Induction and maintenance of protective CD8+ T cells against malaria liver stages: implications for vaccine development

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CD8+ T cells against malaria liver stages represent a major protective immune mechanism against infection. Following induction in the peripheral lymph nodes by dendritic cells (DCs), these CD8+ T cells migrate to the liver and eliminate parasite infected hepatocytes. The processing and presentation of sporozoite antigen requires TAP mediated transport of major histocompatibility complex class I epitopes to the endoplasmic reticulum. Importantly, in DCs this process is also dependent on endosome-mediated cross presentation while this mechanism is not required for epitope presentation on hepatocytes. Protective CD8+ T cell responses are strongly dependent on the presence of CD4+ T cells and the capacity of sporozoite antigen to persist for a prolonged period of time. While human trials with subunit vaccines capable of inducing antibodies and CD4+ T cell responses have yielded encouraging results, an effective anti-malaria vaccine will likely require vaccine constructs designed to induce protective CD8+ T cells against malaria liver stages.

Key words: malaria - CD8+ T cell - vaccine - sporozoites

Given the enormous disease burden of malaria, effective control strategies, such as the development of a protective vaccine, are urgently needed. The liver stage of malaria is an attractive vaccine target because a small number of parasites productively invade the liver to establish infection in hepatocytes which express class I major histocompatibility complex (MHC) and, therefore, are ideal targets of protective immunity mediated by CD8+ T cells. This review highlights recent studies conducted in our laboratory, and by colleagues in the field, that have expanded our knowledge of protective CD8+ T cell responses against liver stages. In addition, we will discuss the implications of these studies on the development of an effective malaria vaccine and will critically examine the advantages and disadvantages of existing vaccine strategies.

Priming of CD8+ T cells - Protective immunity to rodent malaria parasites was first achieved in mice by immunization with radiation attenuated sporozoites (Nussenzweig et al. 1967). These studies were later extended to humans using irradiated mosquitoes infected with *Plasmodium falciparum* sporozoites and have provided the rationale for the development of an irradiated sporozoite vaccine (Clyde et al. 1973). In experimental models, protection against live sporozoite challenge was shown to require antigen-specific CD8+ T cells as in

vivo depletion of CD8+ T cells completely abrogated sterile immunity in mice infected with rodent malaria parasites (Schofield et al. 1987, Weiss et al. 1988). Most importantly, we have shown that CD8+ cells against defined epitopes of *Plasmodium berghei* and *Plasmodium yoelii* circumsporozoite (CS) protein strongly inhibited the development of liver stage parasites (Romero et al. 1989, Rodrigues et al. 1991). Subsequently, transgenic mice expressing a T-cell receptor (TCR) specific for the MHC l restricted epitope of the CS of *P. yoelii* have been developed and used to characterize the induction of effector CD8+ T cells (Sano et al. 2001, Carvalho et al. 2002). Using this system, we demonstrated that malaria-specific CD8+ T cells are primed in the skin-draining lymph nodes of mice (Chakravarty et al. 2007). Following immunization by irradiated *P. yoelii* infected mosquitoes, interferon gamma (IFN-γ) producing CD8+ T cells were detected in the ear-draining lymph nodes as early as 48 h after immunization; however, significant responses in the spleen, liver and liver-draining lymph nodes were not observed until 72 h post-immunization. A significant reduction in the anti-sporozoite CD8+ T cell response was observed in animals that had their draining lymph nodes removed prior to sporozoite immunization. Together, these results demonstrate a critical role for the skin-draining lymph nodes in the priming of CD8+ T cells protective against pre-erythrocytic stage parasites, but do not exclude a possible contribution of liver associated antigen presenting cells in the presentation of parasite antigens. A subset of liver resident dendritic cells (DCs), CD8α+CD11c+, was shown to activate CD8+ T cells, as determined by the acquisition of the CD44hiCD45RBlo phenotype and IFN-γ production in vitro, following prime-boost intravenous immunizations with irradiated *P. berghei* sporozoites (Jobe et al. 2009).

