cells, DCs, and macrophages were significantly higher in allicin-treated mice. Allicin also promoted the maturation of CD11c+ DCs, while it did not cause major changes in IL-4 and the level of anti-inflammatory cytokine IL-10 (54–56).

Fenugreek is a common household item in special food preparation of Indians. Alkaloids and flavonoids derived from Fenugreek have been shown to inhibit the hemozoin formation and possess anti-plasmodial activity against chloroquine sensitive and resistant *P. falciparum*. It was found that fenugreek extract in a dose of 50, 100, or 250 mg/kg showed immunomodulatory property through various mechanisms including weight of thymus, delayed type of hypersensitivity response, humoral immunity and phagocytosis (57, 58, 65, 66). Peanuts, grapes, grape juice, and berries are common food consumed all over the world. Resveratrol, derived from the same have been shown to significantly reduce the ability of infected RBCs to adhere to the body's cells lining small blood vessels. Such phenomenon is predicted to greatly lessen the probability of developing severe clinical manifestations of malaria, according to the study (59).

Frequent consumption or decoction of medicinal plants by the people in malaria endemic areas has been shown to help fight the infection. Evidence for this came from the longitudinal study undertaken by Foundation for Revitalization of Local Health Traditions (FRLHT) in endemic regions of Odisha, Andhra Pradesh, and Chhattisgarh of India. It showed that traditional plant decoction taken by people (tribal and non-tribal) reduced the incidence of malaria by more than threefold (in communication, 67–69). Based on the actions of said compounds present in diet and/or in decoction, it is possible that parasite development is largely interrupted at the blood stage; in other words the *Plasmodia* infection among endemic populations, as in case of India, would preferentially be restricted to the liver stage helping provoke immune response against the LSAs. Therefore, we hypothesize that natural diet of people of different subcontinent could attenuate *Plasmodia* at different stages of infection and thus help provoke the immune responses against malaria liver-stage infection enhancing protection (**Figure 3**). Recent findings from the studies of vaccination of host using infectious sporozoite under the cover of chloroquine support the hypothesis.

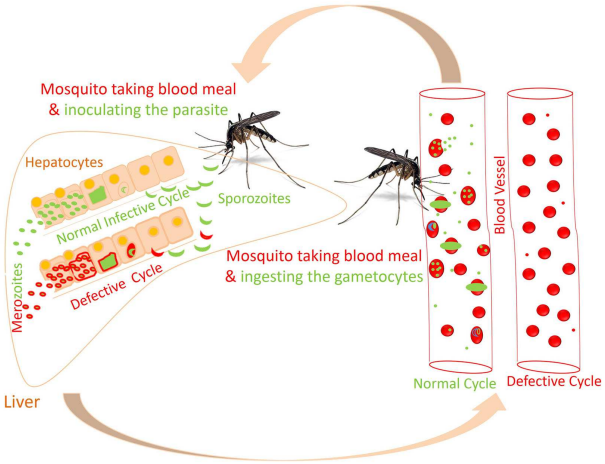


FIGURE 3 | Proposed altered life cycle of *Plasmodium* following consumption of diet containing anti-malarial compounds. Action of various compounds derived from diet is depicted in the figure. Green colored parasite represents normal life cycle while that of red colored parasite represents defective life cycle because of action of compounds present in diet. Possibilities: SPZ not able to infect the hepatocytes; parasite development is interrupted at initial or late stage in hepatocytes; or merozoites released might not able to infect the RBCs. Certain compounds in diet might attenuate the parasite following infection in RBCs.

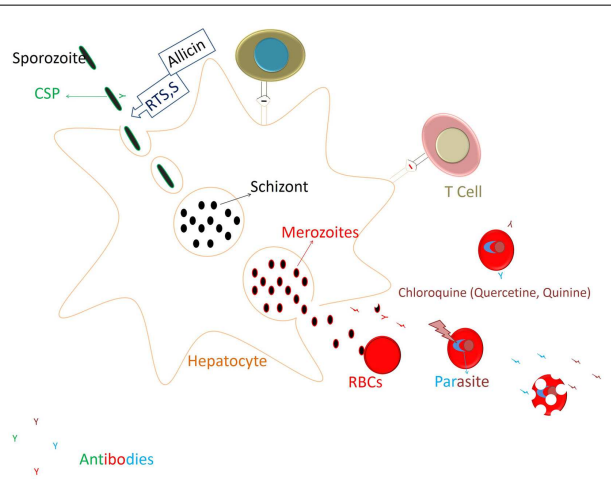


FIGURE 4 | Restricting the parasite development to Liver stage and generation of immune responses. RTS,S vaccines targeting the circumsporozoite protein (CSP) of the parasite generates mainly antibody responses against the CSP that acts on the sporozoite to prevent its invasion into hepatocytes. Allicin from the garlic cloves also would act in similar manner (prevention of hepatocyte invasion by spz) by eliminating CSP processing. After invading the hepatocyte sporozoite will undergo further development, and merozoites released infect the RBCs and start degrading Hb. Heme released is polymerized to curtail its toxicity on the parasite. Chloroquine at this stage blocks the heme polymerization and kills the parasite. Thus, chloroquine (or compounds from food having similar action) would restrict the parasite development. Parasitic antigens released from the infected RBCs might generate the humoral and cellular responses to the blood stage parasite and also against the cross stage antigens from liver stage parasite (69).

LESSONS FROM CHEMOPROPHYLAXIS STUDY WITH CHLOROQUINE AND DETERMINING FACTORS FOR PROTECTIVE IMMUNITY

In endemic areas, people are repeatedly exposed to malaria parasite and control the infection with immune responses directed against the blood stage, as supported by majority of the investigations (2), or with the help of anti-malarial drugs. It is possible that interruption of parasite development at different stages, either in liver or in blood, by natural means or drug usage favors generation of liver-stage specific immune responses (**Figure 4**) (70). This possibility is revealed by the CPS immunization studies in mice and humans. In CPS immunization, three doses of parasite under chloroquine cover induce sterile protection that correlates with $CD8^+$ T cell responses directed against the pre-erythrocytic stage of parasite (7, 8). In these studies, the immunized volunteers have been shown to be protected for up to 2 years. Similarly, it is

possible that compounds from dietary plants, as described above and medicinal decoction (during the diseased condition), would have similar actions like to Chloroquine. In fact, the effective anti-malarial drugs we use today are derived from the plants. In malaria endemic areas, though maternal antibodies help fight the infection during their early life; children are highly prone to infection, which might be due to their developing immune system. Their susceptibility to malaria infection increases further probably for the fact that consumption of herbs and spices as part of food is quite low or rare in children, which increases as they grow. Hence, with the advancement of age, repeated exposure to the parasite, and frequent consumption of the herbs and spices might help to develop the protective immunity against the malaria, which is further supported by the observation that after several years of exposure to parasite children do develop immunity to the severe life threatening malaria (2).

Although blood stage specific immune responses seem to be dominating protection, the presence of liver-stage specific immune responses should not be underestimated. Possible reasons for not having noticeable liver-stage immunity among endemic population could be the low antigen availability and the tolerogenic environment in liver that keeps the inflammation under control. Hence in case of malaria, the parasite load and its frequency of exposure are the key factors to develop protective immunity against liver-stage infection. In natural exposure, the parasite load appears to be lower in endemic population compared to the immunization regimen in experimental studies. However, there is possibility of induction and building up of liver-stage specific immune responses in endemic populations over the years of exposures contributing to the protection (**Figure 5**). Many pre-clinical vaccine studies including ours support the above notion (71–73). In CPS immunization studies, it has been seen that three doses of parasite (sporozoite) immunization under the cover of chloroquine through i.v. route within short time period induce the sterile protection (26). While two doses of 20,000 sporozoite (spz) immunization protect 40% of mice, three doses of the same protect 100% of mice following infectious sporozoite challenge. Similar results were also found in our experiments using attenuated parasite (γ-spz) immunization strategy (manuscript in preparation, 71). The higher level of protection correlates with the presence of higher number of multifunctional $CD8^+$ T cells suggesting that multiple exposure to parasite antigens is required to achieve the protecting numbers of multifunctional $CD8^+$ T cells (74). It is also possible that $CD8^+$ T cell repertoire would be broadened for the subdominant antigens (antigens from various stages of development in liver) with repeated Ag exposure and ensue the protection against liver-stage malaria. Interestingly, it has been demonstrated that mice immunized with three doses of 10,000 spz under the cover of chloroquine were partially protected in contrast to mice that were given three doses of 20,000 spz (26). Further support came from the comparative studies in which the immunizations of attenuated parasites were done through intradermal (ID) vs. intravenous (IV) routes. ID route of immunization was thought to mimic the natural route of delivery of sporozoite through mosquito bite. It was

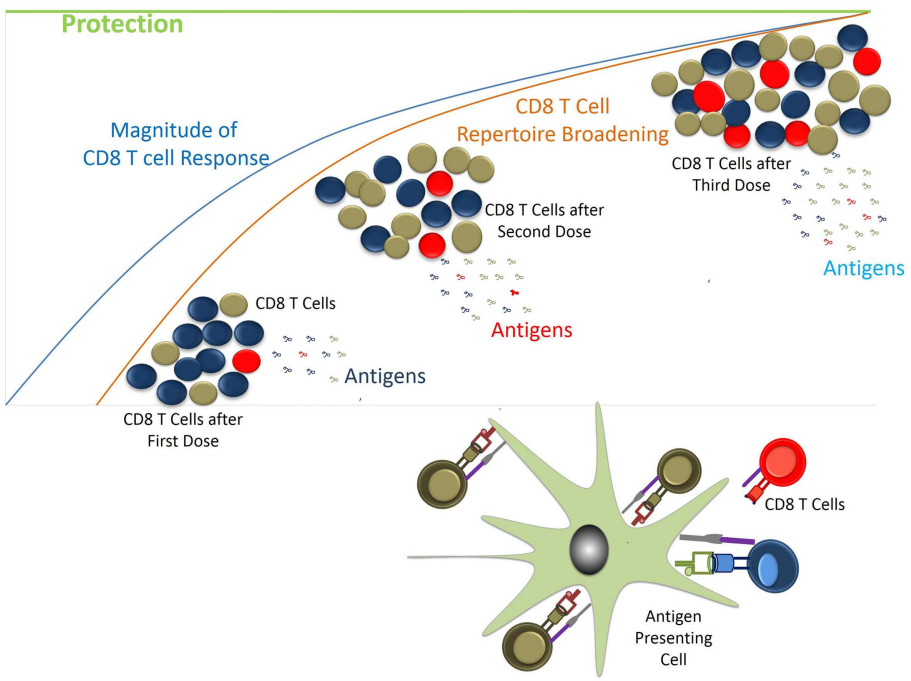


FIGURE 5 | Generation of CD8 T cell response vs. protection. CD8 T cells are generated following exposure to parasite antigens through natural infection. Depending on the availability and affinity of antigens CD8 T cell clonotypes would have access to MHC/p complexes. Initially MHC/p in excess or with high affinity would preferentially induce the specific T cells. During subsequent antigen exposures other antigens would come into picture while response to previously generated CD8 T cells will be boosted. Gradually over time the desired repertoire of T cells with required frequencies would be generated helping protect the host from the incoming infection.

found that immunization of mice through IV route was protective, while the same through ID route failed to protect the mice upon challenge (75). It has been demonstrated that parasite load in liver in case of ID immunization is very low compared to the IV (75) reflecting the low levels of the availability of antigens, which might be not sufficient to induce the desired magnitude of $CD8^+$ T cells and to broaden the repertoire. To better understand the liver-stage specific immune responses, protected individuals in endemic areas should be included in large scale studies while CPS immunizations in humans could be taken as alternative positive control. Screening of protective antigens from those individuals will help the efforts of subunit vaccination.

CONCLUSION

Over a decade ago, the malaria genome was sequenced, which has revealed that the parasite contains over 5000 genes. Researchers around the globe are actively engaged in identifying and characterizing the genes. We have not yet been very successful in making effective vaccines, which could generate the protective immune responses against various developmental stages of *Plasmodia* infection. It is well known that multiple immune mechanisms are required to prevent the infection, e.g., $CD8^+$ T cells are critical for liver-stage infection as parasite remains inside the hepatocytes while humoral response is the key mechanism in blood stage infection where antibodies are required to prevent the free merozoites to infect the RBCs (27). In addition, to prevent the sporozoite from invading the hepatocytes, we require neutralizing antibodies. Hence, a vaccine should be made, which could generate a diverse immune response. RTS,S is the most successful subunit vaccine available that produces the neutralizing antibodies against the CSP, present on the surface of sporozoite. Currently, whole sporozoite vaccines (RAS or GAS) are also becoming popular as they confer the sterile protection at least in experimental models by activating $CD8^+$ T cells. Here, RAS are mainly restricted to initial stage while GAS are designed to be restricted at early, mid, or late in liver-stage development. Interestingly, chemoprophylaxis studies with chloroquine have shown the activation of both $CD8^+$ T cells and antibody response in which parasite is prevented at early blood stage (26).

Although we have understood mechanistically immune responses generated in animals or in experimental trials in humans, we have very little idea about the antigenic targets against which immune responses seems to be protective. And this is particularly true for liver-stage infection. Because there is lack of understanding of induction of protective immune response among endemic population against liver-stage infection, it has been very difficult to identify the targets. Therefore, systemic efforts must be made to understand liver-stage specific protective immune responses, particularly, in those populations living in malaria endemic area who are protected from the malaria by getting repeated exposure to the parasite. A recombinant protein of liver-stage parasite in adjuvant formulation will be an ideal formulation for mass scale immunization. A pre-erythrocytic stage vaccine should induce strong cellular immune response as well as develop long lasting immunological memory. New insight into parasite biology, stage specific expression profile, and characterization of carefully selected new antigens would provide new targets for interventions. Research institutes with focused R&D and skilled manpower must take the lead in the efforts to make vaccine against malaria by targeting antigens from multiple stages of *Plasmodia* infection.

