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A retrospective evaluation of the role of T cells in the development of malaria vaccine

Moriya Tsuji

HIV and Malaria Vaccine program, Aaron Diamond AIDS Research Center, The Rockefeller University, New York, NY 10016

Abstract

Due to the fact that the life cycle of malaria parasites is complex, undergoing both an extracellular and intracellular phases in its host, the human immune system has to mobilize both the humoral and cellular arms of immune responses to fight against this parasitic infection. Whereas humoral immunity is directed toward the extra-cellular stages which include sporozoite, erythrocytic and sexual stages, cell-mediated immunity (CMI), in which T cells play a major role, targets intracellular stages, i.e. hepatic stages - liver stages - of the parasites. In this review, the role of T cells in protective immunity against liver stages of the malaria infection is being reevaluated. Furthermore, this review intends to address how to translate the findings regarding the role of T cells obtained in experimental systems to actual development of malaria vaccine for humans.

Keywords

malaria; CD8+ T cells; CD4+ T cells; hepatic stages; immunity; vaccine

Role of CD8+ T cells against murine malaria

The first observation of a successful induction of sterile immunity against malaria infection, i.e. protective immunity that can totally protect against subsequent infection, was made by Nussenzweig, R. and Vanderberg, J. in mice immunized with radiation-attenuated sporozoites (IrSp) of rodent malaria parasites, *Plasmodium berghei* (Nussenzweig, et al., 1967). Initially this powerful protective immunity induced by IrSp was shown to be primarily mediated by humoral immunity - neutralizing antibodies against sporozoites (Potocnjak, et al., 1980, Yoshida, et al., 1980). More recently, however, two independent studies involving the depletion of CD8+ T cells from immunized mice *in vivo*, demonstrated that the immunity induced by IrSp was also, in part, contributed by CD8+ T cells (Schofield, et al., 1987, Weiss, et al., 1988). In these studies, a group of IrSp-immunized mice were depleted of CD8+ T cell population just prior to malarial challenge, and it was found that CD8+ T cell-depleted mice, unlike untreated mice, failed to mount protective immunity against malaria. This observation presents strong evidence of the significant role performed by CD8+ T cells in protective immunity against pre-erythrocytic stages of rodent malaria, consisting of the sporozoite and liver stages. Soon afterwards, the protective role of CD8+ T cells was further defined by adoptive transfer studies (Rodrigues, et al., 1991, Romero, et al.,

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*Corresponding authors, Tel: 1-212-448-5021, fax: 1-212-725-1126, mtsuji@adarc.org.

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1989). In these studies, CD8⁺ T cell clones against an immunodominant CD8⁺ T cell epitope of the circumsporozoite (CS) protein, a major sporozoite antigen, were generated and then transferred into naïve mice followed by infection with live malarial sporozoites. Adoptive transfer of CS antigen-specific CD8⁺ T cell clones could confer protection against subsequent malarial challenge, indicating that CD8⁺ T cells play a key role in conferring protection against rodent malaria, including *P. berghei* and *P. yoelii*. In the latter study, the parasite load in the liver was measured by determining the amount of parasite-specific ribosomal RNA (rRNA), and it was found that CD8⁺ T cell clones displayed their protective effect by inhibiting the parasite development in the liver (Rodrigues, et al., 1991). This indicates that CD8⁺ T cells are indeed capable of attacking the hepatic stages of malaria parasites. It is noteworthy that the subsequent study by Rodrigues et al. found that adoptive transfer of only CD44^{high}, but not CD44^{low}, CD8⁺ T cell clone specific for the CS protein could confer protection. This study also found that the protective capacity of the CD44^{high} CD8⁺ T cell clone is associated with their ability to home in on the proximity of malaria-infected hepatocytes upon adoptive transfer *in vivo* (Rodrigues, et al., 1992). Interestingly, there was no clear correlation between the ability of the CD8⁺ T cell clones to confer protection and their ability to secrete IFN- γ , TNF- α , serine esterase or perforin, nor was there any link to their ability to lyse the target cells.