It is well established that DCs play a critical role in the priming of *Plasmodium* specific CD8^{+} T cells (Jung et al. 2002, Plebanski et al. 2005, Chakravarty et al. 2007, Jobe et al. 2009). Furthermore, several lines of evidence suggest a vital role for cross presentation in the priming of CD8^{+} T cells by DCs. Pre-treatment with Toll-like receptor (TLR) ligands can cause pre-maturation of DCs and subsequently inhibit cross presentation to CD8^{+} T cells (Radhakrishnan et al. 2005, Wilson et al. 2006). Accordingly, activation of CD8^{+} T cells was significantly reduced in animals that had been treated with CpG (a TLR-9 ligand) prior to immunization with irradiated *P. yoelii* sporozoites. In a recent study, we expanded on the requirement for cross presentation using two different in vivo methodologies and a mutant transgenic parasite. To study the in vivo processing requirements of CS by DCs and hepatocytes, our laboratory generated *P. berghei* parasites expressing a mutant CS protein with the H2-K[b] SIINFEKL epitope (*P. berghei CS[SM]*). Studies with this parasite and TCR transgenic CD8^{+} T cell specific for this H-2K[b] epitope (Hogquist et al. 1994) revealed a requirement for the TAP transporter and the endosome-to-cytosol pathway in antigen presentation by DCs. In this study, the induction phase of CD8^{+} T cells was evaluated in mice defective in endosomal function and cross-presentation (3d) (Tabeta et al. 2006) and also in mice depleted of cross-presenting DCs following in vivo cytochrome *c* (cyt *c*) treatment (Lin et al. 2008, Farrand et al. 2009, Qiu et al. 2009). Significant reductions in CD8^{+} T cell priming were observed in both 3d mice and cyt *c* treated mice (Cockburn et al. 2011).

Cross presentation is greatly enhanced by microbial molecular patterns, especially TLR ligands (Beutler et al. 2003, Kopp & Medzhitov 2003, Hemmi & Akira 2005, Burgdorf et al. 2008). Following receptor recognition of microbial moieties, DCs undergo maturation and migrate to the secondary lymphoid organs where they present antigen to T cells. DC maturation, characterized by high levels of MHC and T cell costimulatory molecules, is critical for the optimal priming of naïve T cells to pathogen-derived antigen (Janeway & Medzhitov 2002, Wilson & Villadangos 2005, Steinman & Hemmi 2006, López-Bravo & Ardavín 2008). To date, a sporozoite-derived TLR ligand has not been identified, although TLRs have been shown to recognize different components of malaria blood stages of *P. falciparum* and *P. berghei* (Pichyangkul et al. 2004, Coban et al. 2005, Krishnegowda et al. 2005, Parroche et al. 2007, Couper et al. 2010, Wu et al. 2010). In addition to TLRs, intracellular pathogens and "danger signals" are sensed by cytosolic Nod-like receptors and result in the formation of the inflammasome, a multi-protein complex responsible for the processing of the pro-inflammatory cytokines interleukin (IL)-1β and IL-18 (Fritz et al. 2006, Martinon et al. 2007, Lamkanfi & Dixit 2009, Schroder & Tschopp 2010). Prior to establishing infection in the liver, sporozoites must traverse several cells and thus may conceivably trigger an innate immune response via these intracellular receptors. An understanding of the innate immune signaling pathways activated against sporozoite antigens will provide critical insights into the induction of protective CD8^{+} T cell responses and may influence the selection of an effective vaccine adjuvant.

