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Protective CD8+ T cells against *Plasmodium* liver stages: immunobiology of an 'unnatural' immune response

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Summary

Immunization with high doses of irradiated sporozoites delivered by the bites of infected mosquitoes has been shown to induce protective responses against malaria, mediated in part by $CD8^+$ T cells. In contrast, natural transmission involving low exposure to live sporozoite antigen fails to elicit strong immunity. In this review, we examine how irradiated sporozoite immunization breaks the natural host-parasite interaction and induces protective $CD8^+$ T cells. Upon biting, the malaria-infected mosquitoes deposit parasites in the skin, many of which eventually exit to the bloodstream and infect hepatocytes. However, certain antigens, including the circumsporozoite protein, remain in the skin and are presented in the draining lymph node. These antigens prime specific $CD8^+$ T cells, which migrate to the liver where they eliminate parasitized hepatocytes. We discuss the relevance of the different tissue compartments involved in the induction and effector phases of this response, as well as the cellular requirements for priming and memory development of $CD8^+$ T cells, which include a complete dependence on dendritic cells and a near absolute need for $CD4^+$ T-cell help. Finally, we discuss the impact of the immunodominant circumsporozoite protein on this protection and the implications of these findings for vaccine design.

Keywords

$CD8^+$ T cells; memory; malaria; sporozoite; liver; lymph node

Introduction

The *Plasmodium* parasite carries out an intricate life cycle with obligatory developmental stages in the mammalian hepatocytes and erythrocytes, as well as within the mosquito vector. Since the first demonstration 40 years ago that protective immunity to malaria can be induced by immunization with irradiated sporozoites both in mice 1, 2 and in humans 3–6, considerable work has been done to reveal the mechanisms of this protection and identify the antigens involved. While the contributions of antibody and T-cell-mediated immunity have been demonstrated by depletions and passive and adoptive transfers 7–18, a comprehensive understanding of how protective immunity is induced by irradiated sporozoites remains elusive. Antibodies have been shown to be effective at limiting the number of parasites that successfully reach the liver, but this protection is demanding, requiring high titers of high affinity antibodies from long-lasting durable memory B cells to be effective8. $CD8^+$ T cells have been shown to be important for eliminating parasites that successfully invade and replicate within hepatocytes. Thus, the liver stage is the primary

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target for the vaccine-inducible T-cell responses and is the presumed target of the irradiated sporozoite vaccination model. Importantly, however, this protection is not observed after parasite exposure under natural transmission and can only be induced after immunization with large numbers of parasites 5, 19. Indeed, vaccination of humans with sporozoites requires exposure to nearly 1,000 infected mosquito bites – a condition unlikely to be found in endemic areas, where individuals are exposed to a single infected mosquito bite every few days or weeks. Clearly, this immune response is better characterized as a vaccine-induced response than one developed after natural exposure to parasite infection. In fact, little is known about $CD8^+$ T-cell responses induced under conditions of natural transmission. Studies in endemic areas appear to show that humans exposed to malaria transmission harbor circulating $CD8^+$ T cells specific for parasite-derived epitopes 20–22. However, these cells are present at extremely low frequency, and it is unclear if these cells have any anti-parasite activity.

While the time spent inside host hepatocytes following inoculation by the infected mosquito varies between host and parasite (approximately two days for rodents strains and five to seven days for *P. falciparum* in humans), this stage represents the only time the parasite is vulnerable to recognition and elimination by $CD8^+$ T cells. A major difficulty in detecting and eliminating the liver stage parasites during natural infection is the 'silent' nature of this process, with a very limited number of sporozoites delivered by the mosquito (approximately 10–100 sporozoites per mosquito) 23, resulting in a small number of infected hepatocytes. While this quiet stage may be an effective parasite strategy for subverting its susceptibility to $CD8^+$ T cells, it also creates an attractive target for elimination due to the small number of parasites and apparent vulnerability within a cell expressing class I major histocompatibility complex (MHC). In the context of immunization with attenuated sporozoites, artificially increasing the number of parasites amplifies the quiet infection to a full immunological insult capable of inducing protective immunity. Significant work has revealed much of the basic biology of the induction of the protective $CD8^+$ T cells induced by attenuated sporozoites 12, 13, 17. Protective T-cell clones specific for an immunodominant epitope of the circumsporozoite (CS) protein were identified in our laboratory, allowing for the generation of a T-cell receptor (TCR)-transgenic mouse that has facilitated the tracking and study of the induction of parasite-specific $CD8^+$ T cells, as well as the development of protective memory populations 24–30. This review discusses recent work from our laboratory and other studies in the field that have advanced our knowledge of protective CS-specific $CD8^+$ T cells, as well as implications that these studies may have on the future of malaria vaccine design.