The protective role of CD8⁺ T cells was also shown in mice having received immunization of other forms of malaria vaccines. The first group to show that recombinant vaccine induces CD8⁺ T cell mediated-protection against malaria was the group led by Sadoff (Sadoff, et al., 1988). This group showed that oral immunization with a recombinant *Salmonella typhimurium* expressing *P. berghei* CS protein induces protective immunity against a *P. berghei* sporozoite challenge and that this immunity is mediated by CD8⁺ T cells, since depleting the CD8⁺ T cells *in vivo* abrogated the protection (Sadoff, et al., 1988). Later, the same group showed that immunization of mice with a recombinant vaccinia virus expressing *P. berghei* CS protein could elicit a high level of protection, which did not correlate with CS repeat-specific antibody responses and was abrogated by *in vivo* CD8⁺ T cell depletion (Lanar, et al., 1996). We have shown that immunization with a single immunizing dose of a recombinant adenovirus expressing the CS protein of *P. yoelii* could induce a very potent protective immunity against the liver stages of malaria and that the level of protective immunity was diminished to a large degree, i.e. 60%, upon depleting the CD8⁺ T cell population, whereas CD4⁺ T cell depletion reversed the level of protective immunity to a lesser degree, <20% (Rodrigues, et al., 1997). More recently, Jobe et al. have found that three doses of vaccination with genetically attenuated *P. berghei* sporozoites induced a sterile immunity in mice and that the sterile immunity observed in wild-type mice was abolished in mice lacking β 2-microglobulin (β 2m) (Jobe, et al., 2007). And most recently, Schmidt et al. were able to elicit a very high frequency of CS antigen-specific CD8⁺ T cells that last more than a year. This was achieved by priming mice with dendritic cells (DCs) pulsed with a peptide corresponding to the CD8⁺ T cell epitope of the CS protein followed by boosting with a recombinant *Listeria monocytogenes* expressing the same CS antigen-derived CD8⁺ T cell epitope. The CS antigen-specific memory CD8⁺ T cells mediated a life-long protection in mice (Schmidt, et al., 2008). Subsequently, Schmidt et al. have also shown that the induction of a high level of CS antigen-specific CD8⁺ T cell response by this immunization regimen permitted mice to develop sterilizing sporozoite-specific antibodies after repeated asymptomatic challenges with physiologic numbers of viable sporozoites (Schmidt, et al., 2009). Altogether these studies further confirm the key role of CD8⁺ T cells in protective anti-malaria immunity, regardless of immunogens used under different experimental settings.

A group led by Zavala generated T cell receptor (TCR)-transgenic mice expressing a T cell receptor (TCR), based on the TCR sequence of CS antigen-specific CD8⁺ T cell clone

recognizing the immunodominant CD8+ T cell epitope of the *P. yoelii* CS antigen as used in the study described above (Sano, et al., 2001). Using the TCR-transgenic mice, this group yielded several key findings. First, they showed that the *in vivo* presentation of the CD8+ T cell epitope of the CS protein occurs within a relatively short period of time, i.e. <48 hrs (Hafalla, et al., 2002). Second, the proper development of CS antigen-specific, protective CD8+ T cell response requires IL-4 secreted by CD4+ T cells (Carvalho, et al., 2002). Lastly, after an infectious mosquito bite, CS antigen-specific, protective CD8+ T cell response induced are primed by dendritic cells (DCs) that reside in skin-draining lymph nodes (Chakravarty, et al., 2007).