Another major question in the priming of anti-sporozoite CD8^{+} T cell responses is the precise site of antigen capture by DCs and its delivery to the skin-draining lymph nodes. In 2006, Amino et al. (2006) performed a quantitative analysis of sporozoite movement in the skin using green fluorescent protein-tagged sporozoites. Intriguingly, 25% of inoculated sporozoites were found to associate with DCs in the lymph nodes. The majority of intradermally inoculated sporozoites resides in the skin for over 1 h and exit the skin at a slow trickle (Sinnis & Coppi 2007, Yamauchi et al. 2007). These studies indicate that sporozoite migration to the draining lymph nodes, in addition to parasites deposited in the skin, can provide antigens to the lymph nodes draining the site of inoculation. This finding raises the following questions: how and where do DCs acquire sporozoite antigen? One possibility is that sporozoite antigen is acquired in the dermis by skin-resident DCs that then migrate to the lymph nodes and present antigen directly to naive CD8^{+} T cells. At least three distinct subsets of skin-resident migratory DCs have been characterized: Langerhans cells, dermal DCs, and langerin^{+}CD103^{+} dermal DCs (Heath & Carbone 2009). The recent identification of langerin^{+}CD103^{+} dermal DCs, a subset of migratory DCs that plays a key role in cross-presenting viral and self antigens (Bursch et al. 2007, Ginhoux et al. 2007, Poulin et al. 2007, Bedoui et al. 2009), is especially intriguing given the requirement for cross-presentation in the priming of anti-sporozoite CD8^{+} T cells. Alternatively, dermal DCs could transfer skin-derived antigen to lymph node resident DCs for CD8^{+} T cell priming as shown in studies using skin infection with herpes simplex virus (Allan et al. 2006). Finally, it is also possible that CD8^{+} T cell priming does not require skin-derived DCs, but instead, requires direct processing and presentation of sporozoite antigen by lymph-node resident DCs. Determination of the site of antigen capture and the identification of the DC subset(s) responsible for inducing protective CD8^{+} T cell responses are important questions for malaria research.

Antigen recognition by CD8^{+} T cells in the liver and effector mechanisms - Following priming in the skin-draining lymph nodes, activated CD8^{+} T cells migrate to the liver where they recognize antigen presented by hepatocytes and eliminate the infected cell (Chakravarty et al. 2007). A proteasome-dependent pathway was shown to be required for the in vitro processing of *P. berghei* CS by infected and traversed mouse hepatocytes (Bongfen et al. 2007, 2008). Our studies using bone marrow chimeras revealed a requirement for direct peptide recognition between effector CD8^{+} T cells and host parenchymal liver cells as memory CD8^{+} T cells were unable to eliminate infected hepatocytes bearing a non-cognate MHC. Importantly, antigen presentation by cells of the hematopoietic lineage was not required for the effector function of CD8^{+} T cells (Chakravarty et al. 2007). Using the transgenic *P. berghei* parasite expressing SIINFEKL, the H-2K[b] restricted epitope (*P. berghei CS[SM]*)

mentioned above (Cockburn et al. 2011) and genetically deficient mice of the C57Bl/6 background we were able to gain further insight into the antigen presentation pathway in hepatocytes. We evaluated the requirement for the TAP dependent pathway for antigen presentation in hepatocytes by transferring activated SIINFEKL specific CD8^{+} T cells to TAP-1 deficient animals. Effector CD8^{+} T cells were unable to inhibit parasite development in the livers of TAP-1 deficient animals. In contrast, effector cells efficiently eliminated liver stages in mice deficient in endosome function and cross-presentation (3d and cyt *c* treated mice). Therefore, endosomes are not required for the presentation of sporozoite antigen in infected hepatocytes. Additional studies also indicated that the conserved *Plasmodium* export element and vacuolar translocation signal, which have been shown to be required for the export of *Plasmodium* proteins into the erythrocyte cytosol (Hiller et al. 2004, Marti et al. 2004), may not be required for CS antigen presentation to CD8^{+} T cells (Cockburn et al. 2011). Further investigation of the mechanism by which CS enters the cytosol of infected hepatocytes is critical for a fundamental understanding of the antigen presentation requirements for CD8^{+} T cell mediated elimination of liver stages.