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Corrigendum: Liver-stage specific response among endemic populations: diet and immunity

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A corrigendum on

Liver stage specific response among endemic populations: diet and immunity
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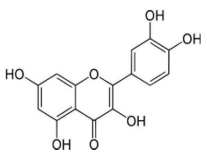
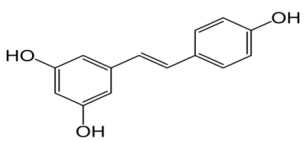
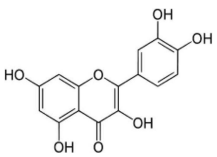
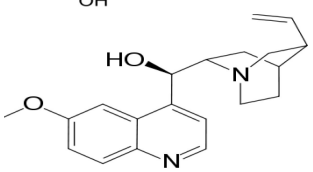
In the original article on page 5 and 6 there are two errors in Table 3. The corrected figures are given in following table.

Compound name	Incorrect figure	Correct figure	Reference
Resveratrol			(1)
Quinine			(2, 3)

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Chloroquine neither eliminates liver stage parasites nor delays their development in a murine Chemoprophylaxis Vaccination model

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Chemoprophylaxis Vaccination (CVac) confers long lasting sterile protection against homologous parasite strains in humans, and involves inoculation of infectious sporozoites (SPZ) under drug cover. CVac using the drug chloroquine (CQ) induces pre-erythrocytic immunity in humans that includes antibody to SPZ and T-cell responses to liver stage (LS) parasites. The mechanism by which CVac with CQ induces strong protective immunity is not understood as untreated infections do not confer protection. CQ kills blood stage parasites, but its effect on LS parasites is poorly studied. Here we hypothesized that CQ may prolong or perturb LS development of *Plasmodium*, as a potential explanation for enhanced pre-erythrocytic immune responses. Balb/c mice with or without CQ prophylaxis were infected with sporozoite forms of a luciferase-expressing rodent parasite, *Plasmodium yoelii*-Luc (*Py*-Luc). Mice that received primaquine, a drug that kills LS parasites, served as a positive control of drug effect. Parasite burden in liver was measured both by bioluminescence and by qRT-PCR quantification of parasite transcript. Time to appearance of parasites in the blood was monitored by microscopic analysis of Giemsa-stained thick and thin blood smears. The parasite load in livers of CQ-treated and untreated mice did not significantly differ at any of the time points studied. Parasites appeared in the blood smears of both CQ-treated and untreated mice 3 days after infection. Taken together, our findings confirm that CQ neither eliminates LS parasites nor delays their development. Further investigations into the mechanism of CQ-induced protection after CVac are required, and may give insights relevant to drug and vaccine development.

Keywords: ***Plasmodium*, chloroquine, liver-stage, CVac, prepatent**

Introduction

The most successful vaccination approach against malaria involves immunization with whole organism, generally by the liver-infective form called sporozoites (SPZ). SPZ attenuated by radiation (RAS; Nussenzweig et al., 1967; Clyde et al., 1973) or by genetic alterations (GAP;

Mikolajczak et al., 2010; Vaughan et al., 2010) infect hepatocytes but arrest during liver stage (LS) development and do not emerge in the blood stream. More recently, experimental infection and drug treatment has achieved impressive protection: in humans, inoculation of SPZ under drug cover, referred to here as Chemoprophylaxis Vaccination (CVac), required much lower SPZ doses than RAS to induce sterile immunity that persisted for >2 years in four of six individuals (Belnoue et al., 2004; Roestenberg et al., 2011). Chloroquine (CQ) prophylaxis has been studied most extensively as drug cover during CVac, and this regimen generates preerythrocytic immunity that includes anti-sporozoite antibody (Behet et al., 2014) and LS-specific T-cells in animals and humans (Belnoue et al., 2004; Renia, 2008; Bijker et al., 2013).

Chloroquine kills blood stage parasites as they reach the trophozoite stage of development (Yayon et al., 1983), but the effect of CQ on LS parasites has received less attention. It is widely believed that CQ does not affect LS development, allowing the immune system to encounter the full repertoire of LS antigens. However, the more durable protection achieved with CVac after ∼16-fold fewer mosquito bites than required for RAS suggests that drug treatment effects might contribute independently to protection.

The effect of CQ on *Plasmodium* LS development is not completely settled. Based on an *in vivo* infection model, Belnoue et al. (2004) reported that CQ does not kill *Plasmodium* LS at 42 h post infection with SPZ. However, the effect of CQ on later time points including blood stage emergence has not been studied. Using an *in vitro* infection model, Fisk et al. (1989) reported a partial schizonticidal effect of CQ on late LS, but the CQ dose was relatively high, and had cytotoxic effects on hepatocytes as well. Here we report a systematic evaluation of the effects of therapeutic concentrations of CQ on late LS development, using a luciferase expressing *P. yoelii* parasite in Balb/c mice.

Materials and Methods

Mice

Female Balb/c mice, 4–6 week old were purchased from Taconic. Mice were housed in the NIH animal facility under pathogen free conditions and fed with autoclaved food *ad libitum*. All experiments were performed once mice reached 16 to 18 weeks of age. All experiments were approved and performed as per the guidelines by the Animal Care and Use Committee (ACUC) of NIAID/NIH.

Parasite and Mosquitoes

Plasmodium yoelii parasites (strain 17XNL), including the parental line and a transgenic line expressing firefly-luciferase and GFP (*Plasmodium yoelii*-Luc, *Py*-Luc), were maintained by cycling between Balb/c mice and *Anopheles stephensi* mosquitoes. Salivary gland SPZs were harvested on days 14–18 as described earlier (Ozaki et al., 1984; Guebre-Xabier et al., 1999).

Infection and Evaluation of LS Development by Bioluminescence Imaging

Female Balb/c mice (16–18 weeks old) were infected with 15,000 freshly dissected SPZ of *Py*-Luc in 100 μl of 1X PBS+2% normal mouse serum by IV injection. Development of LS was monitored by bioluminescence imaging (BLI) at different time points post-infection as described earlier (Mwakingwe et al., 2009; Miller et al., 2013). Briefly, mice were injected intradermally with 150 μl of Rediject D-Luciferin (Caliper Life Sciences, USA), then anesthetized with isoflurane-anesthesia system 8–10 min later to allow measurement of bioluminescence and image acquisition using IVIS-100 animal imager (Caliper Life Sciences, USA). Bioluminescence images were acquired at the following settings: 15 cm Field of View (FOV), medium binning, and exposure time of 30–60 s. Quantification of luminescence was performed using the ROI settings of the Living Image 4.4 software (Perkin Elmer, USA). ROI were drawn around the abdominal area locating the liver. ROI measurements were expressed as total flux photons per second (p/s).

Drug Treatment and LS Burden Estimation by Intravital Imaging

Infected mice comprised three groups that received different treatments in 100 μl of PBS by intraperitoneal (IP) route: one group received 0.8 mg/mouse of CQ diphosphate (SPZ+CQ; Sigma; Belnoue et al., 2004): one group received 1.5 mg/mouse of PQ bisphosphate (SPZ+PQ; Sigma; Putrianti et al., 2009); one group received PBS without drug (SPZ). Two drug control groups were also included, whereby mice received CQ or PQ but no SPZ infection. An additional control group received no drug and no SPZ. Drug was delivered at the time of infection (0 hours post infection, hpi) and at 24 hpi. Bioluminescence images were acquired *in vivo* with IVIS-100 (Perkin Elmer) at different time points after drug injection indicated in the figure legends. BLI was performed on whole livers that had been perfused (10 ml of RNAse free 1x PBS) and isolated at sequential time points from 40 hpi onward. Liver samples were also snap-frozen in liquid nitrogen for qRT-PCR.

Determination of LS Burden by qRT-PCR

Total RNA was extracted from the whole liver as described earlier (Schussek et al., 2013) using RNeasy mini kit (Qiagen Inc). cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA, USA). Gene expression was measured with 1:40 dilutions of cDNA. Standard curve quantitative RT-PCR was performed (Bruna-Romero et al., 2001) in a 20 μl volume, which includes 1X ABI Power SYBR master mix (Applied Biosystems) and 0.25 μM of either *P. yoelii* 18S rRNA primer (forward- GGGGATTGGTTTTGACGTTTT, reverse- AAGCATTAAATAAAGCGAATA) or mouse β-actin primers (Forward- GGCTGTATTCCCCTCCAT; reverse- CCAGTTGGTAACAATGCAAT). PCR reactions were run on ABI 7500 machine (Applied Biosystems), using the following conditions: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s alternating with 60°C for 1 min.

cDNA standards for both 18S rRNA and β-actin were prepared as 10-fold dilutions (10^7–10^3 copies) from purified PCR product. Liver of naïve mouse was used as negative control. Parasite load was normalized to host β-actin as a ratio (absolute copy of *Py* 18S/ absolute copy of mouse β-actin).

Determination of Prepatent Period

Thick and thin blood smears were collected from the infected mice at different time points starting 42 hpi. Blood smears were Giemsa-stained and examined with a bright field microscope with 100× oil-immersion objective and by expert slide readers blinded to the study groups. Blood smears were considered positive if at least two infected RBCs were found in 100 adjacent fields.

Statistical Analysis

Mann–Whitney test was used to compare groups for LS burden measured by either BLI or qPCR. $P \leq 0.05$ was considered statistically significant. GraphPad Prism software (version 6) was used for statistical analysis.

Results

LS Parasites Persist 54 h after SPZ Inoculation into Untreated Mice

Plasmodium undergoes extensive multiplication during LS development, producing tens of thousands of merozoites from an individual sporozoite. Upon completion of LS development, merozoites are released into the blood stream as small merozoite-filled vesicles called merosomes. To quantify the multiplication and subsequent release of parasites in the liver, we infected mice with 1.5×10^4 luciferase expressing *Py*-Luc SPZ and performed BLI at 40, 44, 48, 54, and 62 hpi. Parasite biomass increased with time until 44 h and then gradually declined with the lowest LS parasite burden at 54 hpi (**Figures 1** and **2**; Supplementary Figure S2). We could detect measurable luciferase activity in the liver at 54 hpi with an average total flux of 5.0×10^6 photon/s (**Figure 1**). By 62 hpi, parasites are detected throughout the body, indicating a blood stage infection (Supplementary Figure S1E), and any residual parasites in the liver could not be distinguished from circulating blood stage parasites.

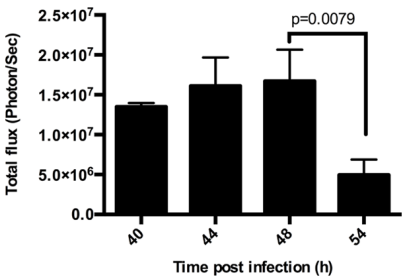

FIGURE 1 | Duration of *Plasmodium yoelii*-Luc (*Py*-Luc) liver stage (LS) growth. Graph representing quantification of total flux from infected and untreated mice imaged at 40, 44, 48, and 54 h after sporozoites (SPZ) injection. $n = 3$ for 40 h, 4 for 44 h, and 5 each for 48, and 54 h. Graph represents Mean ± SD. Mann–Whitney test was performed and $p < 0.05$ considered as significant.

CQ Neither Kills LS Parasites Nor Delays Their Development

To evaluate the effect of CQ on developing LS parasites, we infected mice with 1.5×10^4 *Py*-Luc SPZ, and then treated with either CQ (0.8 mg/mouse), PQ (1.5 mg/mouse), or an equal volume of PBS. Three groups of uninfected mice received same treatments (CQ, PQ, or PBS) but no SPZ. Imaging of whole bodies or of isolated livers was performed at 40, 44, 48, and 54 hpi. CQ-treated (SPZ+CQ) and untreated (SPZ) mice did not differ significantly at any time point (**Figures 2** and **3**; Supplementary Figure S2), whereas the SPZ+PQ group was completely devoid of any bioluminescence, as expected (**Figure 2**; Supplementary Figure S1). Parasite burden estimation with conventional qPCR gave similar results (**Figure 2D**). At 40 hpi, bioluminescence appeared lower in SPZ+CQ group than SPZ group but this trend was not statistically significant ($p = 0.06$; **Figure 2C**), and no such trend was observed in the corresponding qRT-PCR measurements at this time point ($P = 0.86$; **Figure 2D**).

To remove inter-subject variability in a second experiment, we followed individual mice over time, and acquired BLI at sequential time points. We infected each of 20 mice with SPZ of *Py*-Luc; half were treated with CQ while half received an equal volume of PBS. At 42, 44, 46, and 48 hpi, LS parasite burden was measured by whole body BLI. We did not observe significant differences in the parasite burden in the SPZ+CQ *versus* SPZ animals at any time point (Supplementary Figures S3 and S4). In a third experiment, using parental Py-17XNL infection, we confirmed by qPCR that the LS parasite burden at 48 hpi does not significantly differ between SPZ+CQ and SPZ animals ($p = 0.38$, Supplementary Figure S5). Both SPZ+CQ and SPZ mice became blood stage positive at 48 hpi by blood smear microscopy, further indicating that CQ does not delay LS development (**Figure 4**).