Priming of $CD8^+$ T cells

The site of anti-parasite $CD8^+$ T-cell priming was believed to be in the liver, given the liver-homing properties of the sporozoites, but no evidence existed that supported this assumption. Moreover, the tolerogenic nature of the hepatic microenvironment would not make it an ideal site for T-cell priming 31–33. Delivery of irradiated sporozoites by *Plasmodium*-infected mosquitoes results in the migration of the parasites from the deposition site in the skin to the local capillaries where they enter the bloodstream 34–36. Recent studies have shown that the majority of sporozoites delivered in the skin remain there for over one hour, with significant numbers of parasites remaining for up to six hours 37, 38. In addition to entering the bloodstream, sporozoites also drain to the regional lymph node (LN) and can be detected there by polymerase chain reaction (PCR) for up to six hours after inoculation, with a peak at three hours 37, 39. Following intradermal injection of non-irradiated sporozoites, intravenous injection of LN contents into a naive mouse is capable of initiating a blood stage infection, indicating that at least a portion of the parasites in the LN

are intact and infectious 39. Clearly, sporozoites remain in the skin for significant amounts of time and in large numbers, likely initiating anti-parasite immune responses at this site.

To gain insight into the initial site of antigen engagement and priming of parasite-specific $CD8^+$ T cells, a mouse model of malaria, *Plasmodium yoelii*, was used. To detect early events in T-cell priming, transgenic $CD8^+$ T cells (CD8Py) specific for an epitope of the *P. yoelii* CS protein were transferred into normal BALB/c mice, which were then subjected to the bites of irradiated mosquitoes on the ear. The first detectable $CD8^+$ T-cell response [by interferon-γ (IFN-γ) production) was found exclusively in the ear-draining LN (dLN) 48 h post-immunization, prior to any significant level of clonal expansion by these cells 39. No responses were detected in the spleen, liver, or liver-dLNs until the following day, at the first sign of clonal expansion. dendritic cells (DCs) were previously shown to be required to efficiently prime $CD8^+$ T cells following irradiated sporozoite immunization by *in vivo* depletion of $CD11c^+$ cells 40. In addition, *in vitro* experiments have shown that DCs incubated with sporozoites are capable of presenting parasite antigen to $CD8^+$ T cells 41. *Ex vivo* antigen presentation assays demonstrated that the sporozoite-derived epitope was predominantly being presented by DCs from the skin dLN and not in the spleen or liver-dLN of parasite-immunized mice. Taken together, these results suggest that most anti-CS $CD8^+$ T cells are primed by DCs laden with sporozoite antigen in the skin-dLNs.

Early detection of activated T cells in the dLN indicated that the LN is likely the primary site of antigen encounter and clonal expansion and that effector cells found in other tissues originated from that LN. Treatment of mice with FTY720 to prevent lymphocyte egress from the LNs resulted in significantly fewer parasite-activated $CD8^+$ T cells in the spleen and liver, with a concomitant accumulation of activated $CD8^+$ T cells in the dLN 39. Similarly, mice lacking a LN draining the site of inoculation displayed a significant decrease in the number of activated $CD8^+$ T cells in the liver 39. Finally, removal of the spleen and LN completely ablated protective immunity induced by irradiated sporozoites upon challenge with live sporozoites. These results further demonstrate that protective $CD8^+$ T cells are primed primarily in the LN that drains the site of immunization, although some $CD8^+$ T cells are likely to be primed in the spleen by sporozoites that reach the bloodstream. Additionally, our studies do not rule out the possibility that presentation of non-CS antigens occurs in the liver; however, the existence of priming to such antigens and the anti-parasite activity of the resultant $CD8^+$ T-cell response has yet to demonstrated.