Besides the CS protein, thrombospondin-related anonymous protein (TRAP), or sporozoite surface protein 2 (SSP2), is the second T cell antigen that has been well characterized (Rogers, et al., 1992). Antibodies to extracellular domains of TRAP have no effect on sporozoite infectivity, because TRAP is localized in micronemes (Muller, et al., 1993, Rogers, et al., 1992). Therefore TRAP vaccines can only be effective by generating T cell responses. In fact, TRAP has been shown to induce a specific CD8+ T cell response, and cloned TRAP-specific CD8+ T cells were shown to contribute to protection against malaria challenge, upon their adoptive transfer (Khusmith, et al., 1991). The last pre-erythrocytic antigen that can induce “protective” CD8+ T cell response is called PyHep17. This was supported by the study in which immunization of mice with a naked DNA encoding PyHep17 could induce protective immunity that is mediated by CD8+ T cells (Doolan, et al., 1996). Most recently, using CS-transgenic JHT mice, in which immune response to the CS antigen is tolerated and, in addition, generation of antibodies is impaired, Kumar et al. have found that hyper-immunizing these mice with IrSp of *P. yoelii* could induce a very strong protective anti-malaria immunity. This protection is mainly mediated by CD8+ T cells, since the depletion of CD8+ T cells abolished the protection (Kumar, et al., 2006). This finding underscores the presence of protective CD8+ T cell responses against sub-dominant non-CS antigens in IrSp.

As for the questions regarding how CD8+ T cells exert their inhibitory activity against the liver stages, it was earlier believed that IFN- γ might be one of the key players that mediate the anti-plasmodial effects of CD8+ T cells against the liver stages of malaria based on the following three sets of studies. A first study showed that the inoculation of a recombinant IFN- γ into malaria-infected mice resulted in a strong inhibition of the liver stage development in their liver (Ferreira, et al., 1986). The second study showed that the administration of neutralizing anti-IFN- γ antibody to sporozoite-immunized mice abrogated the protection against sporozoite-induced malaria (Schofield, et al., 1987), and in the last study, a single immunizing dose of IrSp, which could mount a protective immunity in wild-type mice, had failed to do so in IFN- γ receptor-deficient mice (Tsuji, et al., 1995). Collectively, these studies highlighted the role of IFN- γ in mediating protective immunity against pre-erythrocytic stages. More recently, however, our group has shown that, in contrast to sporozoite-induced immunity that is mediated by IFN- γ , a single immunizing dose of a recombinant adenovirus expressing the CS protein was able to induce CD8+ T cell-mediated protective immunity against the liver stages, which is independent of IFN- γ (Rodrigues, et al., 2000). These findings were later corroborated by the study, in which CS-specific transgenic CD8+ T cells derived from IFN- γ -deficient mice immunized with a recombinant vaccinia virus expressing the CS protein were able to display anti-liver stages activity upon adoptive transfer (Chakravarty, et al., 2008). I would like to emphasize that, therefore, at present it is still not clear whether IFN- γ plays a vital role in mediating anti-liver stages effects of CD8+ T cells.

Although the role of nitric oxide, which is induced intracellularly by IFN- γ , in protective immunity against the liver stages had been investigated using its antagonists, the conclusions

remain inconclusive (Doolan, et al., 1996, Mellouk, et al., 1991, Mellouk, et al., 1994, Seguin, et al., 1994). In addition, mice lacking perforin or Fas were still able to induce protective immunity against sporozoite-induced malaria, thereby suggesting that these molecules do not mediate anti-parasitic activity of CD8⁺ T cells (Renggli, et al., 1997). However, it is important to be cautious in interpreting the results whenever genetically engineered knock-out mice are used in the experiments, because of the presence of a compensatory mechanism in these mice. In fact, the study by Doolan & Hoffman indicated the complexity of protective immunity against liver-stage malaria, by showing that distinct mechanisms of protection are induced in different strains of inbred mice by a single immunizing dose of IrSp, and in the same strain by different methods of immunization, such as sporozoites and DNA (Doolan and Hoffman, 2000). In conclusion, the effector mechanisms by which CD8⁺ T cells attack the liver stages remain uncovered, and further studies are required to identify the key molecule that mediated anti-liver stages activity of CD8⁺ T cells.