Discussion

The effect of CQ on *Plasmodium* blood stages is well characterized, with the inhibition of heme-polymerase enzyme leading to accumulation of toxic heme products, and parasite death (Slater and Cerami, 1992). CQ-CVac generates strong LS-specific immune responses and more durable protection at a log-fold lower SPZ dose as compared to RAS immunization (Clyde et al., 1973; Bijker et al., 2013). However, it is not clear how CQ treatment helps generate this strong and durable immune response. One school of thought holds that CQ-CVac allows the complete development of LS parasites that express a wide array of LS specific antigens, prior to killing the parasites in their first cycle of blood stage development; however, untreated *Plasmodium* infections also display the full antigen repertoire without inducing sterile preerythrocytic immunity. We hypothesized that

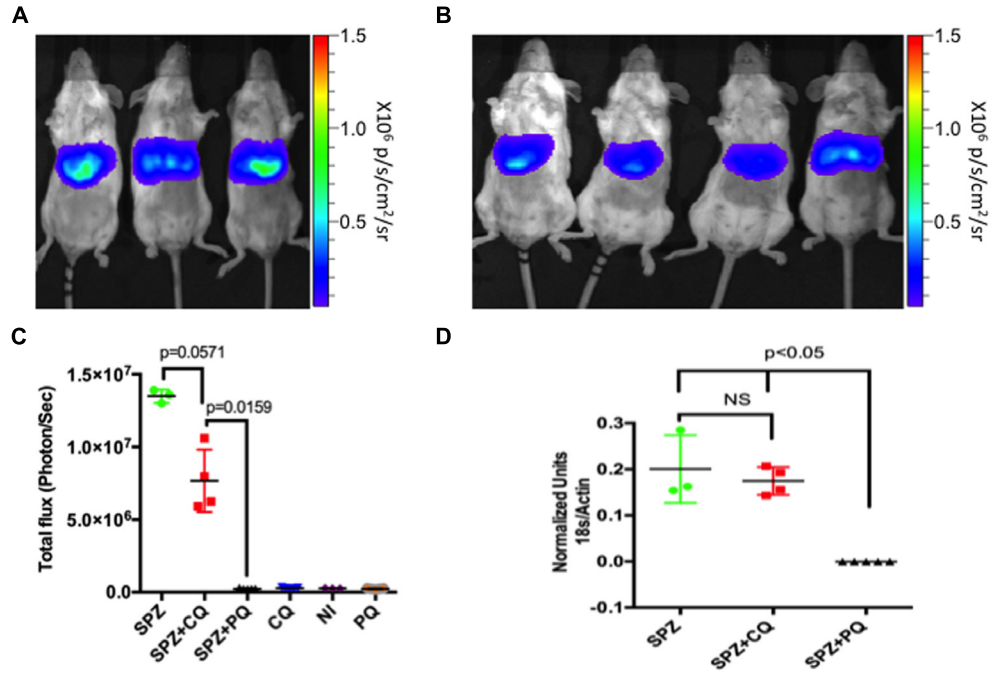

FIGURE 2 | Effect of CQ on LS parasite at 40 hours post infection (hpi). Rainbow images of mice infected and untreated **(A)** or CQ-treated **(B)** showing parasite load in liver 40 h after injection of 1.5 × 10^4 *Py*-Luc salivary gland SPZ. Rainbow scale represents radiance (p/s/cm^2/sr). **(C)** Quantification of total flux from whole body imaging of mice (shown in **A** and Supplementary Figure S1). $n = 3$ for SPZ (infected and untreated) and NI (neither infected nor treated) group, four for SPZ+CQ group and five each for SPZ+PQ and drug control (treated uninfected) groups. **(D)** Quantification of LS parasite burden 40 hpi in livers of SPZ ($n = 3$), SPZ+CQ ($n = 4$), and SPZ+PQ ($n = 5$) mice by qPCR. Graph represents Py-18S RNA normalized to murine β-actin RNA. Data in **(C,D)** represents Mean ± SD, Mann–Whitney test was performed and $p < 0.05$ considered as significant. CQ, Chloroquine; PQ, Primaquine; NI, non-infected and non-treated.

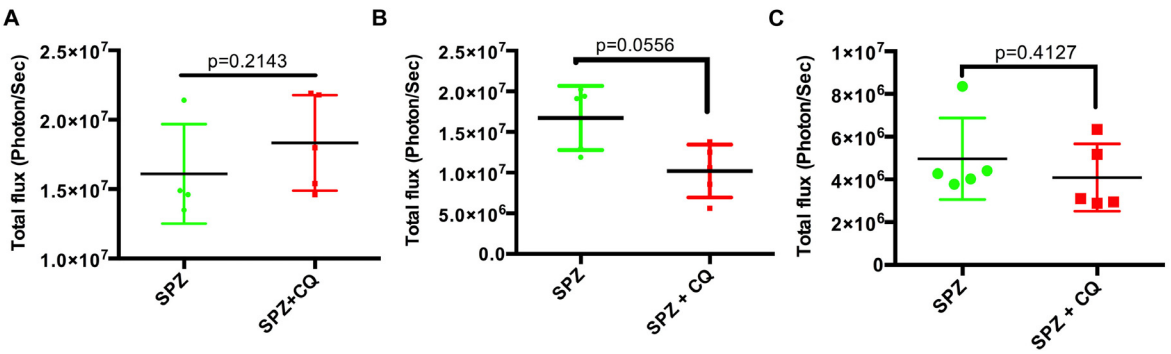

FIGURE 3 | Parasite load in liver at different time points after CQ treatment. Quantification of total flux from whole body imaging of mice (shown in Supplementary Figure S4). $n = 4$ for SPZ group at 44 hpi **(A)**, five each for both SPZ and SPZ+CQ at 48 h **(B)**, and 54 h **(C)** time points. Graph represents mean ± SD. Mann–Whitney test was performed and $p < 0.05$ considered as significant.

CQ may have partial or subtle effects on late LS development that contribute to enhanced immune responses, but we find no evidence that CQ decreases LS burden or delays LS development.

The effect of CQ on the LS of *Plasmodium* has previously been studied *in vivo* in a mouse model (Belnoue et al., 2004) and *in vitro* by infection of non-human primate hepatocytes (Fisk et al., 1989). Belnoue et al. (2004) reported that mice treated with 0.8 mg CQ and untreated mice had similar LS parasite burden 42 hpi, but did not examine effects beyond this time point; the CQ dose (~32 mg/kg) used by Belnoue et al. (2004, and also by us for this study) was based on earlier work (Orjih et al., 1982), and is in the dosing range (30–35 mg/kg) used for children of 10–20 kg body weight (Moore et al., 2011).

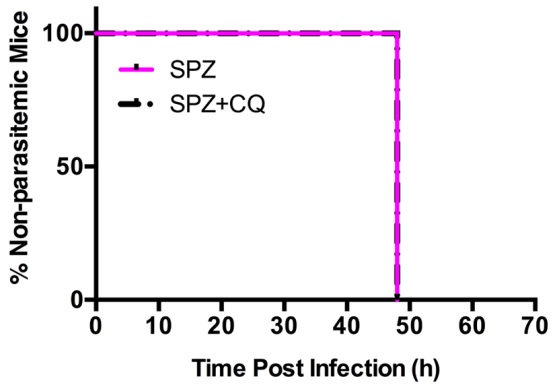

FIGURE 4 | Effect of CQ on prepatent period of *Py*-Luc. Mice ($n = 18$) were infected with 1.5×10^4 *Py*-Luc salivary gland SPZ and either CQ-treated (SPZ+CQ; $n = 9$) or not (SPZ; $n = 9$). Blood smears (both thick and thin smears) were collected starting from 48 hpi. Smears were stained and examined by expert smear reader blinded. Smears are considered positive if minimum two parasites were found in 100 adjacent fields.

Earlier, Fisk et al. (1989) reported that CQ at 1 μg/ml had a partial parasiticidal effect *in vitro* on late LS *P. cynomolgi* and *P. knowlesi* schizonts. However, these drug concentrations were ∼2.5-fold higher than therapeutic concentrations (0.03–0.4 μg/ml) in humans (Tett et al., 1989; Carmichael et al., 2003), and had toxic effects on hepatocytes that may have contributed to parasiticidal activity.

We examined the effect of CQ on LS development *in vivo* by imaging luciferase expressing *P. yoelii* parasites in Balb/c mice, and followed parasite development in the liver, both by BLI and qPCR, at multiple time points from 40 h through 62 h. We observed no difference in the LS parasite load between CQ-treated (SPZ+CQ) and untreated (SPZ) groups at any time point during late LS development. Our observations at 42 hpi are consistent with those of Belnoue et al. (2004) and additionally showed no differences at 44, 46, 48, and 54 hpi.

We hypothesized that CQ might delay LS development, at least for a subset of parasites, thereby creating a condition of prolonged antigen exposure that might enhance LS specific immunity. All mice, both SPZ+CQ and SPZ, had parasites detectable by blood smear microscopy by 48 hpi, suggesting that there had been no delay in LS development with CQ treatment. This was further substantiated by similar LS parasite burdens in both groups of mice at 48 hpi (**Figure 3**; Supplementary Figures S3–S5). Our study cannot exclude the possibility that a small subset of parasite persist in liver of SPZ+CQ mice, because emerging blood stage parasites might mask the presence of LS forms in bioluminescence or qRT-PCR studies.

The potential effect of CQ to delay the prepatent period has also been examined in humans as part of CVac trials, using highly sensitive qPCR techniques to detect, and quantify blood stage parasites at densities below the sensitivity of microscopy (i.e, subpatent parasitemia; Roestenberg et al., 2009; Bijker et al., 2013). During CVac, blood stage parasites are detected by qPCR at the time expected of untreated individuals (Roestenberg et al., 2009; Bijker et al., 2013), although a direct comparison of blood stage parasite burden between CQ-treated and untreated individuals has not been reported. Similarly, we find that blood stage parasites are detected by microscopy at 48 hpi in both SPZ+CQ and SPZ mice. A previous study in mice reported that CQ suppressed blood stage growth during CVac (Peng et al., 2014), as is observed in human studies (Roestenberg et al., 2009; Bijker et al., 2013). Our results in mice confirm that CQ does not delay LS development nor kill LS parasites, and support the notion that the diminished first wave of parasitemia is likely to be solely due to its effects on blood stage growth, rather than the effect on the number of parasites released from liver.

Chloroquine was once widely used as an anti-malarial drug and was highly effective in clearing blood stage forms of sensitive *Plasmodium* parasites, but use of CQ has waned decisively with the global spread of CQ-resistant parasites. Recently, the impressive immunity observed after CVac has sparked renewed interest in CQ as a component of whole organism vaccines, whereby individuals are inoculated with infectious SPZ under CQ drug cover. However, the mechanisms by which drug treatment converts infectious SPZ into an effective vaccine remain unclear. Using a luciferase-expressing parasite and an *in vivo* mouse infection model, we have systematically evaluated the effect of CQ on *Plasmodium* late liver stages. We report that CQ given at a dose used for CVac studies neither kills nor delays LS parasite development. However, our study does not exclude other effects of CQ on *Plasmodium* LS biology that might impact immune responses, such as an altered repertoire of expressed antigens, and hence further investigation is warranted.

Author Contributions

Conceived and designed the experiments: TS and PD. Performed the experiments: TS, LL, JH, SC, SO-G, and DC. Analyzed the data: TS and PD. Wrote the manuscript: TS and PD.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00283/abstract

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REVIEW
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Plasmodium cellular effector mechanisms and the hepatic microenvironment

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Plasmodium falciparum malaria remains one of the most serious health problems globally. Immunization with attenuated parasites elicits multiple cellular effector mechanisms capable of eliminating *Plasmodium* liver stages. However, malaria liver stage (LS) immunity is complex and the mechanisms effector T cells use to locate the few infected hepatocytes in the large liver in order to kill the intracellular LS parasites remain a mystery to date. Here, we review our current knowledge on the behavior of CD8 effector T cells in the hepatic microvasculature, in malaria and other hepatic infections. Taking into account the unique immunological and lymphogenic properties of the liver, we discuss whether classical granule-mediated cytotoxicity might eliminate infected hepatocytes via direct cell contact or whether cytokines might operate without cell–cell contact and kill *Plasmodium* LSs at a distance. A thorough understanding of the cellular effector mechanisms that lead to parasite death hence sterile protection is a prerequisite for the development of a successful malaria vaccine to protect the 40% of the world's population currently at risk of *Plasmodium* infection.

Keywords: *Plasmodium*, liver, antigen-presenting cells, CD8 T cells, liver lymphatics

Plasmodium falciparum malaria remains one of the most serious health problems globally and long-lasting protective malaria vaccine is desperately needed. The ability to interrupt the clinically silent liver phase of the malaria parasite would prevent an estimated 207 million clinical cases every year, leading to the death of one young African child almost every minute (WHO, 2013). Vaccination with attenuated parasites elicits multiple cellular effector mechanisms that lead to *Plasmodium* liver stage (LS) elimination. While granule-mediated cytotoxicity requires contact between CD8 effector T cells and infected hepatocytes, cytokine mediated parasite killing could occur without cell–cell contact. This review aims to put into context the biology of the pre-erythrocytic stages of *Plasmodium*, the unique immunological and lymphogenic properties of the liver, and recent insight into the dynamic behavior of CD8 effector T cells in the hepatic microvasculature to provide a better understanding of the cellular events involved in the blocking of *Plasmodium* LS development.

Abbreviations: APC, antigen-presenting cell; BM, bone marrow; BMT, bone marrow transfer; CSP, circumsporozoite protein; DC, dendritic cell; GAS, genetically attenuated sporozoites; KC, Kupffer cell; LSEC, liver sinusoidal endothelial cell; RAS, radiation-attenuated sporozoites; TCR-Tg, T cell receptor transgenic.

Immunity against Pre-Erythrocytic *Plasmodium* Antigens

While T cell priming against sporozoite antigens is thought to occur in the LNs draining the mosquito bite skin site (Chakravarty et al., 2007), the liver draining LNs are the most likely site of T cell activation against late-LS and early blood stage antigens. However, T cell priming may also occur in the liver itself, for example by direct recognition of infected hepatocytes and or via cross-presentation by the various non-parenchymal antigen-presenting cell (APCs) including hepatic dendritic cell (DCs; Jobe et al., 2009; Crispe, 2011; Bertolino and Bowen, 2015). For an overview on the induction phase of immunity against pre-erythrocytic *Plasmodium* antigens, the reader is referred to recent reviews (Crispe, 2014; Van Braeckel-Budimir and Harty, 2014; Radtke et al., 2015). Here, we focus on the effector phase of the disease and discuss how the various cellular effector mechanisms might operate in the liver, upon first infection of a naïve host leading to disease versus repeated exposure or vaccination resulting in immunity. We present this review in the context of the unique immunological and lymphogenic features of the liver.

The Liver, a Metabolic Organ with Unique Tolerogenic and Lymphogenic Properties

The liver is known as a lymphatic organ with unique immunological properties (Knolle and Limmer, 2001; Sheth and Bankey, 2001; Bertolino et al., 2002; Mackay, 2002; Racanelli and Rehermann, 2006; Crispe, 2009). Its tolerogenic properties, necessitated by continuous natural exposure to innocuous food antigens and commensal microbial products from the gastrointestinal tract, are now widely recognized (Racanelli and Rehermann, 2006; Crispe, 2009; Jenne and Kubes, 2013). It seems likely, therefore, that by choosing the liver as the initial site of multiplication, *Plasmodium* is able to exploit the tolerogenic properties of the liver (Frevert et al., 2006; Crispe, 2011; Bertolino and Bowen, 2015). Less appreciated is the generation of lymph in this large metabolic organ. Plasma flows continuously through the sinusoidal sieve plates and enters the space of Disse (**Figure 1**). Once in the perisinusoidal space, the lymph travels in a retrograde fashion around the sinusoids toward the periportal space of Mall (Reid et al., 1992). Despite more than half of the lymph of the entire body being of hepatic origin (Henriksen et al., 1984; Magari, 1990; Trutmann and Sasse, 1994; Ohtani and Ohtani, 2008), the contribution of lymph formation to liver immunology has been surprisingly underappreciated to date (reviewed in Frevert and Nacer, 2013). By influencing cytokine dissemination, the unique hepatic blood-lymph counterflow principle has important implications for the effector phase of immunity against *Plasmodium* LS.