To date, the effector mechanisms used by CD8^{+} T cells to eliminate liver stage parasites are not clearly defined and are likely to be redundant. The two main cytotoxic mechanisms of CD8^{+} T cells, release of perforin/granzyme and Fas/FasL interactions, are dispensable for parasite elimination. Mice deficient in these two mechanisms (Renggli et al. 1997) as well as CD8^{+} T cells lacking one or both mechanisms (Morrot & Zavala 2004) were still able to eliminate *Plasmodium* liver stages. In addition, a recent study indicates a *Plasmodium*-species specific difference in the effector mechanisms used by memory CD8^{+} T cells to eliminate liver stage parasites. In particular, protection against *P. berghei* and *P. yoelii* liver stage parasites was dependent on IFN-γ and tumor necrosis factor alpha (TNF-α); however, perforin was also required to confer protection against *P. yoelii* (Butler et al. 2010). Other studies using genetically attenuated *P. yoelii* parasites revealed a partial requirement for perforin as well as IFN-γ for protective immunity (Trimnell et al. 2009). These studies, along with several others (Weiss et al. 1992, Seguin et al. 1994, Doolan & Hoffman 2000, Mueller et al. 2007), demonstrate a critical role for IFN-γ in CD8^{+} T cell mediated protection. However, the authors base their conclusions on the global ablation of IFN-γ using neutralizing antibodies or genetic knockouts that are severely affected in other aspects of the immune response downstream of IFN-γ deficiency. Our studies determined that despite systemic inhibition of IFN-γ and TNF-α, mice were still protected against live *P. yoelii* sporozoites (Rodrigues et al. 1991). Similar results were obtained using adoptive transfer of IFN-γ deficient CS-specific CD8^{+} T cells (Chakravarty et al. 2008). In summary, the protective mechanisms of CD8^{+} T cell mediated immunity have yet to be precisely defined and may differ between model systems and the genetic background of the host.

Generation of CD8^{+} T cell memory population: role of CD4^{+} T cells and persisting antigen - Sporozoite development in the liver is brief, lasting 48 h in rodent malaria infections, and precedes the time required for the infiltration of memory CD8^{+} T cells. It seems clear that memory T cell populations residing in the liver of the immunized host provide the first line of defence against subsequent infection and are therefore the key players of the recall response. Over the past few years, our laboratory has determined critical factors in the generation of a robust, stable memory population. In particular, CD4^{+} T cell help was shown to be required for CD8^{+} T cell memory responses against malaria (Carvalho et al. 2002, Overstreet et al. 2011). CD4^{+} T cell depletion or treatment with IL-4 neutralizing antibodies prior to irradiated sporozoite immunization did not impair CD8^{+} T cell expansion, but resulted in premature contraction of the effector pool (Carvalho et al. 2002, Morrot et al. 2005). Therefore, CD4^{+} T cell help appears to be important for maximal clonal expansion of CD8^{+} T cells. Previous reports in other systems (Janssen et al. 2003, Shedlock & Shen 2003, Sun & Bevan 2003) suggest that an absence of CD4^{+} T cell help contributes to defective cytokine production, killing and re-expansion of memory CD8^{+} T cells. We tested whether the "helpless" CD8^{+} T cells generated by irradiated sporozoite immunization also have defective functional properties. Surprisingly, although the memory size of the "helpless" CD8^{+} T cells was much smaller, the functionality of "helpless" CD8^{+} T cells was not impaired, as the production of IFN-γ, TNF-α and IL-2, as well as cytotoxic degranulation, were similar between "helped" and "helpless" CD8^{+} T cells (Overstreet et al. 2011). This implied that CD4^{+} T cells, while being critical to achieve a large CD8^{+} T cell response, play a minor role, if any, in the development of the functional properties of these cells. Although these "helpless" CD8^{+} T cells were functional, they failed to protect the host from live parasite challenge because of their low numbers. This finding is consistent with recent reports indicating that a large number of circulating anti-malaria T cells is necessary for sterile immunity (Schmidt et al. 2008).