A major question raised by these findings is how and where the antigen is acquired by the DCs in the LN. Given that *Plasmodium* sporozoites have the ability to traverse a wide range of non-hepatic host cells prior to establishing infection in the liver 42, it is possible that the traversal of DCs and deposition of antigen inside these cells is sufficient for presentation of parasite antigen and $CD8^+$ T-cell priming. However, mice immunized with sporozoites following systemic shut down of antigen uptake 43, 44 display a compromised $CD8^+$ T-cell response, strongly suggesting that phagocytosis and subsequent cross-presentation of parasite antigen occurs during sporozoite immunization 39. Simple engulfment and cross-presentation of parasite protein appears to be insufficient for T-cell priming though, as immunization with heat-killed (HK) sporozoites induce minimal $CD8^+$ T-cell responses 39, 45. The necessity of live parasites to the induction of protective immunity is clear, but the reasons for this are not. It could be related to the nature of antigen deposition by the active parasite, the amount of antigen produced, mechanical stimulation by parasite, etc. While the true need for a live parasite is likely to include many variables, the requirement of a live pathogen for an optimal immune response is not entirely unexpected, as maximal $CD8^+$ T-cell responses in other microbial systems have been shown to require a biologically active pathogen 46–48.

The presence of live parasites in the dLN may be a red herring for the actual DCs presenting antigen that may have migrated from the skin. Studies of parasite activity in the skin have shown that sporozoites slowly 'trickle' out of the dermis and into circulation over several hours, suggesting the presence of an intermediate 'skin stage' of *Plasmodium* that has been previously unappreciated 37. Skin-resident DCs may acquire sporozoite antigen *in situ* during the initial inoculation by the mosquito and carry the processed antigen to the dLN for presentation to T cells – a model that has been suggested by previous data in other systems 49–51. Alternatively, various studies have shown that antigen-laden migratory DCs may serve as a transport vessel to carry antigen and pass it off to LN-resident DCs, which in turn prime naive T cells 52–56. Proper identification of the DC population presenting antigen to T cells and the manner in which it was acquired will provide important insight into the biology of anti-sporozoite T-cell priming as well as clues related to the reasons for the need for a live motile parasite for efficient induction of protective immunity.

A second major unknown in the priming of anti-parasite $CD8^+$ T cells is the role of innate immune signaling. It is well known that optimal priming of naive T cells to pathogen-derived antigen is critically dependent on the maturation of DCs, expression of appropriate costimulatory proteins, and secretion of cytokines that allow for proper T-cell activation 57–59. Direct activation of DCs by Toll-like receptor (TLR) ligands has been shown to be of paramount importance in many models of infection 60–62. Glycosylphosphatidylinositol (GPI) anchors from certain protozoans have been shown to bind TLRs and have potent immunostimulatory properties 63, 64. The precise receptor mediating this response has not yet been defined in *Plasmodium*. Some studies of TLR stimulation by *Plasmodium* have been done using ligands purified from blood stage malaria parasites, but little is known about the role of innate signaling in response to pre-erythrocytic stages. Maturation of DCs through sporozoite-derived TLR ligands is possible, given the efficient and early priming of naive $CD8^+$ T cells by cross-presented antigen, a process that has been shown to be dependent on TLR ligation 65–67. Since the sporozoite can actively access the cytoplasm of host cells prior to settling in the liver, there may also be intracellular receptors that are activated during the traversal process. The role that known nucleotide oligomerization domain (NOD) receptors play in the induction of $CD8^+$ T cells to sporozoites has not been investigated and would provide further insight into the induction of protective immunity.

Protective $CD8^+$ T-cell responses can be induced with comparable efficiency by large numbers of radiation-attenuated or live sporozoites, indicating that the development within the liver, or lack thereof, does not influence the induction of protective $CD8^+$ T-cell responses 68. These findings also provide insight into the effect of blood stage parasites on the development of T-cell responses to liver stage parasites. Sporozoite invasion of hepatocytes and subsequent development of liver stage parasites results in the release of merozoites and infection of red blood cells, thus beginning the erythrocytic cycle. This blood stage malaria infection is characterized by systemic inflammation and pathology that has been shown to have mixed effects on immune function 44, 69, which were believed to be detrimental to the development of anti-liver stage T-cell responses. However, recent studies suggest that the blood stage infection after sporozoite infection does not affect the induction of anti-malaria $CD8^+$ T cells 68. Indeed, mice that were immunized with either irradiated or live sporozoites mounted equivalent $CD8^+$ T-cell responses in terms of kinetic expansion, IFN-γ production, memory formation, and protection from secondary challenge. Importantly, these results confirm that high doses of live sporozoites are equally immunogenic and efficient at inducing $CD8^+$ T-cell responses as irradiated sporozoites 28. These results are in agreement with recent longitudinal studies in humans that found blood stage parasitemia following T-cell priming did not affect established T-cell responses 44, 70. They did, however, show that T-cell priming amidst a concurrent blood stage infection hindered IFN-γ enzyme-linked immunospot assay (ELISPOT) responses, presumably

impacting the effector phase or memory development through modulation of DC function, in agreement with other studies using *P. berghei* in mice 44. In total, these studies showed that $CD8^+$ T-cell responses to live sporozoites can be efficiently induced in the face of blood stage parasites that eventually develop from those same sporozoites but that pre-existing blood stage parasites can negatively affect nascent T-cell priming.