Most non-parenchymal liver cells can function as APCs (Jobe et al., 2009; Crispe, 2011), inducing either tolerance or enhanced immune responses amongst liver T cells. The state of immune tolerance rather than immune activation is the more typical and frequent condition under the steady state. It prevents liver pathology arising from a constant inflow of bacterial and other microbes from the gastrointestinal tract and blood-borne antigens from the systemic circulation. The state of tolerance observed in the liver is maintained by the production of anti-inflammatory cytokines, in particular IL-10 (Knolle et al., 1995), and other modulators such as PGE2 produced by Kupffer cells (KCs) via ligation of TLR4. Together with nitric oxide, these mediators down-regulate antigen uptake by liver sinusoidal endothelial cells (LSECs) and DCs, which leads to a decreased T cell activation (Roland et al., 1994; Groux et al., 1996). However, KCs have also been shown to play a role in fighting infections by producing IL-12 and IL-18 and upregulating MHC I and II molecules as well as the co-stimulatory molecules CD40 and CD80 needed for activation of T cells to produce IFN-γ (Burgio et al., 1998). It appears therefore that signals received by KCs propel them either into poor and ineffective or efficient APC, promoting a reversal of immune tolerance. Tolerance induction or maintenance is also a particular property of the LSECs (Berg et al., 2006; Ebrahimkhani et al., 2011) with their typical fenestrations. Although LSECs express MHC I and II molecules as well as costimulatory molecules involved in T cell activation, they produce IL-10 upon TLR4 ligation; the ensuing down-regulation of IL-12 restricts the IFN-γ production by T cells thus reducing the size of the response (Berg et al., 2006; Ebrahimkhani et al., 2011). The anatomical location of the LSECs and KCs in the sinusoidal lumen makes them excellent scavengers of a plethora of exogenous antigens and enables them to present these efficiently in the context of MHC I and II molecules *in vivo* (Limmer et al., 2000) for the induction of tolerance. The tolerizing effects of anti-inflammatory responses of liver APCs is typically observed *in vivo* to low levels of LPS. Both human and rodent LSECs upregulate MHC I and express MHC II molecules in response, for example, to viral and bacterial infections or exposure of the liver to IFN-γ (Steinhoff et al., 1988; Steiniger et al., 1988; Steinhoff, 1990). Similar to KCs and classical splenic DCs, LSECs can cross-present hepatocyte-associated antigens to CD8 T cells (Berg et al., 2006; Ebrahimkhani et al., 2011). In contrast, several functional populations of DCs, the professional APCs, are located in the periportal connective tissue of the liver lobule (Sumpter et al., 2007), and they respond to virus infection by elaborating type I IFN, which ultimately leads to the induction of a pro-inflammatory environment in the liver. Apart from the most abundant plasmacytoid DCs, the mouse liver also harbors myeloid DCs and conventional $CD8\alpha^+$DCs; thus far, the $cCD8\alpha^+$DCs have no counterpart in the human liver. Amongst the three liver DC subsets, the $cCD8\alpha^+$DCs have the highest APC capacity.

The Hepatic Cycle of *Plasmodium*—Implications for Antigen Presentation in the Liver

Following *Plasmodium* infection, mammalian hosts are exposed to *Plasmodium* antigens in multiple ways: (1) sporozoite surface antigens released from viable migrating parasites, (2) sporozoite antigens from parasites that degrade or are eliminated by phagocytosis before they reach their final destination in the liver, (3) early or late LS antigens from parasites that fail to complete the hepatic development cycle, and (4) early or late LS antigens, and potentially blood stage antigens, left behind in the remains of dead infected hepatocytes after successful merosome release.

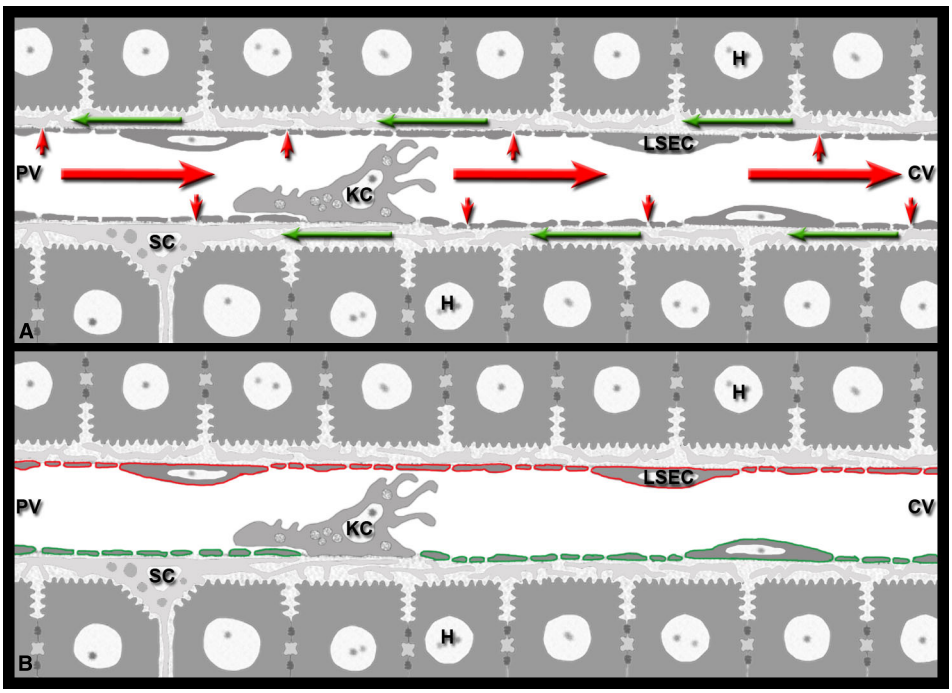

FIGURE 1 | Immunological implications of the hepatic blood-lymph countercurrent. (A) The liver generates lymph by filtering blood plasma (small red arrows) through the sieve plates of the sinusoidal endothelial cells (LSEC) into the perisinusoidal space of Disse formed by LSECs and hepatocytes. The lymph (green arrows) flows inside the space of Disse around the perisinusoidal stellate cells (SC) toward the portal field, while the blood (red arrows) continues its path in the opposite direction, from the portal venule (PV) to the central venule (CV). **(B)** LSECs represent the blood-lymph barrier of the liver: they express both the vascular marker PECAM-1 (red) and the lymphatic marker LYVE-1 (green). The two markers are depicted separately for clarity.

It has been widely accepted that responses to pre-erythrocytic stage antigens are rather low in persons residing in *P. falciparum* endemic areas (Doolan and Martinez-Alier, 2006). The current model suggests amongst others, that the few invading sporozoites leave inadequate antigen levels or that the sporozoites or the LS antigens that are invisible to the host undergo incomplete or faulty antigen processing for presentation to T cells. Consequently, induction of protective T cell and antibody responses by the erythrocytic stage antigens is rarely observed in naturally infected persons. It is also possible that the pre-erythrocytic antigens themselves induce some regulatory T cells or tolerance, both of which can dampen potential protective responses (Espinoza Mora Mdel et al., 2014; Wilson et al., 2015). Immunization with *P. falciparum* sporozoites, however, can induce high levels of sporozoite-neutralizing antibodies (Nardin, 2010) thus emphasizing the relevance of studying the presentation of sporozoite antigens such as circumsporozoite protein (CSP). Protective immunity requires CD8 memory T cells and CD4 helper T cell-derived cytokines such as IFN-γ that elicit sporozoite-neutralizing antibodies and inhibit LS development mainly through upregulation of iNOS and induction of NO in the infected hepatocytes (Doolan and Martinez-Alier, 2006; Nardin, 2010; Dups et al., 2014). Clinical trials conducted during the past decades revealed that synthetic CSP-derived peptide vaccines induce CD4 and CD8 T cells of similar fine specificity and function (Calvo-Calle et al., 2006; Oliveira et al., 2008; Othoro et al., 2009; Nardin, 2010). Another sporozoite surface antigen, the thrombospondin-related adhesive protein (TRAP), induces transient cytokine responses under natural exposure conditions (Moormann et al., 2009) and central memory T cell responses against TRAP are associated with a significantly reduced incidence of malaria (Todryk et al., 2008). These findings support recent vaccination strategies aimed at inducing durable protective T cell responses against TRAP (Ewer et al., 2013). Interestingly, it was shown that long-term residents of hyperendemic areas in Africa, who received protracted daily anti-malarial prophylactic chloroquine treatment to prevent the development of blood stage infection, developed a strong antibody response to sporozoites and LSs, but only a weak response to blood stages (Marchand and Druilhe, 1990; Gruner et al., 2003). In fact, sera from the individuals displaying responses to sporozoites and immunity against LS were used successfully to identify two LS-specific antigens in *P. falciparum* (Guerin-Marchand et al., 1987; Sangani et al., 1990). A similar example of T cell responses to pre-erythrocytic stage antigens comes from the *P. vivax* malaria. A recently published study shows that persons, who are negative for the Duffy antigen and hence are refractory to *P. vivax* blood-stage infection, do develop stronger T cell responses to sporozoite and LS antigens than Duffy antigen-positive subjects (Wang et al., 2005). These studies suggest that responses to the pre-erythrocytic stage antigens, be it those associated with sporozoites or with LS, are indeed inducible. As suggested by others, the presence of blood stage infection, which vastly exceeds the antigenic load of the sporozoites and the LS, may negatively influence the induction of T and B cell responses. Inhibition of antigen presentation by

blood stage antigens has indeed been amply demonstrated in human (Urban et al., 2005) and animal (Ocana-Morgner et al., 2003) *Plasmodium* infections. In the following, we discuss how the main liver cell types could be involved in the acquisition and presentation of different *Plasmodium* antigens.

Sinusoidal Endothelia

In vitro studies revealed that during parasite invasion and traversal, *Plasmodium* sporozoites continuously release surface antigens such as CSP and TRAP as part of gliding motility (Stewart and Vanderberg, 1988, 1991, 1992; Hügel et al., 1996; Spaccapelo et al., 1997). In addition, sporozoites can also translocate CSP into the cytosol of mammalian cells by mere membrane contact (Hügel et al., 1996; Frevert et al., 1998; Pradel and Frevert, 2001). The implications for antigen processing and presentation of this phenomenon, which occurs in the absence of invasion, are discussed below. Various cell types of the liver are likely exposed to sporozoite-released antigens. As sporozoites glide extensively along the sinusoids before infecting hepatocytes (Frevert et al., 2005), a substantial number of LSECs are likely exposed to released CSP (**Figure 2**), which they can present for a recall of both CD8 and CD4 effector T cells (Knolle and Gerken, 2000; Crispe, 2011). LSECs have also been established as the primary cross-presenting APCs of the liver (Limmer et al., 2005). However, cross-presentation by LSECs can also lead to CD8 T cell tolerance (Berg et al., 2006). Thus, while LSECs are candidate APCs, their actual contribution to protective immunity against *Plasmodium* CSP or other sporozoite-associated antigens has not been fully investigated and remains rather difficult to predict.

Kupffer Cells

The vast majority of sporozoite entry events into the hepatic parenchyma involve KCs (Baer et al., 2007b; Tavares et al., 2013), resident macrophages and professional APCs of the liver (Crispe, 2011; **Figure 2**). Early evidence supporting this notion came from electron micrographs showing *P. berghei* sporozoites that stretch from the sinusoidal lumen through KCs all the way into hepatocytes (Meis and Verhave, 1988). Subsequently, *P. yoelii* sporozoites were shown to recognize KC-specific surface proteoglycans (Pradel et al., 2002, 2004) and to pass through KCs on their way into the hepatocytes (Frevert et al., 2005). The use of KC-deficient mouse models initially implied an obligatory role of these hepatic macrophages for liver infection (Baer et al., 2007b). A more recent *in vivo* study confirmed the involvement of KCs in the vast majority of all cell traversal events (Tavares et al., 2013), but also demonstrated that some intravenously injected sporozoites use a paracellular pathway for liver infection (Tavares et al., 2013). Thus, while there is no absolute requirement for KC passage, most sporozoite traversal events involve KCs, either directly or indirectly.

Based on the finding that the vast majority of SPECT$^{-/-}$ *P. berghei* sporozoites were trapped in lasting interactions with KCs, it was proposed that sporozoites must use cell traversal to avoid clearance by KC in the liver (Tavares et al., 2013). Of note, once intracellular, these mutant parasites cannot egress from the KC, and therefore die. This is in contrast to viable WT *Plasmodium* sporozoites, which are not killed by KCs, or other macrophages, from naïve mice (Pradel and Frevert, 2001; Frevert et al., 2006; Klotz and Frevert, 2008). Further, the infectious dose for naïve mice is often less than three intravenously injected *P. yoelii* sporozoites (Conteh et al., 2010). Thus, sporozoite infection of the liver is highly efficient, which renders the possibility unlikely that KCs from naïve mice kill sporozoites *in vivo*. On the other hand, 10% of intravenously injected WT *P. berghei* sporozoites were reported to be degraded during KC traversal (Tavares et al., 2013). This disparity could be due to the different parasite species used for the two studies. Alternatively, as the viability of isolated sporozoite preparations varies, some of the *P. berghei* parasites might have been already dead when they arrived in the liver resulting in their clearance by phagocytosis. In general, all available evidence suggests that viable WT sporozoites survive the interaction with naïve KCs unharmed.