In addition to CD4^{+} T cell help, prolonged antigen presentation is also crucial for maximal expansion of effector T cells. We demonstrated that continuous antigen presentation occurs, up to two months after immunization with irradiated sporozoites (Cockburn et al. 2010). This observation is quite striking considering that irradiated sporozoites are not able to undergo proliferation, and they are not known to differentiate beyond early liver stages (Silvie et al. 2002). Apparently, the parasite antigen does not persist as other forms of parasite or exo-erythrocytic remnants because treatment with primaquine to eliminate early liver stage parasites has no effect on continuous antigen presentation. We also determined that professional antigen presenting cells are responsible for trapping antigens, although the identity of the cell types involved in presenting persisting antigens is unclear and remains an area of further investigation. Persisting antigen may be required for renewing and maintaining the memory CD8^{+} T cell population as naïve cells, such as recent thymic emigrants,

can be primed by persisting antigens. It is important to highlight that this prolonged antigen presentation does not induce CD8$^+$ T cell exhaustion as described in some chronic viral infection models (Klenerman & Hill 2005, Shin & Wherry 2007). On the contrary, prolonged antigen presentation is fully capable of inducing effector T cell differentiation. Given that optimal development of protective immunity appears to require prolonged antigen persistence, this result has direct implications for immune responses in endemic areas where people are exposed to sporozoite antigen on a regular basis.

Implications for the development of pre-erythrocytic vaccines - It is well established that immunization with irradiated sporozoites, in both rodent models and limited human studies, remains the most effective malaria vaccine. However, preparing sporozoites for immunization is a difficult task and a very labor-intensive process. More importantly, all procedures leading to sporozoite purification must ensure the full viability of the sporozoite preparation as dead parasites do not induce effector CD8$^+$ T cell responses (Hafalla et al. 2006). These technical limitations have favoured the development of subunit vaccines composed of protective malaria antigens such as CS. CS-based subunit vaccines can be formulated as the following: synthetic peptides coupled to carrier proteins, synthetic peptide polymers containing the B and/or T cell epitope of CS, DNA constructs and recombinant proteins.

One of the simplest subunit vaccine concepts is peptide-based vaccines. When mice were immunized with the SYVPSAEQI peptide (the CD8$^+$ T cell epitope in CS of *P. yoelii*) alone, the CD8$^+$ T cells strongly proliferated, indicating that the peptide itself is strongly immunogenic. However, the expanded CD8$^+$ T cells did not survive (Overstreet et al. 2010). In all likelihood, certain innate signals required for the induction and development of effector CD8$^+$ T cells are likely absent in peptide-based vaccines. In recent studies we evaluated whether the administration of TLR agonists can rescue the widespread cell death observed after initial priming. Interestingly, CpG is capable of enhancing the magnitude of CD8$^+$ T cell priming, yet CpG treatment did not enhance the survival of CD8$^+$ T cells. In addition, we saw a greater effector population 10 days after peptide with CpG immunization in B cell deficient mice, which suggests that B cells have an inhibitory role in peptide immunization (Overstreet et al. 2010). Indeed, the suppressive role of B cells, through the up-regulation of IL-10 and TGF-β, has been suggested in other systems (Parekh et al. 2003, Lenert et al. 2005). The exact mechanisms by which B cells regulate CD8$^+$ T cell priming and survival after peptide immunization remain to be elucidated.

Another example of a CS-based subunit vaccine is the RTS,S vaccine: a subunit vaccine composed of the repeat domain and C-terminal flanking regions (amino acids 207-395) of the *P. falciparum* CS expressed in the hepatitis B virus-like particle (VLP) (Cohen et al. 2010). When administered with adjuvant, the protection provided by the RTS,S vaccine only achieved about 30-50% efficacy at best. The protective mechanisms mediated by RTS,S appear to be antibody-dependent; however, IFN-γ secreting CD4$^+$ T cells may also contribute to protection (reviewed in Good & Doolan 2010). Moreover, the RTS,S vaccine fails to induce significant CD8$^+$ T cell responses (Kester et al. 2009), possibly due to the fact that VLPs are known to be poor primers of CD8$^+$ T cells.