Effector phase of $CD8^+$ T cells

Immunization with irradiated sporozoites is able to protect from malaria infection, but this protection is strictly liver stage specific 9, 24. One advantage of this approach is that the sporozoite stage is a bottleneck in the lifecycle of the parasite, with relatively few parasites to eliminate during infection. However, a drawback of this liver stage-specific immunity is that the parasite's residence in the liver is quite brief: less than two days in for *P. yoelii* in rodents and five to seven days in *P. falciparum* in humans. Because of this fact, liver-resident effector memory $CD8^+$ T cells are essential for the rapid elimination of liver-stage parasites 29. The infection and exit from the liver by the parasite may be too rapid for lymphoid-resident central memory T cells to become activated, expand, and migrate to patrol the tissues 71. Liver-resident $CD8^+$ T cells are capable of responding rapidly to live sporozoite challenge, with nearly half of antigen-specific cells producing IFN-γ *in situ* within four hours of challenge 39. Selective loss of memory T cells in the periphery demonstrates their importance to protective immunity, as protection was lost in mice lacking liver-resident T cells despite having memory $CD8^+$ T cells in the spleen and LNs 29. Since residence in the liver is essential for $CD8^+$ T-cell-mediated protection, it is likely that intimate contact and cognate peptide recognition between the effector T cell and the parasitized hepatocyte is necessary for protection. This hypothesis was confirmed in bone marrow chimera studies that demonstrated parasite-specific memory $CD8^+$ T cells in the liver were incapable of eliminating infected hepatocytes bearing a mismatched MHC, despite harboring hematopoietic cells capable of presenting antigen to the T cells 39. In the inverse chimera, where the memory $CD8^+$ T cells could recognize cognate antigen on surface of the hepatocytes but not the lymphoid cells, protective immunity remained intact. In total, these data emphasize the need for memory $CD8^+$ T cells to migrate within the liver parenchyma and eliminate parasitized hepatocytes directly and not through paracrine release of cytokines after antigen recognition on local antigen-presenting cells (APCs).

The necessity of liver-resident memory cells for protection raises some fundamental questions about T-cell trafficking that have yet to be fully elucidated. Characterization of $CD8^+$ T cells migrating to intestinal mucosa, skin, and other non-lymphoid organs have shown that activated cells homing to peripheral organs are phenotypically and functionally distinct from cells found in lymphoid organs 72–76. This finding suggests either a selective migration of these cells to peripheral sites or alteration of phenotype by the unique tissue microenvironment. The existence of populations of memory cells with differential expression of homing molecules supports the notion that subsets of memory T cells may exhibit tissue-specific homing patterns mediated by distinct combinations of surface molecules which direct migration/homing to different tissue compartments. It is likely that memory $CD8^+$ T cells that migrate to the liver express a unique set of chemokine receptors and integrins in their surface that mediate their homing and long term residence in this organ. The chemokine receptors CXCR3 77, 78 and CXCR6 77, 79 as well as the integrins $a_E b_7$ 80 and $a_L b_2$ 81, 82 have been implicated in T-cell homing to the liver. Investigation into the role that these and other tissue-homing receptors will be most valuable in strengthening the understanding of protection against malaria and other tissue-tropic pathogens.

The exact effector mechanisms utilized by the protective memory $CD8^+$ T cells to eliminate liver-stage parasites have yet to be identified. While perforin, Fas ligand (FasL), and IFN-γ are established mediators of $CD8^+$ T-cell cytotoxic function 83–86, parasite-specific memory $CD8^+$ T cells selectively lacking perforin 87, 88, FasL 87, or IFN-γ89 are all capable of mediating protective immunity. Antibody neutralization of tumor necrosis factor-α (TNF-α) has also been shown to have no effect on protective immunity 90. Some studies have reported results that suggest a protective role for IFN-γ based on IFN-γ-deficient mice or neutralizing antibodies. However, IFN-γ has many pleiotropic effects on cells of the immune system as well as parenchymal tissue, and therefore the assumption that $CD8^+$ T cell-derived IFN-γ directly kills parasitized hepatocytes may be inaccurate. While the exact cellular mechanism of protective immunity has yet to be clearly defined, the search for a single anti-parasite killing mechanism may be misguided. It is possible, if not likely, that a compilation of all of the above-mentioned effectors contribute to parasite killing in a redundant fashion. Anti-parasite $CD8^+$ T cells readily degranulate and produce IFN-γ and TNF-α simultaneously in response to *ex vivo* antigen restimulation (unpublished observations), indicating that selective deletion or neutralization of a single effector function may be easily overcome by the others and may explain the puzzling results of the single knockout experiments.