In a separate study assessing the *in vivo* responses of KCs to radiation-attenuated *P. berghei* sporozoites (Pb-RAS), infectious sporozoites were shown to down-modulate MHC I and IL-12p40 expression (Steers et al., 2005). However, infectious sporozoite challenge of mice previously immunized with Pb-RAS has the opposite effect in that the expression of MHC I, co-stimulatory molecules, and IL-12 are upregulated. In addition, *in vitro* assessed APC function of these KCs was significantly enhanced in relation to KCs obtained from naïve mice, naïve mice exposed to infectious sporozoites or even mice immunized thrice with Pb-RAS (Steers et al., 2005). Thus, although the prevailing state of immune-tolerance in the liver might attract *Plasmodium* sporozoites to invade hepatocytes where they remain "incognito" as they expand in number, induction of an inflammatory milieu by Pb-RAS reverses the hospitality of the liver to a state of immunologic conflagration that is needed to eliminate the *Plasmodium* parasite. Exposure of mice to doses of IL-12 has a similar, albeit short-lived effect on *Plasmodium* LS (Sedegah et al., 1994; Hoffman et al., 1997). By contrast, Pb-RAS immunization induces lasting protection that becomes refractory to multiple challenges. The reason why KCs respond differentially to RAS versus viable sporozoites is unknown to date. Immunization with the similarly protective genetically attenuated sporozoites (GAS, Khan et al., 2012; Nganou-Makamdop and Sauerwein, 2013) or chemically attenuated sporozoites (CAS, Purcell et al., 2008) may provide insight into the response of KCs, or other hepatic APCs, to sporozoite encounters that are required for protection. Further, careful monitoring of location and fate of the various attenuated parasites may also provide valuable information.

Thus, the hepatic microenvironment from naïve and immune animals is functionally distinct. While KCs from naïve mice become deactivated upon contact with viable sporozoites and eventually succumb to apoptosis (Steers et al., 2005; Klotz and Frevert, 2008), activated KCs may phagocytose sporozoites, in particular if immunization has generated high antibody levels. As in other hepatic diseases (Nagy, 2003), the pro-inflammatory microenvironment created by activated KCs could further enhance the elimination of subsequent cohorts of sporozoites in a local fashion. Depending on the number of attenuated sporozoites used for immunization, challenge with viable sporozoites may allow a few parasites to enter the liver. The finding that challenge of immune mice with viable sporozoites resulted in a very low in

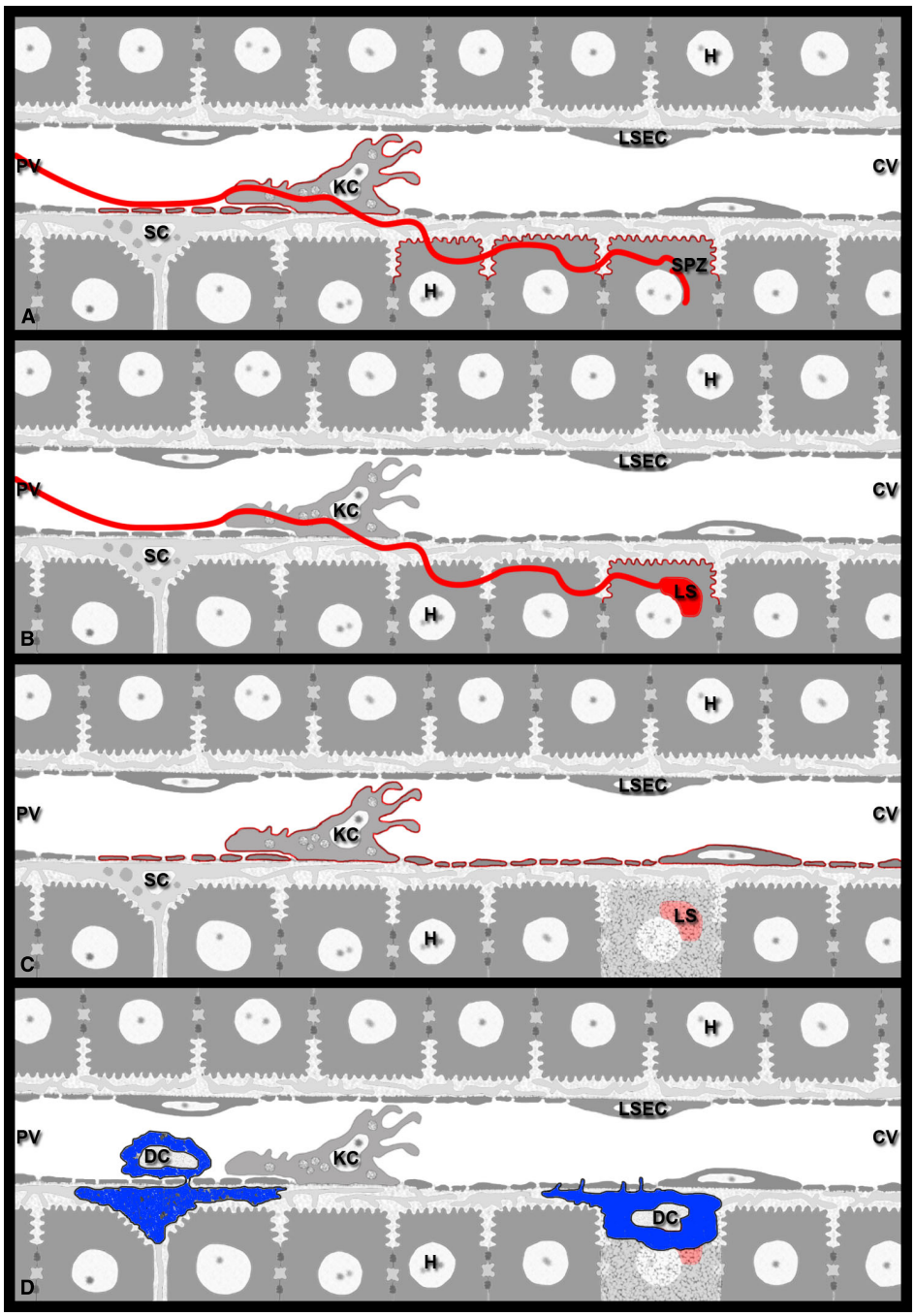

FIGURE 2 | Model for *Plasmodium* antigen presentation in the liver. **(A)** Sporozoites continuously release antigens such as CSP and TRAP from their surface. On their way from the sinusoidal lumen into the liver parenchyma, sporozoites glide along LSECs, traverse KCs, and migrate through several hepatocytes before infecting a final one (red line). In naïve mice, contact-mediated translocation of sporozoite antigens into the cytoplasm of these liver cells may result in antigen presentation by LSECs, KCs, as well as traversed and infected hepatocytes (red outline). **(B)** Sporozoite infection of naïve mice should allow late-LS antigen expression exclusively on infected hepatocytes, i.e., those cells in which the parasites develop (red outline). **(C)** During repeated exposure to viable sporozoites or multiple rounds of immunization with attenuated parasites, infected hepatocytes die after completion of LS development, resulting in the release of debris and leftover late-LS antigens into the environment. These late-LS antigens may then be internalized by nearby APCs such as LSECs and KCs and cross-presented to CD8 effector T cells monitoring the liver. **(D)** Of the various DC subsets that are involved in inflammatory processes of the liver, the conventional CD8α^+DCs are required for the CD8 T cell-mediated elimination of *Plasmodium* LS. While their exact location in the liver is unknown, *Plasmodium* infection likely attracts immature cCD8α^+DCs to the hepatic sinusoids, where they interact with KCs and acquire sporozoite antigens. Extravasation into the space of Disse would allow these DCs (blue) to communicate with CD8 effector T cells patrolling the sinusoids. DCs may also internalize cellular and parasite debris released from dead infected hepatocytes and subsequently cross-present these antigens to CD8 effector T cells, either from within the space of Disse or after traveling to the LNs that drain the liver.

number of very small LS supports this idea (Cabrera et al., 2013). In conclusion, the argument that sporozoites must avoid KC contact to successfully infect the liver (Tavares et al., 2013) could potentially be valid for the immune, but not for the naive host.

Whether KCs act as APCs to recall effector T cells in the *Plasmodium* infected liver is unknown to date (reviewed in Frevert, 2004; Frevert et al., 2006, 2008b, 2014; Frevert and Nardin, 2008). Owing to the unique immunological properties of the liver (Knolle and Limmer, 2001; Sheth and Bankey, 2001; Bertolino et al., 2002; Mackay, 2002; Racanelli and Rehermann, 2006; Crispe, 2009), KCs may respond to sporozoite contact with tolerance rather than inflammation and immunity (Frevert et al., 2006). *P. berghei* and *P. yoelii* sporozoites enter KCs actively by formation of a vacuole and release CSP into the cytosol of these APCs *in vitro* (Pradel and Frevert, 2001), suggesting that KCs can present sporozoite surface antigens to both CD8 and CD4 effector T cells (**Figure 2**). However, a number of reports support the notion that KCs and other macrophages do not survive sporozoite contact (Danforth et al., 1980; Smith and Alexander, 1986; Vanderberg et al., 1990; Klotz and Frevert, 2008). *In vitro* studies revealed that *P. yoelii* sporozoite CSP binds to the low density lipoprotein receptor-related protein (LRP) and proteoglycans on the surface of KCs, which leads to blockage of NADPH oxidase activation, suppression of the respiratory burst, and generation of an anti-inflammatory cytokine secretion profile (Usynin et al., 2007; Klotz and Frevert, 2008). Thus, traversed KCs may not be able to present sporozoite antigens, because they lose APC function and succumb to apoptosis (Klotz and Frevert, 2008). This notion is supported by data from other experimental systems, in which KCs generate tolerance by inducing apoptosis in naïve CD8 T cells in the absence of inflammation (Holz et al., 2012).

Another important finding was that sporozoites are able to introduce CSP into mammalian cells without invading them (Frevert et al., 1996; Hügel et al., 1996). CSP translocation across the cell membrane into the cytosol requires neither the metabolic nor the endocytic machinery of the affected cell (Hügel et al., 1996; Frevert et al., 1998; Pradel and Frevert, 2001) suggesting that it involves a PEXEL motif (Singh et al., 2007). Once in the cytosol, CSP binds to ribosomal RNA (Hügel et al., 1996), which interferes with initiation step of protein synthesis (Frevert, 1999). In contrast to the large number of enzymatically active cytotoxic plant, fungal, and bacterial proteins, which require only a few molecules in the cytosol to kill a cell (Stirpe et al., 1992; Barbieri et al., 1993; Lacadena et al., 2007), CSP has a stoichiometric mode of action (Frevert et al., 1998; Frevert, 1999). The effective concentration of CSP leading to complete inhibition of translation likely varies with the cell type, but complete blockage of the protein synthesis machinery renders uninfected mammalian cells moribund (Frevert et al., 1998). For example, large and metabolically highly active cells such as hepatocytes, which are filled with a huge amount of free and rough endoplasmic reticulum-associated ribosomes, are necessarily less sensitive to the stoichiometric ribotoxic effect of CSP than the much smaller LSECs and KC with a much less developed protein synthesis machinery. In agreement with this notion, macrophages appeared to be particularly sensitive to the ribotoxic action of CSP (Frevert et al., 1998). Nothing is known about the possibility that sporozoite-mediated CSP translocation induces apoptosis in LSECs and KCs. The resulting loss of these APCs from the normally continuous sinusoidal cell layer would expose the underlying parenchyma at the site of sporozoite gliding and KC traversal, similar to the gaps generated by clodronate-mediated KC removal from the sinusoidal cell layer (Baer et al., 2007b), albeit to a much larger extent. As a consequence, effector T cells might gain direct access to traversed and/or infected hepatocytes.

In conclusion, we are only beginning to understand the contribution of KC to sporozoite infection of the liver and the effector phase of immunity against *Plasmodium* LS. KC may be deactivated or even killed by contact with viable sporozoites or by the presence of cytosolic CSP. Alternatively, KC may be activated by exposure to opsonized sporozoites or immunization with attenuated parasites and turned into a source of pro-inflammatory cytokines. A better understanding of these very different scenarios will reveal whether KC (and LSEC) are able to process and present sporozoite antigens for priming of naïve CD8 and CD4 T cells and for induction of recall responses in effector memory T cells.

Dendritic Cells

Hepatic DCs are thought to play a major role in the cross-presentation of hepatocyte-derived antigens (Bertolino and Bowen, 2015). Under resting conditions, however, hepatic DCs are immature. Due to their location in the periportal interstitium and draining hepatic LNs, these professional APCs are not directly accessible to CD8 effector T cells patrolling the hepatic sinusoids (**Figure 2**). Pathological or inflammatory conditions trigger the recruitment of blood-borne DC precursors from the bone marrow to the liver sinusoids (Yoneyama and Ichida, 2005). In a murine model of *Propionibacterium acnes*-induced granuloma model, 6 h were required for a significant number of myeloid DCs and plasmacytoid DC precursors to accumulate in the liver of mice with preexisting granulomata (Yoneyama and Ichida, 2005). After antigen uptake, DCs enter the space of Disse, travel via the portal Glisson's capsule to the draining LNs for antigen presentation to T cells followed by their activation (Sato et al., 1998). Thus, unlike liver-resident APCs (LSECs and KCs), DCs must first travel to the liver, which takes time. Other immune cells also arrive in the liver with a considerable delay. For example, increased numbers of $Ly6C^{hi}$ monocytes, which enter sites of inflammation and get activated locally, were detectable 1 day after systemic infection with *Listeria monocytogenes* (Shi et al., 2010). Further, continuous intravital monitoring revealed that myelomonocytic cells begin to arrive in and extravasate out of hepatic sinusoids roughly an hour after surgical preparation of the liver for IVM (Frevert et al., 2005). Since leukocyte accumulation occurred independently of sporozoite infection, the recruitment of the cells is interpreted as a response to injury.