Another key issue on the effector mechanisms of anti-malarial CD8⁺ T cells is how CD8⁺ T cells are able to recognize or identify malaria-infected hepatocytes among so many uninfected hepatocytes in order to exert their anti-parasite activity. In early studies, CS antigen-specific CD8⁺ T cells were isolated from mice receiving immunization with IrSp. When these isolated CS antigen-specific CD8⁺ T cells were co-cultured *in vitro* with MHC-matched primary hepatocytes infected with live sporozoites, they could inhibit the liver stage development only in the presence of a peptide corresponding to the CD8⁺ T cell epitope of the CS protein (Weiss, et al., 1990). This was the first demonstration uncovering the mechanisms underlying how malaria-immune CD8⁺ T cells display their anti-parasitic activity. The essential role of MHC class I molecules in mediating CD8⁺ T cell-dependent anti-malaria immunity was shown by the study in which adoptively transfer of malaria-immune splenocytes into $\beta 2m$ -deficient mice failed to confer protection (White, et al., 1996). A most recent study using TCR-transgenic mice showed that CS protein-specific CD8⁺ T cells can display anti-parasitic activity in the absence of MHC-matched hematopoietic cells in the recipient bone-marrow chimeric mice (Chakravarty, et al., 2007). This indicates that malaria-specific CD8⁺ T cells do not require bone marrow-derived antigen-presenting cells for protection, but instead, they recognize antigen on parenchymal cells - presumably parasitized hepatocytes. Future studies will hopefully yield compelling evidence for the indispensable role of MHC class I molecules expressed by hepatocytes for CD8⁺ T cells to recognize the parasites and exert their anti-plasmodial activity *in vivo*.

The final question with regards to the role of CD8⁺ T cells in protective anti-malaria immunity is how malaria antigen-specific CD8⁺ T cells get elicited *in vivo*. One group has shown that DCs, particularly those reside in cutaneous lymph nodes, are responsible for priming malaria antigen-specific CD8⁺ T cells after an infectious mosquito bite (Chakravarty, et al., 2007). This was shown by the fact that the ablation of the skin draining lymphoid sites impairs subsequent development of protective immunity. However, another group has shown that liver DCs, particularly those express CD8 α , present malarial antigen to hepatic CD8⁺ T cells (Jobe, et al., 2009). It is interesting to note that this group immunized mice through intravenous inoculation of IrSp. Lastly, there are a couple of studies presenting evidence that hepatocytes are the ones that present malarial antigens to CD8⁺ T cells. In one study, livers were isolated from mice immunized with IrSp, followed by being adoptively transferred to intrasplenically to mice (Renia, et al., 1994). These mice could mount a protective immunity that prevented some of the mice from malaria infection. In a more recent study, primary hepatocytes were infected with live sporozoites *in vitro*, and then their ability to process and present the CS antigen to CD8⁺ T cells had been investigated (Bongfen, et al., 2007). They showed that infected primary hepatocytes are able to process and present the CS antigen to specific CD8⁺ T cells, which is largely dependent

on proteasomes. Although these studies together demonstrated the capability of hepatocytes as an antigen-presenting cell (APC) to process and present exoerythrocytic antigens, it is unclear to which extent hepatocytes contribute as APCs *in vivo* after malaria-infected mosquito bites. Overall, the major cell types that process and present exo-erythrocytic antigens remain unknown and further studies are required to solve this important issue.