$CD8^+$ T-cell memory and recall responses

An understanding of the cellular and functional requirements for parasite killing by $CD8^+$ T cells is clearly useful for the development of T-cell vaccines. A basic problem facing vaccinologists is the lack of effective strategies to develop large number of protective T cells. To generate a sufficiently large T-cell response, most putative vaccine strategies rely on prime-boost regimens using one or more different vectors carrying the same target epitopes 91, 92. Nonetheless, memory $CD8^+$ T-cell responses have proven generally difficult to expand by secondary immunization, though some vectors have proven more effective than others at boosting immune responses 92–95. The difficulty of expanding T-cell responses is illustrated by the failure of individuals from malaria-endemic areas to develop robust epitope-specific immune responses to sporozoite antigens despite regular exposure to infected mosquito bites. Indeed, the $CD8^+$ T-cell responses in naive individuals who have received one or two large doses of irradiated sporozoites are often stronger than those of people who have lived their whole lives in endemic areas 96, 97. Similar results were found in mice that received 100,000 sporozoites either as a single dose or as four doses of 25,000 parasites on consecutive days 28. The mice that received the sporozoites in a single large dose had higher CS-specific T-cell responses, suggesting that the primary response to antigen is ‘set’ very early on and was loosely dependent on primary antigen load. Nonetheless, people in endemic areas might be expected to have robust responses from a lifetime of ‘boosting’ T-cell responses by infected mosquito bites. However, mice that were immunized with irradiated sporozoites weekly for four weeks had antigen-specific responses no larger than control mice received a single immunization at the final time point 93.

Even though repeated exposure to sporozoites does not expand antigen-specific T-cell responses, sporozoite-induced $CD8^+$ T cells can be expanded by heterologous boost with a recombinant virus. Of vaccinia, influenza, and adenovirus vectors expressing the protective SYVPSAEQI epitope from *P. yoelii*, only the recombinant vaccinia virus was able to significantly expand the antigen specific memory $CD8^+$ T-cell population 93, even though all induced similarly sized primary $CD8^+$ T-cell responses in naive mice, confirming previous data from our group and others 95, 98–101. This boosting was strictly dependent on DCs, as has been previously reported 102, which was surprising given the previous finding that protective anti-parasite activity of memory $CD8^+$ T cells does not require DCs

39. Additionally, unlike priming but similar to effector responses, boosting appears to be independent $CD4^+$ T-cell help 93. It is possible therefore, that memory T cells may respond in a spectrum of ways upon secondary encounter with antigen: seeing antigen on an infected parenchymal cell may induce effector function, however seeing antigen presented by a DC with accompanying costimulatory signals may stimulate the memory cell to undergo secondary expansion.

Given the requirement for DCs for memory T-cell expansion 102, it is possible that T-cell boosting requires a threshold amount of antigen to reach DCs for presentation to memory T cells. In studies exploring this possibility, *ex vivo* antigen presentation assays using purified splenic DCs from virally immunized mice with carboxyfluoresein succinimidyl ester (CFSE)-labeled CD8 transgenic (Tg) cells as detectors of antigen led to a number of striking observations. First, recombinant vaccinia virus induced robust antigen presentation by DCs within 24 h, whereas influenza and adenovirus took 48 h for the first modest sign of processed antigen. Second, in sporozoite-immune mice, antigen presentation following secondary immunization with recombinant influenza virus was severely reduced compared to naive mice. This trend was also seen with recombinant vaccinia virus, though to a much lesser degree than with influenza. Third, depletion of $CD8^+$ T cells from the sporozoite-immunized mice prior to boosting with influenza restored antigen presentation to the same levels seen in naive mice, suggesting that compromised antigen presentation by influenza in immune mice was due to rapid control of viral replication and antigen load by memory $CD8^+$ T cells.