With respect to malaria, the relative contribution of liver resident versus recruited inflammatory DC is unknown to date (Mauduit et al., 2012). Several studies have demonstrated a critical role of $CD8\alpha^+$DCs in the activation of effector CD8 T cells responding to an epitope of CSP. Mice that are missing $CD8\alpha^+$DC succumbed to infection in the aftermath of exposure to infectious sporozoites (Layseca-Espinosa et al., 2013). In a murine model of protective immunity induced by *P. berghei* RAS, $CD8\alpha^+$DC

accumulated in the liver coincident with the exposure to Pb-RAS sporozoites and induced CD8 T cells *in vitro* to produce IFN-γ, an activity that is IL-12 and MHC I dependent (Jobe et al., 2009). Several possible scenarios have been proposed for the cellular mechanisms by which CD8α$^+$DCs contribute to protective immunity (Jobe et al., 2009). CD8α$^+$DCs might activate effector CD8 T cells in the portal fields or in the draining hepatic LNs, similar to plasmacytoid DCs, which normally do not remain in the Disse spaces for extended periods of time (Yoneyama and Ichida, 2005). Alternatively, CD8α$^+$DCs might be recruited by KCs located in the vicinity of infected hepatocytes and extravasate into the spaces of Disse, similar to what has been observed for myeloid DCs in the *P. acnes* model (Yoneyama and Ichida, 2005). This would provide the advantage that *Plasmodium*-specific CTLs could be activated directly in the sinusoid and that the resulting release of cytokines and chemokines could kill LS at relatively close range. Quantitative aspects are worth considering as well. It could be argued that DC accumulation in the liver may not be necessary for CD8 T cell activation, because *in vitro* studies indicate that one CTL is sufficient to kill infected hepatocytes or *Plasmodium* peptide-primed target cells (Bongfen et al., 2007; Frevert et al., 2008a; Trimnell et al., 2009). However, sterile protection against *Plasmodium* infection requires extreme numbers of CD8 effector T cells in the mouse model (Schmidt et al., 2010) and the recruitment of large numbers of CD8 T cells to the vicinity of LS (Cockburn et al., 2013). Together with the apparent absence of direct CTL contact with the dying infected hepatocytes (Cockburn et al., 2013), these data suggest that parasite elimination is relatively inefficient *in vivo* (see below). Therefore, the recruitment of large numbers of DC to the liver may be required for optimal antigen uptake to assure efficient presentation to and activation of T cells in the draining LN. This unusual situation where antigens are loaded onto APCs (in this case DCs) in the liver for subsequent presentation in another organ, the draining LN, may cause a large fraction of the malaria antigens to undergo extensive proteolysis, resulting in a rather limited repertoire of antigenic peptides to activate T cells. Further, if effector memory CD8 T cells are activated in the draining LN they might return to the liver with a considerable delay to kill LS and prevent the erythrocytic phase of the infection.

In conclusion, a combination of DC recruitment to the liver, subsequent DC migration to the draining LN resulting in a limited peptide repertoire, delayed T cell migration back to the liver, and the impossibility to use direct contact-mediated cytotoxicity (see below) may indeed render the CTL response quite ineffective in conferring sterile protection to the host. Clearly, more work is required to elucidate the relative contribution of recruited versus resident hepatic DCs to the presentation of *Plasmodium* antigens to T cells. Dynamic *in vivo* studies should be particularly suitable to shed light onto the local cellular interactions involved in immunity.

Hepatocytes

After crossing the sinusoidal cell layer, sporozoites traverse several hepatocytes before selecting a final cell for multiplication and differentiation to thousands of merozoites (Mota et al., 2001; Frevert et al., 2005). As mentioned above, sporozoites continuously release surface antigens while migrating (Stewart and Vanderberg, 1988, 1991, 1992; Spaccapelo et al., 1997). The first microscopic evidence for the presence of sporozoite-released CSP in the cytosol of infected hepatoma cells came from the Frevert lab (Hügel et al., 1996; Frevert et al., 1998). Temporal analysis of infected HepG2 cells revealed that the amount of cytosolic CSP decreases with increasing maturity of the intracellular LS. Further, because only a few of the skin-deposited sporozoites get a chance to infect hepatocytes, the amount of CSP available in the liver for either direct presentation to CD8 T cells or for cross-presentation involving non-parenchymal cells is limited (Chakravarty et al., 2007). Nevertheless, as *Plasmodium* increases its chances for survival in the liver by preventing the death of its host cell (van de Sand et al., 2005; Sturm et al., 2006; Rennenberg et al., 2010; Graewe et al., 2012; Kaushansky et al., 2013), antigen presentation to CD8 T cells might continue until merozoite replication and merosome release are accomplished. Two groups proposed that *Plasmodium* LS antigen presentation to CD8 effector T cells is restricted to hepatocytes. First, by transplanting bone marrow from C57BL/6 donor mice into lethally irradiated BALB/c recipient mice, Chakravarty et al. (2007) created C57BL/6 → BALB/c (H-2K^b → H-2K^d) bone marrow chimera. Adoptive transfer of CS-TCR Tg CD8 T cells (H-2K^d) into these chimera inhibited LS development, but not in the reverse chimeric mice (Chakravarty et al., 2007). On the basis of these observations, the authors suggested that CD8 effector T cells must recognize CSP-derived antigen on hepatocytes to eliminate *Plasmodium* LS from the liver. However, a role of radio-resistant LSECs and stellate cells (SCs) cannot be excluded, because these cells do not originate from the bone marrow (Bertolino and Bowen, 2015). Further, sessile KCs are radiation-resistant (Klein et al., 2007) and kinetic studies revealed that 85% of the recipient KCs remain in the liver 2 months after standard bone marrow transfer (BMT); even after 1 year, less than half of the original KC population is replaced (Kennedy and Abkowitz, 1997; Seki et al., 2009; Inokuchi et al., 2011). Therefore, to fully reconstitute bone marrow derived cells in the liver, KCs must be depleted with clodronate liposomes prior to irradiation and BMT (Van Rooijen and Sanders, 1996). This strategy could be used to accurately address the question of the role of KCs as APCs for *Plasmodium*-specific effector T cells. In this scenario, KC may activate CD8 effector T cells to release inflammatory cytokines, such as IFN-γ, to eliminate *Plasmodium* parasites within infected hepatocytes. In a separate study involving OT-I cells, it was observed that these T cells significantly inhibited the parasite burden in C57BL/6 mice and bone marrow TAP$^{-/-}$ → C57BL/6 chimeras infected with *P. berghei*-OVA transgenic parasites, but not in C57BL/6 → TAP$^{-/-}$ chimeras and TAP$^{-/-}$ mice infected with the same parasites. Kimura et al. (2013) concluded that infected hepatocytes process and present the OVA epitope via the classical cytosolic MHC I pathway.

Two decades ago, immunoelectron microscopy demonstrated CSP in the cytosol of HepG2 cells harboring *P. berghei* sporozoites (Hügel et al., 1996). In retrospect, we know now that these sporozoites were traversing the hepatoma cells (Mota et al., 2001; Frevert et al., 2005), because they were not enclosed in a parasitophorous vacuole as required for productive infection (Mota et al., 2001). Based on these *in vitro* data, it seems likely

that both traversed and infected hepatocytes contain sporozoite surface antigens in the liver (**Figure 2**). *In vivo* studies, on the other hand, suggest that hepatocytes do not survive sporozoite traversal, but undergo necrosis (Frevert et al., 2005). As a consequence, sporozoite antigens associated with the remains of these disintegrating hepatocytes are likely phagocytosed and could be cross-presented by liver APCs. In contrast, only infected hepatocytes would contain early-late-LS, and eventually merozoite antigens within the PV (Butler et al., 2011). Indeed, *in vitro* studies with primary hepatocytes indicate that both traversed and infected cells are able to process and present antigen to induce IFN-γ secretion in primed CD8 T cells (Bongfen et al., 2007). However, *in vitro* exposure of primary hepatocytes to either WT or cell traversal-deficient Pb-RAS revealed that only infected hepatocytes process and present CSP via the proteasome pathway (Bongfen et al., 2008), which led to the proposal that only infected hepatocytes could induce their own elimination (Balam et al., 2012). Results from *in vivo* experiments in a murine model revealed the capacity of hepatocytes to induce protection by priming naïve T cells and by presenting *Plasmodium* CSP to antigen-specific primed CD8 T cells (Balam et al., 2012). As mice immunized with WT and traversal-deficient sporozoites exhibited similar CD8 T cell responses to CSP, the authors suggested that infected hepatocytes play a dominant role in CD8 T cell priming *in vivo*. This interpretation appears to contrast with the observation that hepatic $CD8\alpha^+$DCs play a critical role in the activation of CD8 effector T cells after immunization with Pb-RAS (Jobe et al., 2009). Although it is entirely possible that different cells act as APCs during CD8 T cell priming in response to infection with attenuated sporozoites, these APCs should express MHC-I:peptide complexes, co-stimulatory signals and produce inflammatory cytokines (Zarling et al., 2013). In addition, priming of CSP-specific responses may also depend on the source of peripheral DCs (Chakravarty et al., 2007; Cockburn et al., 2011). Together with the finding that apoptotic infected hepatocytes can provide antigens to hepatic DCs (Leiriao et al., 2005), and the notion that antigen presentation by hepatocytes tends to have a tolerizing effect (Crispe et al., 2006; Bertolino and Bowen, 2015), these data underscore the need for more work to elucidate the cellular events within the complex architecture of the intact liver with its large number of non-parenchymal cells (Racanelli and Rehermann, 2006; Frevert and Nardin, 2008; Crispe, 2011), some of which exhibit greater APC function than hepatocytes (Bertolino and Bowen, 2015).

Several different scenarios can be envisioned for cell-mediated immunity directed against late-LS antigens in the naïve versus the immunized host. For example, first-time infection of a naïve host with WT sporozoites leads to expression of late-LS antigens only in hepatocytes, the only liver cell type that supports parasite growth and differentiation. Consequently, under experimental conditions that involve adoptive transfer of late-LS antigen-specific CD8 CTLs into first-time infected recipient mice, these CTLs should be able to recognize late-LS antigens exclusively on infected hepatocytes (**Figure 2**). Although most merozoites are successfully released into the blood, some parasite debris and LS remnant bodies are left behind and inflammatory cells infiltrate the exhausted former host cell (Baer et al., 2007a). If some of these late-LS antigens are picked up by liver APC, then CTL responses could be activated that would take effect against subsequent generations of incoming parasites. For example, in mice immunized with parasites under drug cover (Belnoue et al., 2004, 2008; Renia et al., 2013), which have experienced several complete rounds of LS development, late-LS antigens left behind after merosome release are likely internalized and cross-presented by hepatic APCs, in particular LSECs and DCs (**Figure 2**). Similarly, repeated exposure of humans to infected mosquito bites in endemic areas may cause hepatic APCs to cross-present late-LS antigens. Thus, under natural infection conditions, CTLs patrolling the sinusoids might recognize late-LS antigens on LSECs, DCs, and/or KCs in addition to infected hepatocytes. Finally, hepatic APCs may also present late-LS antigens after vaccination with late-stage arrested GAS. We anticipate that advances in longitudinal imaging with sensitive molecular tools will reveal the time of parasite antigen persistence and the fate of GAS-infected host cells.

In conclusion, a few parasites engage with a minute number of hepatic APC in a highly local fashion within a huge organ. Consequently, the fate of the LSECs and KCs that have been touched or traversed by migrating sporozoites *in vivo* remains unknown to date. We are confident that tools can be developed and strategies designed to identify the few APCs at a time when the parasites have already left, be it by imaging or otherwise, for analysis of phenotypical changes in response to infection and immunization.

Contact-Dependent Mechanisms against *Plasmodium* Liver Stage Parasites

The mechanism by which effector cells eliminate *Plasmodium* LS from the liver has been a matter of contention for decades (Doolan and Hoffman, 2000). While *Plasmodium*-specific CTLs can induce apoptosis in target cells by formation of an immunological synapse (Frevert et al., 2008a; Trimnell et al., 2009), followed by contact-mediated cytotoxicity, the situation is more complex in the liver, where infected hepatocytes are hidden behind a layer of sinusoidal cells. Histological liver sections, although suggestive (Rodrigues et al., 1992), are inadequate to confirm CD8 T cell contact with infected hepatocytes and granule-mediated cytotoxicity. Electron microscopy offers the resolution to visualize CD8 T cell extravasation into the space of Disse, formation of an immunological synapse, and chromatin condensation indicating apoptotic target cell death (Frevert et al., 2008a). However, comprehensive analysis of the small number of LS developing in the large liver requires a more efficient method. For example, dynamic *in vivo* imaging has been used successfully to visualize subcellular details on the liver phase of *Plasmodium* including sporozoite invasion, merozoite maturation, and merosome release into the blood (Sturm et al., 2006; Baer et al., 2007a). However, identification of the exact position of effector T cells in the hepatic microenvironment by intravital imaging requires simultaneous labeling of several crucial structural elements: the vascular lumen, the sinusoidal endothelium, the space of Disse, hepatocytes, and the intracellular parasites. With the

recent introduction of a novel imaging technique, intravital reflection recording, it is now possible to monitor the behavior of T cells in the hepatic microvasculature of non-fluorescent mice (Cabrera and Frevert, 2012). Combined with vascular tracers, cellular or molecular markers, and fluorescent reporter mice, intravital reflection recording represents a powerful technique to define the exact location of effector T cells with respect to infected hepatocytes and neighboring non-parenchymal cells, sinusoidal endothelia and KCs.

Three recent studies aimed to identify the mechanism by which CD8 effector T cells kill *Plasmodium* LS in the liver. Using a 70–90% pure preparation of PyCSP TCR-Tg CD8 T cells specific for the *P. yoelii* CSP epitope SYVPSAEQI, Cockburn et al. (2013) showed that CD8 effector T cells cluster around *Plasmodium* LS in the BALB/c mouse liver signaling via G protein-coupled receptors. Both antigen-specific and antigen-unrelated OT-I cells exhibited the same low velocity inside the cluster. Based on this report, PyCSP TCR-Tg cells were proposed to recruit antigen-unrelated OT-I cells to the site of *P. yoelii* LS development (Bayarsaikhan et al., 2015) supporting the argument that all patrolling CD8 T cells will slow down in an area where adhesion molecules are upregulated, because there is no mechanism for recruitment to be antigen specific, only engagement. Surprisingly, however, both the parasite-specific T cells and the OT-I cells moved at slow speed also outside the cluster (Cockburn et al., 2013). Other experimental systems showed that when non-lymphoid tissue resident memory CD8 T cells encounter cognate antigen, they recruit preformed circulating memory CD8 T cells of the same specificity to the site of infection (Schenkel et al., 2013). This specialized feature of tissue resident CD8 T cells allows for an accumulation of a large number of effector CD8 T cells prior to the protracted process re-activation and arrival of antigen-specific cells induced in the secondary lymphoid organs, e.g., the draining LN (Schenkel et al., 2013). Another example suggesting that recruitment of CD8 T cells is strictly antigen-dependent comes from studies of tumor killing by activated CTLs. The tumor antigen-specific CD8 T cells migrate at high velocity (∼10 μm/min) in the periphery of tumors expressing cognate antigen, deeply infiltrate the tumors, and kill tumors in a contact-dependent fashion (Boissonnas et al., 2007). In contrast, the same CTLs do not infiltrate unrelated tumors and neither arrest nor kill tumor cells that fail to present cognate antigen.