It has been suggested that protection depends on the size of the memory CD8$^+$ T cell pool (Schmidt et al. 2008). Expansion of existing memory populations may therefore enhance the protective immunity conferred by CD8$^+$ T cells. Though subunit vaccines have achieved modest success, they remain a valid vaccine option if their effectiveness can be enhanced. In fact, we have demonstrated that CD8$^+$ T cell mediated protection against sporozoite challenge could be achieved with recombinant viral vectors using heterologous prime-boost regimens (Li et al. 1993). Antigen persistence may enhance the efficacy of subunit vaccines. This is an interesting area of future research and it would be necessary to determine how long antigen is presented after immunization with current subunit vaccine formulations. In summary, the partial protection provided by subunit vaccines suggests that improved adjuvant formulations, vaccine constructs and heterologous prime-boost regimens are required to enhance the immunogenicity of CS-based vaccine approaches.

In addition to the continued development and optimization of subunit vaccines, there has been a renewed interest in whole parasite vaccines. Although these vaccines are difficult to manufacture, they may allow the generation of a broad, protective immune response against multiple parasite antigens at once, rather than the few targeted in subunit vaccines. The development of an irradiated *P. falciparium* vaccine, based on cryopreserved sporozoites extracted from salivary glands of infected mosquitoes, has been proposed (Luke & Hoffman 2003) and is currently being considered. However, poor immunogenicity due to poor survival of parasites after freezing may present a major hurdle for the implementation of this type of vaccine.

Recent knowledge in *Plasmodium* genome sequences and advancement in transfection technologies have led to the development of genetically attenuated sporozoites (GAS), which are theoretically similar to the radiation attenuated sporozoite approach. In fact these mutant parasites are capable of inducing long-lasting immunity in mice (Mueller et al. 2005, Tarun et al. 2007). When targeted appropriately, some mutant parasites are able to develop into large schizonts before undergoing growth arrest in the liver. These parasites may allow more liver stage antigens to be presented to the immune system and perhaps induce a more polyclonal immune response than irradiated sporozoites (Vaughan et al. 2010). However, evidence indicates that incomplete attenuation of certain strains of GAS represents a serious problem to this approach (Mueller et al. 2005, van Dijk et al. 2005). In addition, as is the case for the preparation of a radiation attenuated vaccine, large-scale manufacturing, extraction of parasites from infected mosquitoes, cryopreservation and delivery of the vaccine are major technical challenges for the production of whole parasite vaccines.

Since *Plasmodium* parasites have several life stages, antigen selection will be crucial for the development of an effective vaccine. Ideal protective antigens should be those that are recognized by CD8^{+} T cells during priming in the lymph nodes and on infected hepatocytes. Thus, the distinct requirements for antigen presentation in DCs and hepatocytes have important implications for vaccine research. Specifically, DCs are capable of acquiring antigens by phagocytosis and can therefore induce CD8^{+} T cell responses to secreted as well as non-secreted antigens. In contrast, hepatocytes can only present antigens in the cytosol of infected or traversed cells. Therefore, optimal targets of protective immunity should include pre-erythrocytic antigens that are both processed by DCs and presented by infected hepatocytes to effector cells, the latter being the most critical requirement for parasite elimination. It is generally accepted that there are other protective antigens in addition to CS. In fact, mice tolerized to CS still develop protective immunity after immunization with irradiated sporozoites (Kumar et al. 2006). Moreover, immune responses to a wide range of parasite antigens were observed in human volunteers immunized with irradiated sporozoites (Doolan et al. 2003). There is a consensus that an efficient malaria vaccine should target blood stages and sexual stages in addition to pre-erythrocytic stages of the parasite. The advancement in transcriptional profiling of all *Plasmodium* stages may help to identify novel antigens that can be incorporated into the development of multi-stage vaccines against human malaria infection.

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