Overall, the data suggest that $CD8^+$ T cells are the major regulators of the ability of a virus to boost those same T cells. The exact mechanism by which $CD8^+$ T cells regulate antigen presentation is unclear, though it may occur at the level of the DC: effector T cells may directly kill DCs presenting cognate antigen 103–105, strip peptide from surface MHC molecules, or down modulate MHC-peptide complexes on the surface of Daces 106, 107. Additionally, memory $CD8^+$ T cells might quickly clear antigen in the periphery, preventing it from reaching lymphoid tissues and being cross-presented by DCs. There is some evidence that vaccinia viruses may be able to directly infect DCs and induce repaid direct presentation of antigen 108, while influenza viruses preferentially infect epithelial cells and rely on cross presentation by DCs for antigen presentation 44, 93, 109. Imaging experiments using vaccinia viruses expressing both the K^b epitope SIINFEKL and green fluorescence protein (GFP) showed infected DCs interacting with cognate OT-1 $CD8^+$ Tg cells as early as eight hours following infection 108. Other experiments using vaccinia vectors carrying the human cytomegalovirus proteins US2 and US11, which interfere with MHC class I processing and block direct antigen presentation, were less immunogenic that parental virus 110, 111, suggesting direct infection of professional APCs.

While heterologous prime-boost regimens have emerged as a promising approach to T-cell vaccination, there may be fundamental problems with the approach itself: $CD8^+$ T cells can powerfully regulate the presentation of their own cognate antigen, both during priming and boosting. In the context of sporozoite immunizations, it may be possible that effector/memory CS-specific $CD8^+$ T cells regulating the presentation of the CS antigen also prevent the presentation of other sporozoite antigens. This prevention may lead to the development of a strong but narrowly focused response, which may not be as protective as a more diverse response, and that is also vulnerable to mutations in the protective epitopes. Interestingly, four immunizations of irradiated sporozoites does not lead to expansion of SYVPSAEQI-specific T cells compared to one immunization 93, but a similar regimen results in sterile immunity (even in B-cell-deficient mice), meaning that other T-cell responses (of unknown specificity) are likely to be primed and expanded 112. This apparent repertoire expansion of protective T cells occurs only after three or more immunizations, suggesting that antigen

may be limiting in priming cells to certain antigens. Regardless, the increasing ability to protect with multiple immunizations without observing increasing frequency of SYVPSAEQI-specific $CD8^+$ T cells suggests that multiple antigenic targets are important for protection mediated by parasite-specific T cells. Unfortunately, it is also possible that $CD8^+$ T cells of differing specificities will compete with each other, possibly lessening the overall efficacy of the immune response. This idea was suggested by studies in which DNA and pox-virus vectors were engineered to carry at least epitopes from six different *P. falciparum* proteins 113. Clearly while progress is being made in understanding the cellular and molecular processes involved in T-cell effector function and expansion, we still are not able to 'rationally' design vaccines. Empirical testing of vectors and constructs remains our best approach to this problem.

CD4 dependence of $CD8^+$ T-cell responses

In the absence of helper T cells, primary $CD8^+$ T-cell responses to pathogens typically proceed normally through expansion, contraction, and memory formation, only to exhibit profound defects in secondary recall responses 114–116. CD4 help in models of inflammatory pathogen infection appears to be mediated by direct or indirect (presumably through the APC) 'imprinting' of effector $CD8^+$ T cells in a way that allows for the formation of competent memory cells 117 or, alternatively, in the maintenance of memory $CD8^+$ T cells 118, 119. However, not all anti-pathogen $CD8^+$ T-cell responses are independent of $CD4^+$ helper T cells 26, 120–122. $CD8^+$ T-cell responses to irradiated sporozoites display striking impairment in the absence of CD4 help as early as four days post-immunization, when the helpless effector $CD8^+$ T-cell population begins a premature contraction 26. Experiments using CFSE-labeled CD8Tg cells demonstrate that normal and helpless cells proliferate equally well, showing that antigen-specific proliferation can occur rapidly in the absence of CD4 help. After three days, the helpless CD8 response beings to crash, quickly falling to ~10% of the normal response over the next several days. This reduced memory population remains stable and can be detected in all lymphoid and non-lymphoid tissue for a least one month. The crippled primary response to sporozoites in the absence of CD4 help may closer resemble responses to tumors and other immunogens that do not produce severe systemic inflammation, where primary $CD8^+$ T-cell responses are limited in the absence of survival signals to effector T cells during the primary expansion, as well as providing these the cells the imprint to allow formation of competent memory cells. The basis for this abnormally early dependence on helper T cells during anti-sporozoite T-cell priming is unknown and has been a topic of interest in our laboratory.