Role of CD8+ T cells against human malaria

In contrast to the amassed experimental proof of the protective role of CD8+ T cells in anti-malaria immunity in a mouse model, there are only a few circumstantial evidence for their role in human malaria. One of the first studies to demonstrate the protective role of CD8+ T cells against human malaria was done by a group led by Hill, who showed that HLA-B53 MHC class I allele was associated with protection from severe forms of *P. falciparum* malaria infection in African children (Hill, et al., 1992). In this study, HLA-B53-restricted CD8+ T cells were found to recognize a conserved nonamer peptide from LSA-1 antigen among malaria-immune Africans. The same group later identified HLA-B53-restricted CD8+ T cell responses against several epitopes present in LSA-3 antigen, as well as in Exp-1, the homologue of PyHEP17 that induces a CD8+ T cell-mediated protection in a rodent malaria model as described above (Aidoo, et al., 2000). In the same year, a CD8+ T cell clone specific for HLA-A2-restricted CD8+ T cell epitope of the *P. falciparum* CS protein was established from an adult living in malaria endemic area in Africa (Bonelo, et al., 2000), and it was shown that the CD8+ T cell clone could efficiently get activated upon recognizing human hepatocytes infected with a recombinant vaccinia virus expressing the *P. falciparum* CS protein (Bonelo, et al., 2000). More recently, taking advantage of newly available genomic information, HLA-supertype considerations, and *in vitro* IFN- γ Elispot assay, one study elegantly identified several novel antigens that can induce a significant CD8+ T cell response in people immunized with IrSp and protected from malaria challenge (Doolan, et al., 2003). Surprisingly, although CD8+ T cell responses were detected against well known pre-erythrocytic antigens including CS protein, TRAP, LSA-1 and Exp-1, these responses did not seem to be associated with protection, since the CD8+ T cell responses against these antigens were higher in sporozoite-immunized individuals who were not protected from malaria challenge. This is most likely due to the widely diversified nature of HLAs in humans, which prevents against skewing toward a few immunodominant antigens. In any cases, the use of cutting-edge bioinformatics and other modern immunological tools may lead us to identify key antigens that can elicit protective CD8+ T cell responses in humans, thereby ultimately providing us the evidence to support the protective role of CD8+ T cells against human malaria.

Role of CD4+ T cells against murine malaria

The role of CD4+ T cells as anti-malarial effector CD4+ T cells was first shown by our study in which a CD4+ T cell clone was established that recognizes an antigen shared by both the sporozoite and blood stages of rodent malaria parasites and adoptively transferred into mice for the purpose of determining the protective effect of the CD4+ T cell clone (Tsuji, et al., 1990). Upon its adoptive transfer, mice were protected from subsequent malarial sporozoite challenge and did not develop parasitemia. This protection is stage specific, since an adoptive transfer of this CD4+ T cell clone failed to confer protection against blood stages of malaria infection (Tsuji, et al., 1990). Taken together, this study indicates that this CD4+ T cell clone specific for yet unidentified antigen plays a definite role in mediating protective immunity against pre-erythrocytic stages, but not blood stages of malaria. Soon after, CD4+ T cell clones against either immunodominant or cryptic epitopes of the CS protein were established and adoptively transferred to naïve mice prior to sporozoite challenge (Renia, et al., 1993, Takita-Sonoda, et al., 1996). Interestingly,

regardless of Th1 or Th2 profile of the CD4⁺ T cell clones, they were able to confer protection against malaria.

The protective role of CD4⁺ T cells against rodent malaria was also shown in other antigens besides the CS antigen. It was demonstrated that protective anti-malaria immunity induced by immunization with synthetic peptides corresponding to either TRAP or Hep17 protein of *P. yoelii* was abolished by way of depleting CD4⁺ T cell population (Wang, et al., 1996).

The relative contribution of CD4⁺ T cells in protective immunity against pre-erythrocytic stages of malaria was first shown by Weiss et al, who depleted CD4⁺ T cells from a group of mice prior to sporozoite immunization (Weiss, et al., 1993). The CD4⁺ T cell depletion treatment caused loss of protection in sporozoite-immunized mice against malaria challenge. Because this CD4⁺ T cell depletion had also abolish the ability of B cells to produce neutralizing antibodies against sporozoites, they infused hyperimmune sera but had no effect on restoring immunity in CD4⁺ T cell-depleted mice. It still remains possible that the absence of CD4⁺ T cells might have some influence on the competent function of CD8⁺ T cells. Nevertheless, this study demonstrated the overall contribution of CD4⁺ T cells as helper CD4⁺ T cells, as well as cytotoxic CD4⁺ T cells, in protective immunity against pre-erythrocytic stages of rodent malaria. For the purpose of determining the relative contribution of CD4⁺ and CD8⁺ T cells in mediating protective anti-malaria immunity, Rodrigues et al. depleted B cells from B10 mice, carrying H-2^b haplotype, before giving a single immunizing dose of IrSp, and then, a few days prior to challenging the mice with live sporozoites, they further depleted CD4⁺ T cells or CD8⁺ T cells (Rodrigues, et al., 1993). They found that the depletion of either CD4⁺ T cells or CD8⁺ T cells inhibited partially – 30% - the protective immunity induced by IrSp, indicating that CD4⁺ T cells and CD8⁺ T cells equally contributed to the protective immunity in B10 mice. Since H-2^b mice do not have an immunodominant CD8⁺ T cell epitope, this mice model may resemble more closely to humans rather than H-2^d mice. This finding was later corroborated by the study in which β 2m-deficient mice were used (Oliveira, et al., 2008). In this study, the depletion of CD4⁺ T cells from sporozoite-immunized, β 2m-deficient mice with C57BL/6 background that also carries H-2^b haplotype, prior to malaria challenge, abolished the protective anti-malaria immunity, indicating that in the absence of CD8⁺ T cells, CD4⁺ T cells play a predominant role in mediating protective immunity against pre-erythrocytic stages.