One reason for the large size of the PyCSP TCR-Tg CD8 T cell clusters (80 μm diameter) may be that the CD8 T cells received MHCI:SYVPSAEQI signals from sporozoite-traversed cells in addition to sporozoite-infected hepatocytes. This possibility is difficult to evaluate, however, because the exact location of the PyCSP TCR-Tg cells with respect to the sinusoidal walls is not visible in that study (Cockburn et al., 2013). Whether these cells were intra- or extravascular in this experimental setting based on a single CSP-derived peptide remains to be shown. In addition, whether large numbers of effector T cells also accumulate around *Plasmodium* LS in natural infections or after immunization with attenuated sporozoites, in particular in the large human liver, also remains to be shown. Immunization with a viral prime boost regimen against multiple epitopes in the *P. falciparum* TRAP revealed that much lower levels of IFN-γ producing CD8 T cells were required for protection of humans (Ewer et al., 2013) compared to the high CD8 T cell frequencies necessary for protection in mice (Schmidt et al., 2010). According to the authors (Ewer et al., 2013), the significantly longer duration of *P. falciparum* LS development, which lasts about 7 days compared to the 2 days for rodent species, could be a plausible explanation for this difference.

In agreement with the concealed location of hepatocytes in the liver, CD8 T cells appear to use different mechanisms for *Plasmodium* LS elimination *in vitro* and *in vivo*. Similar to classical granule-mediated target cell killing, which is typically triggered within seconds of CTL conjugation (Berke, 1995; Keefe et al., 2005; Pipkin and Lieberman, 2007), *P. yoelii* specific CD8 T cells rapidly induce apoptosis in infected hepatocytes *in vitro* and kill the parasites efficiently using a contact- and perforin-dependent, but IFN-γ independent mechanism (Trimnell et al., 2009). In contrast, *P. yoelii* LS killing *in vivo* is a protracted process that is contact-independent and require multiple CD8 T cells per parasite (Cockburn et al., 2013). Together with the finding that CD8 T cells from *P. yoelii* GAS-immunized mice rely partly on IFN-γ for protection (Trimnell et al., 2009), these intravital observations suggest that cytokines are responsible for the PyCSP TCR-Tg cell-mediated LS killing (Cockburn et al., 2013). Compared to classical granule-mediated cytotoxicity, cytokine-mediated effector mechanisms operate slower and, depending on the local cytokine concentration, may range from mere growth stagnation to parasite death.

Another study showed that OT-I CD8 T cells accumulate in very large numbers around mature LS in the livers of naïve C57BL/6 mice 40–48 h after infection with *P. berghei*-gfpOVA (Kimura et al., 2013). Whether or not these T cell clusters formed around all LS was not reported, but as LS development is highly asynchronous, some LS had likely matured and released merosomes, which is typically followed by infiltration of the remains of the dead hepatocytes with inflammatory cells (Khan and Vanderberg, 1991a; Baer et al., 2007a). Alternatively, the clusters were formed by T cells specific for late-stage antigens that appear in the hepatocyte cytoplasm, in this case OVA (Bayarsaikhan et al., 2015). Depending on the host-parasite combination, mice can also mount inflammatory responses prior to LS maturation. For example, *P. berghei* sporozoites are 2000-fold less infectious to BALB/c mice than to C57BL/6 mice (Scheller et al., 1994). On the other hand, *P. yoelii* is 50–100 times more infectious to C57BL/6 mice than *P. berghei* (Briones et al., 1996). As a result, the *P. berghei* parasite biomass in the C57BL/6 mouse liver is significantly lower than that of *P. yoelii* suggesting that *P. berghei* induces an inflammatory response not only in BALB/c mice, but also in C57BL/6 mice. Granuloma formation in naïve *P. berghei*-infected mouse livers starts at 24 h after infection and is therefore clearly independent of adaptive immune mechanisms. The finding that *P. yoelii* LS develop basically undetected in the mouse until merosome release is accomplished (Khan and Vanderberg, 1991b; Liehl et al., 2014) supports this notion (see caveat below). Taken together, CD8 effector T cells appear to participate in granuloma formation around moribund *P. berghei*-infected hepatocytes (Kimura et al., 2013). The alternative hypothesis, namely that CD8 T cells cluster around healthy LS that successfully develop

in the liver of naïve mice or humans, requires experimental confirmation.

A third study presents novel imaging techniques that proved essential for visualization of the exact location of CD8 effector memory T cells in the *Plasmodium*-infected liver (Cabrera and Frevert, 2012). Intravital reflection recording revealed that immunization of BALB/c mice with radiation-attenuated *P. yoelii* sporozoites (Py-RAS), early-stage genetically attenuated (Py-uis4$^{-/-}$), or late-stage genetically attenuated (Py-fabb/f$^{-/-}$) parasites significantly increased the velocity of CD8 T cells patrolling the hepatic microvasculature compared to naïve mice (Cabrera et al., 2013). After adoptive transfer, CD8 T cells from these immunized donor mice unexpectedly remained immobile in the hepatic microvasculature for at least 3 days, whether or not the recipient mice were infected (Cabrera et al., 2013). The same low velocity was observed after transfer of TCR-tg CD8 T cells specific for the *P. yoelii* CSP$_{280-288}$ epitope (Butler et al., 2010; Schmidt et al., 2011). Instead of migrating with a leading edge and a trailing uropod as in immunized mice, the transferred CD8 T cells were rounded (Cabrera et al., 2013) and met the definition of local confinement (Friedl and Weigelin, 2008). In fact, an 28% pure PyCSP TCR-tg CD8 effector memory T cells exhibited a similarly low average velocity after adoptive transfer as the 70–90% pure PyCSP TCR-Tg CD8 effector T cells mentioned above (Cockburn et al., 2013). Although recipient mice were infected with one million sporozoites, a parasite density that should have facilitated CTL encounters with LS, none of the transferred CD8 T cells, whether of hepatic or splenic origin, approached or made contact with infected hepatocytes under any of the experimental conditions used (Cabrera et al., 2013). Because neither immunized donor mice nor infected recipient mice provided any evidence for CD8 T cell extravasation into the space of Disse, the CD8 effector memory T cells from the immunized mice likely eliminated infected hepatocytes via release of soluble mediators, cytokines and/or chemokines (Frevert and Nacer, 2013; Miller et al., 2014). The finding that adoptive transfer of two million purified CD8 T cells from Pb-uis3$^{-/-}$ immunized mice conferred sterile protection to challenge with 50,000 viable sporozoites supports this notion (Mueller et al., 2007).

In conclusion, while conducted under very different conditions in terms of *Plasmodium* species, CD8 T cell specificity, and time of imaging, common denominators that emerge from these studies are (1) the low velocity of adoptively transferred CD8 T cells, whether antigen-specific or not and whether in proximity to the parasites or not, in the liver of recipient mice, (2) the lack of direct CD8 T cell contact with infected hepatocytes, and (3) the considerably slower LS killing *in vivo* compared to hepatocyte monocultures, where CTLs have direct access infected hepatocytes.

Adoptive Transfer-Associated Phenotypical CD8 T Cell Changes in Other Systems

Much of our current understanding of T cell functions comes from experiments using adoptive transfers of either enriched T cell subsets displaying a certain phenotype or T cells bearing a TCR-Tg for a given specificity. Recent advances in the use of intravital imaging have provided us with a much clearer understanding of T cell functions vis-à-vis the patterns of migratory behavior within the various lymphoid and non-lymphoid organs. The microarchitecture of each organ and the expression of MHC:peptide complexes, chemokines, adhesion molecules and cytokines provide signaling pathways that prompt specific movement and velocity patterns, as well as changes in T cell function. Collectively, these observations suggest a certain plasticity of most T cells is needed mainly to prevent tissue pathology, while simultaneously enhancing T cell effector function against invading pathogens. For example, liver CD8 T cells specific for HBV modulate their IFN-γ production and cytolytic function in an oscillatory and sequential fashion, which culminates in effective reduction of viral load (Isogawa et al., 2005). Th17 cells convert to IFN-γ producing Th1-like cells after adoptive transfer into NOD/SCID recipient mice (Bending et al., 2009; Martin-Orozco et al., 2009). Further, adoptively transferred resting memory CD8 T cells acquire an effector phenotype upon entry into non-lymphoid tissues as indicated by induction of lytic activity and granzyme B expression (Marzo et al., 2007). Some of these observed changes may simply reflect a programmed T cell differentiation, e.g., from resting memory to effector cells, upon reinfection. Changes in the profiles of cytokine production or lytic function may also indicate that anatomical location plays a role in local T cell differentiation.

Cytokine-Mediated Effector Mechanisms against *Plasmodium* Liver Stages

Nonspecific responses to microenvironmental changes could provide an alternative explanation for the finding that antibody-mediated blockage of IFN-γ abrogated protection against *Plasmodium* LS within 2 days (Schofield et al., 1987b; Rodrigues et al., 1991; Weiss et al., 1992), but neither 8 days after CD8 T cell transfer (Chakravarty et al., 2008) nor in immunized mice in the absence of adoptive transfer (Doolan and Hoffman, 2000). Whatever the cause of the immobility of adoptively transferred CD8 effector T cells, this phenomenon is useful to demonstrate that direct contact is not necessary for CTL-mediated protection against *Plasmodium* LS. Blockage of IFN-γ within 2 days after transfer abrogates protection (Schofield et al., 1987b; Rodrigues et al., 1991; Weiss et al., 1992), a result that supports the concept that soluble factors are obligatory for protection when CTLs are unable to approach infected hepatocytes. A role of cytokines, in particular IFN-γ and TNF-α, in parasite killing is clearly documented, both *in vivo* and *in vitro* (Ferreira et al., 1986; Mellouk et al., 1987, 1991; Schofield et al., 1987a; Rodrigues et al., 1991; Weiss et al., 1992; Seguin et al., 1994; Renggli et al., 1997; Doolan and Hoffman, 2000; Jobe et al., 2007; Mueller et al., 2007; Butler et al., 2010). For example, C57BL/6 mice deficient in both perforin and the CD95/CD95L pathway were protected after immunization with Pb-RAS suggesting that parasite-specific CD8 effector T cell-derived cytokines activate mechanisms responsible for the elimination of the intracellular LS (Renggli et al., 1997).

IFN-γ is now considered the central mediator of protection against LS (McCall and Sauerwein, 2010). However, the finding that (1) blockage of IFN-γ does not abolish protection in actively immunized mice (Doolan and Hoffman, 2000), (2) CD8 T cells

from IFN-γ deficient mice protect mice challenge 8 days after transfer (Chakravarty et al., 2008), when the CTLs have likely regained motility, and (3) CD8 T cells protect IFN-γ KO mice against infection (Butler et al., 2010) all suggest that IFN-γ is not the only soluble factor involved. CD8 T cells monitor hepatocytes with small cytoplasmic projections that reach into the space of Disse (Warren et al., 2006), but they appear not to extravasate into the liver tissue or form immunological synapses with hepatocytes *in vivo* (Crispe, 2011). Interestingly, while perforin-dependent cytotoxicity plays an important role in the clearance of virus infections from extrahepatic organs, this mechanism was not involved in the elimination of hepatocytes infected with a non-cytopathic adenovirus from the liver (Kafrouni et al., 2001). Because hepatic CD8 T cells exhibited similar IFN-γ and TNF responses and were able to kill virus-infected cells *in vitro*, this finding was interpreted as a relative resistance of hepatocytes to perforin-mediated cytotoxicity (Kafrouni et al., 2001). Alternatively, however, CD8 T cells may be unable to use contact-dependent cytotoxicity because they cannot exit the hepatic sinusoid (Cabrera et al., 2013; Frevert and Nacer, 2013). Of note, CTLs can form two different types of immunological synapses (1) lytic synapses, used for target cell killing and (2) stimulatory synapses with APCs to induce cytokine secretion (Faroudi et al., 2003; Depoil et al., 2005; Wiedemann et al., 2006). It seems therefore possible that CTLs specific for *Plasmodium* sporozoite proteins recognize—from within the sinusoidal lumen—antigens at the site of parasite entry into the parenchyma, form stimulatory synapses with sinusoidal APC, in particular KCs, secrete IFN-γ in a multidirectional fashion (Kupfer et al., 1991; Huse et al., 2006; Sanderson et al., 2012), and trigger the secretion of additional cytokines such as IL-6 and IL-12 that then contribute to LS killing (Nüssler et al., 1991; Pied et al., 1991, 1992; Vreden et al., 1992; Belnoue et al., 2004). Synapse formation with sinusoidal APCs could involve cytotoxic granule proteins (Doolan and Hoffman, 2000; Butler et al., 2010), which might explain the requirement for perforin in protection of vaccinated humans (Seder et al., 2013) and mice (Butler et al., 2010). This scenario could also pertain to CTLs that recognize late-LS antigens on cross-presenting APCs.

How can CTL-derived cytokines reach infected hepatocytes (**Figure 1**)? Considering the high sinusoid-to-lymph filtration rate (Greenway and Lautt, 1970; Laine et al., 1979; Henriksen et al., 1984), the enhanced pressure gradients created by leukocytes moving through the sinusoidal lumen (Wisse et al., 1983), and the highly anastomosed sinusoidal microvasculature, CD8 T cells could conceivably exploit both the anterograde blood flow and the retrograde lymph flow to take control of a large portion of the liver lobule—without extravasation and granule-mediated cytotoxicity (Frevert and Nacer, 2013). This hypothesis is supported by the finding that intravenous IFN-γ inoculation reduced the *P. berghei* liver burden independently of the sporozoite challenge dose (Ferreira et al., 1986). Further, CD8 T cells protect against various viral liver infections and *in vivo* data indicate that virus replication is blocked without CD8 T cell extravasation and in the absence of hepatocyte death (Guidotti et al., 1999; Kafrouni et al., 2001; Guidotti, 2002; Crispe, 2011). Consistent with the notion that CTL-derived cytokines traverse the sieve plates of the sinusoidal endothelia and disseminate via the lymphatic conduits of the liver, adoptively transferred-specific CTLs acted against infected hepatocytes, but had no effect on not infected renal tubule or choroid plexus epithelia (Ando et al., 1994a), both of which are shielded from the bloodstream by a continuous layer of non-fenestrated endothelia.