A previous study in our laboratory demonstrated that interleukin-4 (IL-4) was critical to the formation of anti-parasite memory $CD8^+$ T cells 29, inducing a complex pattern of intracellular signaling in these cells 123. Selective deletion of the IL-4 receptor α (IL-4Rα) on the anti-CS $CD8^+$ T cells allowed us to examine the role of IL-4 signaling on these cells in an intact animal. We found that the peak of the response of the IL-$4R^{-/-}$ CD8Tg cells to irradiated sporozoites mirrored that of the IL-$4R^{+/+}$ CD8Tg cells, indicating that IL-4 was not likely to serve a crucial role in allowing maximal expansion by $CD8^+$ T cells. The kinetics of contraction in the spleen, however, were more rapid in the IL-$4R^{-/-}$ cells but stabilized to match the population size of the normal $CD8^+$ cells by 25 days post-immunization. Curiously, profound decreases in population size of IL-$4R^{-/-}$ memory cells were observed in non-lymphoid tissue as early at 10 days post-immunization, indicating a role for IL-4 for either inducing the stable formation of effector memory $CD8^+$ T cells or in the peripheral maintenance of these cells. Antibody depletion experiments showed that the IL-4 was required within the first week of immunization to presumably allow for the stable formation of peripheral effector memory cells and was not necessary afterwards for maintenance of these cells in the periphery. Thus, while IL-4 appears to be critical in the

formation of stable effector memory $CD8^+$ T cells in the periphery, it cannot account for all helper functions from $CD4^+$ T cells, as lymphoid resident memory $CD8^+$ T cells are established normally in the absence of IL-4 signaling.

To further characterize the role that $CD4^+$ T cells play in $CD8^+$ T-cell priming to irradiated sporozoites, we examined the functionality of the helpless memory population. Given that other studies have found functional defects in memory $CD8^+$ T cells generated in the absence of $CD4^+$ T cells, we sought to investigate whether helper T cells provided help in many ways – both in sustaining primary expansion and also by imprinting competency on the memory cells. Upon *ex vivo* restimulation of memory CD8Py cells with peptide-coated target cells, helpless $CD8^+$ T cells displayed profound defects in production of IFN-γ and TNF-α as well as cytotoxic degranulation (measured by CD107a cell surface mobilization), both in frequencies of positive cells and staining intensity per cell (manuscript in preparation). Curiously, normal and helpless memory cells were indistinguishable in terms of their ability to produce IL-2 upon restimulation. Thus, in addition to being drastically reduced in population size, the anti-CS $CD8^+$ T cells were also functionally impaired. These *ex vivo* observations were confirmed by challenge with live sporozoites, where mice lacking helper T cells were conferred no protection from live sporozoite challenge despite an abundance of anti-parasite T cells in the liver (manuscript in preparation).

The $CD8^+$ T-cell response to irradiated sporozoites provides a unique model to study CD4 help. In the absence of $CD4^+$ helper T cells, multiple phenotypic deficiencies can be observed throughout the entire response into the stable memory phase, which is ineffective at protecting from live challenge. The lack of systemic inflammation associated with the sporozoite immunization may allow for investigation of all the possible roles played by helper T cells in orchestrating the immune response in the absence of a compensating widespread cytokine milieu. The profound defects in protective $CD8^+$ T-cell responses in the absence of sufficient T-cell help further underlines the need for investigation into synthetic vaccines given the significant geographical overlap between malaria and HIV/AIDS in Africa. Further work is needed to investigate the missing signals for effector $CD8^+$ T cells in the absence of $CD4^+$ T cells.

Immunodominance of the CS protein

After the demonstration of the ability of irradiated sporozoites to induce sterile immunity, protective epitopes were identified in rodent species of *Plasmodium* 24, 25, 124–126. B-cell and MHC I-restricted epitopes were identified in the CS protein against which specific antibodies and $CD8^+$ T-cell clones were capable of protecting from live sporozoite challenge. Studies showed that, from the viewpoint of antibody 127, 128 and T-cell responses 112, CS is the immunodominant protein involved in protection induced by immunization with sporozoites. Because of its observed immunodominance, the CS protein has been the primary target of a wide array of research into inducing protective immunity with synthetic subunit vaccines 129, as well as bacterial 24, 130 and viral 94, 131–135 vectors encoding *Plasmodium* CS proteins. The current RTS,S vaccine is composed of *P. falciparum* CS subunits expressed in immunogenic hepatitis B particles. This vaccine reduces the frequency of severe illness 136, an outcome likely to be the result of reduced blood-stage parasitemia due to lowered liver-stage burden. However, the protection afforded by this vaccine does not approach the level of sterile protection that is achievable with irradiated sporozoites. These results have led to investigation into the role that CS immunodominance plays in the induction of protective immunity to irradiated sporozoites and for the identification of subdominant antigens that may serve important roles in sterile immunity. The outcomes of these studies have yielded some unexpected results that may prove critical for antigen selection and the success of subunit vaccines.