Role of CD4⁺ T cells against human malaria

In early studies, human volunteers, who received a vaccination with IrSp of *P. falciparum* and protected from malaria infection, were shown to raise CS antigen-specific CD4⁺ T cells having a cytolytic activity in a peptide-specific fashion *in vitro* (Moreno, et al., 1991). When CD4⁺ T cell clones were isolated from the volunteers immunized with a synthetic peptide containing a universal CD4⁺ T cell epitope of the CS protein, they were shown to be predominantly Th1-type CD4⁺ T cell clones that produce high levels of IFN- γ (Calvo-Calle, et al., 2005). In fact, in a recent study, in which peripheral blood mononuclear cells (PBMCs) were used from volunteers immunized with a RTS,S vaccine, a virus-like particle consisting of a portion of the CS protein fused to the hepatitis B virus surface antigen, as well as from individuals with naturally acquired immunity, the presence of IFN- γ -producing CD4⁺ T cells specific for a conserved epitope of the CS protein was detected by a cultured Elispot assay, and this CD4⁺ T cell response has been shown to be correlated with protection of humans in West Africa against natural *P. falciparum* infection and disease (Reece, et al., 2004). One study demonstrated the presence of CS antigen-specific opsonizing antibodies in the sera of RTS,S-immunized volunteers correlates with protection against *P. falciparum* challenge, indicating that these antibodies may facilitate the processing/presenting via exogenous pathway, thereby inducing CS antigen-specific CD4⁺

T cells that inhibit the development of liver stages of malaria parasites in immunized people (Schwenk, et al., 2003). Overall, similarly to CD8+ T cells, the definitive role of CD4+ T cells in protective immunity against human malaria has yet to be determined. I hope that with recent technological advances and the growing amount of key information relate to bioinformatics would be able to facilitate research and reveal the role that CD4+ T cell plays in mediating and/or contributing anti-malaria immunity in humans.

Future prospect toward a T cell-based malaria vaccine

Although the role of CD8+ T cells in protective immunity against malaria is well established in an experimental mouse model, there is still no compelling evidence showing that is the case for human malaria as well. In this regard, it is rather urgent that the role of CD8+ T cells in protective immunity against hepatic stages of human malaria be established. Once the protective role of CD8+ T cells against human malaria is established, then it would become more logical to take an approach to design a vaccine that can elicit a potent malaria-specific CD8+ T cell response in humans. The most advanced clinical trials are based on RTS,S vaccine that seems to elicit primarily humoral response and, to a lesser extent, CD4+ T cell responses specific for the CS protein (Kester, et al., 2009). This indicates that, 1) without eliciting CD8+ T cell response and 2) without having any responses against other antigens than the CS protein, RTS,S is still able to mount some degree of protective immunity in young children living in an endemic area. This is indeed very promising news, because these RTS,S trials indicate to us that there is certainly room for improvement to the RTS,S; making it more potent by adding more “protective” antigens and/or designing the vaccine to elicit a stronger CD8+ T cell response. Any of these strategies would make the RTS,S vaccine more powerful and hopefully increase the efficacy of the vaccine, another step towards the successful eradication of malaria.

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