While the crucial role of perforin expressing CD8 T cells in protection against *Plasmodium* LS is now well documented (Renggli et al., 1997; Doolan and Hoffman, 2000; Trimnell et al., 2009; Butler et al., 2010), the exact mechanism by which this cytotoxic granule protein contributes to the elimination of infected hepatocytes *in vivo* is unclear. CTLs typically release perforin into an immunological synapse (Lieberman, 2003; Pipkin and Lieberman, 2007; Lopez et al., 2013). Unlike Fas/FasL-mediated cytotoxicity, which requires only a few molecules on the target cell and which could therefore potentially be accomplished via small cytoplasmic projections such as TEHLI (Warren et al., 2006), immunological synapse formation involves a much larger area of contact between CTL and target cell (Dustin, 2005; Pipkin and Lieberman, 2007). For this reason, establishment of synapse with the basolateral hepatocyte membrane likely requires extravasation of the CTL. However, CTLs conjugating with hepatocytes from within the space of Disse have not been documented to date, neither in malaria nor in other liver infections. While CTLs specific for the were initially thought to exert a direct cytopathic effect on infected hepatocytes *in vivo* (Ando et al., 1994b), the use of a transgenic mouse model of hepatitis B infection revealed that adoptively transferred virus-specific CTLs abolish gene expression and replication of the virus in the liver in the absence of hepatocyte death (Guidotti and Chisari, 1996; Guidotti et al., 1999; Kafrouni et al., 2001; Wuensch et al., 2006; Giannandrea et al., 2009). Thus, rather than reflecting secretion of cytotoxic molecules and induction of apoptosis, which requires immunological synapse formation with the target cell, the requirement of perforin expression for protection may indicate CD8 T cell maturation in the liver. This model would also link perforin expression to IFN-γ secretion. It is now established that CTLs eliminate the virus by secretion of IFN-γ and TNF-α and that this cytokine-mediated, non-cytopathic mechanism represents a survival strategy of the host to control massive viral infections of vital organs such as the liver (Guidotti, 2002). Clearly, widespread takeover of the hepatocyte machinery by a hepatotropic virus differs from the very focal intracellular development of a large protozoan parasite such as *Plasmodium*. However, all available evidence suggests that both infectious agents can be eliminated via cytokines, i.e., in the absence of CTL extravasation and immunological synapse formation with antigen-presenting hepatocytes. Taken together, CTLs appear to be more likely to engage in stimulatory immunological synapse formation with sinusoidal APCs rather than establishing lytic synapses with infected hepatocytes. However, this aspect of effector CD8 T cell activity will need to be investigated further. Confocal or multi-photon microscopy, combined with cellular and molecular tools for intravital imaging of the hepatic microvasculature (Cabrera and Frevert, 2012), can provide the necessary spatio-temporal resolution to elucidate which liver cell types interact with which *Plasmodium* antigen-specific CD8 T cells and whether discrete epitopes of the still

unknown liver-stage specific antigens require specific hepatocyte and/or APC contacts.

Much is known about the cytotoxic mechanisms associated with immunological synapse formation *in vitro* (Russell and Ley, 2002; Catalfamo and Henkart, 2003; Lieberman, 2003; Trambas and Griffiths, 2003). For example, upon *in vitro* conjugation of human-specific CD8 T cells with cognate target cells, perforin rapidly accumulates at the immunological synapse where it promotes cytotoxicity (Makedonas et al., 2009). Although cellular cytotoxicity against congenic hepatocytes has been studied extensively in various virus hepatitis models (Guidotti et al., 1999; Kafrouni et al., 2001; Wuensch et al., 2006; Giannandrea et al., 2009), CTL conjugation with target hepatocytes in the intact liver has not been documented to our knowledge, neither ultrastructurally nor with the combined use of modern fluorescent tools and state-of-the-art imaging techniques. Unlike the continuous endothelium of other organs, which prevents ready access of soluble mediators to the parenchyma, one of the essential functions of the fenestrated sinusoidal endothelium of the liver is to allow unrestricted passage of soluble and small corpuscular substances to hepatocytes for detoxification and a plethora of anabolic and catabolic processes. Further, the liver is unique in that every hepatocyte is in direct contact with sinusoidal endothelia thus allowing screening by patrolling immune cells and exposure to their secreted cytokines. This is in stark contrast to other organs, which require immune cell infiltration for elimination of infected, malignant, or otherwise abnormal target cells. This applies in particular to organs with a tight blood barrier such as brain or testes or tissues comprised of a dense parenchyma with a relatively low degree of vascularization such as joint or cartilage.

In conclusion, it appears that CD8 effector T cells recognize the site of sporozoite entry into the liver, either by detecting antigen presented on hepatic local APC or by screening hepatocytes via TEHLI. Being unable to extravasate and kill via classical granule-mediated cytotoxicity, CTLs appear to eliminate *Plasmodium* LS via secretion of cytokines, in particular IFN-γ.

Cytokine-Mediated Control of Parasite Growth

Cytokines alone can clearly confer protection against *Plasmodium* LS, further supporting the notion that CTL-mediated cytotoxicity does not require contact with infected hepatocytes. *In vitro* studies document that *P. yoelii* and *P. falciparum* infected hepatocytes can be eliminated by direct exposure to IFN-γ (Ferreira et al., 1986; Mellouk et al., 1991, 1994) and intravenous IFN-γ inoculation reduced liver burden in a *P. berghei* model (Ferreira et al., 1986). Antibodies against CD8 T cells or IFN-γ abrogated protection (Ferreira et al., 1986), suggesting that CD8 T cells either secrete sufficient IFN-γ or produce other cytokines that elicit IFN-γ secretion from other cellular sources (Schofield et al., 1987b). Clearly, IFN-γ mediated protection is dependent on iNOS-mediated production in infected hepatocytes (Mellouk et al., 1991, 1994; Nussler et al., 1993; Seguin et al., 1994; Klotz et al., 1995). However, despite the essential role of IFN-γ in iNOS upregulation in infected hepatocytes (Nussler et al., 1993; Seguin et al., 1994; Klotz et al., 1995), the exact mode of operation of this cytokine *in vivo* is not known (Doolan and Hoffman, 2000; Overstreet et al., 2008; Butler et al., 2010). Interestingly, IFN-γ induced iNOS activation was shown to regulate the replication of various obligatory intracellular microorganisms including *Toxoplasma* (Pfefferkorn, 1984, 1986; Pfefferkorn et al., 1986; Takacs et al., 2012), *Trypanosoma cruzi* (Silva et al., 2003), *Leishmania* (Bogdan et al., 2000), *Mycobacterium* (Herbst et al., 2011), and *Chlamydia* (Zhang et al., 2012). An IFN-γ based regulatory mechanism could explain why challenge of immunized mice results not only in a smaller number of *Plasmodium* LS (as expected for a direct killing mechanism), but also in a reduced LS size (Cabrera et al., 2013). In agreement with this finding, the effect of IFN-γ induced iNOS activation may range from a minor reduction in LS growth to complete cure of the host cell depending on the local CD8 T cell density, the level of IFN-γ secretion, the position of the T cells and the LS relative to the liver lobule, and the distance between them.

Thus, it appears that CTLs exert an IFN-γ mediated growth-regulatory effect on *Plasmodium* LS rather than acting in a parasitocidal fashion. To be efficient, this mechanism requires an organ with sinusoidal endothelia, a large *trans*-endothelial lymph filtration rate, and a high microvascular density that exposes every parenchymal cell to CTL-derived cytokines—properties uniquely combined in the liver. Considering that a similar mechanism of cellular immunity has been proposed to operate against viral infections of the liver (Guidotti, 2002; Guidotti and Iannacone, 2013), IFN-γ induced iNOS activation in hepatocytes (Chen et al., 2003) may have evolved as an efficient strategy to control hepatotropic microbes while promoting hepatocyte survival and preserving the function of this essential organ.

Contribution of Cellular Effector Mechanisms to Protection of Naïve Versus Immune Hosts

Plasmodium has developed multiple parallel strategies to evade detection in the liver: the choice of a tolerogenic environment for its initial round of replication, the limited infection of a minute number of hepatocytes in the huge liver, and the change of protein expression from sporozoite to late-LS antigens. For these reasons, it has been suggested that finding and killing all LS during the brief period of LS development represents the predominant challenge the CD8 T cell response is facing (Bertolino and Bowen, 2015). Indeed, this reasoning clearly applies to the most severe malaria cases, namely to first infections of naïve hosts that result in a fatal outcome after only one round of LS development. In these cases, effective CTL-mediated interruption of LS development would prevent the clinically symptomatic blood infection in young *P. falciparum*-infected children and also allow susceptible mice to survive experimental infection with large numbers of lethal *P. yoelii* XL or *P. berghei* ANKA sporozoites.

After repeated infections, for example with non-lethal parasite species/strains or with sub-lethal inoculation doses, protection increasingly relies on the humoral arm of immunity. Challenge of immunized mice by mosquito bite likely allows antibodies to immobilize most sporozoites in the skin (Vanderberg and Frevert, 2004). Should a few parasites manage to escape from the skin,

they are likely opsonized in the bloodstream and phagocytosed by KCs. In contrast to liver infection of naïve mice, which is very fast and highly efficient (Shin et al., 1982; Conteh et al., 2010), studies with mice that had been vaccinated with *P. yoelii* RAS or GAS showed that the majority of intravenously inoculated sporozoites are unable to enter the liver (Cabrera et al., 2013). Thus, LS development in the immune host is impeded and extremely scarce, which drastically reduces the probability of parasite recognition by *Plasmodium*-specific CD8 T cells, in particular in the large human liver. These considerations emphasize the crucial role of antibodies in protection of the previously exposed host against recurring sporozoite infections (Vanderberg et al., 2007; Vanderberg, 2014).

Contribution of Innate Immune Responses to Protection against *Plasmodium* LS

Recent studies demonstrate a significant impact of the innate immune response on the survival of *Plasmodium* in the murine liver. Both *P. berghei* and *P. yoelii* infected hepatocytes were shown to sense *Plasmodium* LS and induce a type I IFN response that is propagated by hepatocytes in an interferon-a/b receptor dependent fashion and reduces the parasite burden in the liver (Liehl et al., 2014; Miller et al., 2014). In the *P. yoelii* model, primary infection with a late-stage GAP increased the number of IFN-γ secreting CD1d-restricted NKT cells in the liver thus implicating IFN-γ as a crucial innate factor in controlling secondary infection with WT XNL parasites (Miller et al., 2014). Although lymphocytes were required for the innate suppression of secondary liver infections, CD8 T cells, CD4 T cells, and NK cells, the latter of which constituted the largest subset of IFN-γ secreting cells, appeared to be dispensable as effector cells. Type I IFN signaling was responsible for the recruitment of both CD8 T cells and $CD49b^{+}CD3^{+}$ NKT cells to the liver, but only NKT cells reduced the LS burden significantly (Miller et al., 2014), which is in line with earlier work (Gonzalez-Aseguinolaza et al., 2000).

Certain differences between the two murine models are noteworthy. For example, the innate immune response to *P. berghei* infection involved the cytosolic pattern recognition receptor MDA5, the adaptor molecule for cytosolic RNA sensors, MAVS, suggesting parasite RNA sensing, and the transcription factors IRF3 and IRF7 (Liehl et al., 2014). In the *P. yoelii* model, IRF3, but not IRF7, was crucial for the induction of the type I IFN response and neither MDA5 nor MAVS contributed to a functional innate response against secondary infection (Miller et al., 2014). Further, *P. berghei* infection activated a considerably stronger innate immune response compared to *P. yoelii*, and interferon-stimulated gene expression was upregulated at 36 h after infection with WT *P. berghei*, but only at 42 h after infection with WT *P. yoelii* (Liehl et al., 2014). In agreement with earlier histological work (Khan and Vanderberg, 1991b), immune cell infiltration occurred midway through completion of LS development for *P. berghei*, but only at the time of merosome formation for *P. yoelii* (Miller et al., 2014). Finally, IFN-γ signaling pathways were upregulated as early as 24 h after infection with late-stage arrested *P. yoelii* parasites (Miller et al., 2014), while early stage-arrested *P. berghei* parasites (GAS or RAS) failed to induce such a response (Liehl et al., 2014).

Thus, many questions remain. For example, is the intense inflammation and early granuloma formation associated with *P. berghei* infection of the murine liver the cause or the consequence of the type I IFN response? Is parasite death required for RNA sensing and initiation of the innate response? Can LS sensing be improved by parasite attenuation? Addressing these issues may provide crucial clues as to which events in the life cycle reveal the presence of the parasite to the host. Perhaps most relevant for malaria endemic areas: does the human host indeed sense intracellular LS if infected with a well adapted *Plasmodium* species?

Outlook and Future Directions

We hope to have highlighted the vast number of open questions associated with the local cellular events leading to protection against *Plasmodium* LS. While much progress has been made on the biology and nutritional requirements of *Plasmodium* LS (Luder et al., 2009; Vaughan et al., 2009; Rankin et al., 2010; Labaied et al., 2011; Deschermeier et al., 2012; Graewe et al., 2012; Itoe et al., 2014), the liver is still a black box in terms of effector mechanisms mediating the detection and elimination of infected hepatocytes. Recent live attenuated malaria vaccination trials in humans and non-human primates as well as work in mice clearly demonstrate the requirement for protection of large quantities of IFN-γ producing CD8, CD4, and γδ T cells as well as IFN-γ secreting NK and NKT cells in the liver (Epstein et al., 2011; Teirlinck et al., 2011; Seder et al., 2013). Neither location nor behavior in the liver of any of these cells nor their exact modes of action during adaptive and innate immunity has been determined, however. Elucidation of the protective cell-mediated mechanisms elicited by attenuated sporozoite vaccines will aid in the development of a cheap, safe, and easy-to-administer synthetic vaccine that matches this gold standard. As hundreds of millions of people living in endemic areas are in desperate need for protection against malaria, future vaccination protocols should be selected based on their capacity to boost the number of CD8 T cells. Indeed, a multi-epitope malaria vaccine that significantly increases the magnitude of T cell induction has already been developed (Ewer et al., 2013). This so-called ME-TRAP vaccine contains full-length *Plasmodium falciparum* TRAP fused to ME, a string of 20 malarial T and B cell epitopes (McConkey et al., 2003).

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