Transgenic mice expressing the *P. yoelii* CS protein (CS-Tg) were developed to study the role that CS played in protective immunity induced by irradiated sporozoites 112. CS-Tg mice are tolerant to CS and are thus unable to mount T-cell responses to this protein. Priming and boosting of B-cell-deficient CS-Tg mice with irradiated *P. yoelii* sporozoites did not induce protective immunity. However, unlike two doses of irradiated sporozoites, immunization of these mice three times induced sterile protection that was mediated by $CD8^+$ T cells. The ability of a third immunization to induce protective $CD8^+$ T cells demonstrates that non-CS reactive $CD8^+$ T cells are present in these mice but appear to require a the third antigen exposure to sufficiently expand to numbers capable of completely eliminating liver-stage parasites.

The basis of immunodominance hierarchies to pathogens in general are unclear but has been postulated to be related to antigen abundance, availability, and processing, as well as variations in affinity and precursor frequencies of antigen-specific $CD8^+$ T cells. In the context of *Plasmodium*, CS is expressed at extremely high levels on the surface of the sporozoites and is constantly sloughed off and released to the extracellular environment and cytoplasm of traversed cells as the parasite migrates through the skin, LN, and liver parenchymal tissue 42. The availability of large amounts CS antigen to multiple tissue and cellular compartments creates the potential for a skewing of the immune response toward this highly expressed protein. Additionally, CS is likely to be among the first parasite antigens exposed to the immune system, so the timing of antigen exposure and T-cell priming could impact the hierarchy of immunodominance. Large amounts of soluble CS from the sporozoites may be the primary source of parasite antigen if the sporozoites are able to avoid processing by DCs, further limiting the number of parasite antigens available for recognition by the immune system. A narrowing of the focus of the $CD8^+$ T-cell response to select parasite antigens may further enhance the self-regulation of T-cell priming and expansion (discussed in a previous section) and reinforce an immunodominance profile favoring those few proteins.

The consequences of immunodominance of CS may have implications in vaccine design and, in particular, antigen selection. CS has been commonly targeted by pre-erythrocytic subunit vaccines, and given the immunodominance of CS, this approach appears to be a logical one. However, in light of the modest but encouraging results of RTS,S, additional antigens are likely to be needed if these subunit approaches are to be successful. Protective subdominant antigens are likely to be expressed both by the sporozoite and, to some extent, by the liver-stage parasites to allow recognition by $CD8^+$ T cells primed in the LNs by the sporozoites. This expression may be critical to properly induce protective $CD8^+$ T cells, as LNs are ideal sites for priming naive T cells and liver parenchymal cells may not be. Nonetheless, should liver-stage-specific antigens be identified that are expressed to high enough levels in the infected hepatocyte to be readily detected by $CD8^+$ T cells, such antigens may serve as good targets of subunit vaccines even if they are not targets of $CD8^+$ T cells induced by irradiated sporozoites.

Concluding remarks

Natural exposure to the bites of malaria-infected mosquitoes is unable to induce protective immunity in people living in endemic areas, leaving them susceptible to lifelong re-infection. This evolutionary subversion of the immune system by the invading parasite can be overcome by immunization with large numbers of irradiated sporozoites, artificially increasing the parasite load to sufficiently prime protective T cells and B cells. $CD8^+$ T-cell-mediated protection is particularly demanding, requiring complete and swift elimination of only a handful of parasites during their ‘silent’ infection of the liver. While many of the basic tenets of $CD8^+$ T-cell biology hold true for responses to most pathogens, certain

unique requirements exist for memory formation to sporozoite antigen that necessitate careful examination in this system. Unraveling these complex interactions to yield a better understanding of how protective $CD8^+$ T cells can be primed and the mechanisms by which they act to eliminate the parasite are essential to the creation of an effective vaccine.

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