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You Shall Not Pass: Memory CD8 T Cells in Liver-Stage Malaria

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Abstract

Each year over 200 million malaria infections occur with over 400 thousand associated deaths. Certain vaccines based on attenuated whole parasites can induce protective memory CD8 T cell responses against liver-stage malaria, however, widespread administration of such vaccines is logistically challenging. Recent scientific advances are delineating how protective memory CD8 T cell populations are primed and maintained, and how such cells mediate immunity to liver-stage malaria. Memory CD8 T cell anatomical localization and expression of transcription factors, homing receptors, and signaling molecules appear to play integral roles in protective immunity to liver-stage malaria. Further investigation of how such factors contribute to optimal protective memory CD8 T cell generation and maintenance in humans will inform efforts for improved vaccines.

Keywords

Liver-Stage Malaria; Radiation-Attenuated Sporozoite Immunization; Adaptive Immunity; Sterilizing Immunity; Memory CD8 T Cell; Liver Tissue Resident Memory Cell

A Cellular Immunity-Based Malaria Vaccine: Renewed Promise Through Liver-Stage Immunology

Currently, there are no fully effective, readily deployable vaccines for malaria, a devastating mosquito-borne disease caused by *Plasmodium* parasites that is responsible for greater than 200 million annual cases and 400,000 associated deaths[1]. Humans living in malaria endemic areas are repeatedly infected by *Plasmodium* yet never develop the ability to prevent blood-stage infection, termed sterilizing immunity[2]. Current anti-malarial drugs can effectively target the symptomatic blood-stage, however, emerging parasite resistance has the potential to compromise the efficacy of front line drugs[3]. Existing vaccines targeting protective cellular[4, 5] or humoral[6–9] immune responses suffer from either lack

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of potency and long-term efficacy in endemic areas or field delivery constraints. However, recent advances in the understanding of protective cellular immune responses against liver-stage malaria, which precedes the symptomatic blood-stage, offer renewed promise for vaccination as a tool to combat this global health burden.

Sterilizing immunity to liver-stage malaria can be mediated by memory **CD8 T cells** (see Glossary) in mouse and non-human primate (NHP) malaria animal models, and these cells are thought to participate in vaccine-induced immunity in humans[10]. Intravenous (i.v.) injections of early liver-stage-arresting radiation-attenuated **sporozoites** (RAS) generate memory CD8 T cells that mediate sterilizing immunity to liver-stage malaria[4, 11]. However, this whole parasite vaccination is difficult to deliver on a global scale because it requires (1) i.v. administration of hand-dissected parasites, (2) a “cold-chain” to preserve vaccine viability, and (3) multiple high-dose boosters[12]. Although efforts to address these limitations are underway, including the use of low dose sporozoite immunization under anti-malaria drug cover, success in these endeavors is not certain[5, 9]. Here we discuss recent discoveries from animal models about the mechanisms of memory CD8 T cell generation, maintenance, and protective function, and we analyze how these and other findings may inform liver-stage vaccine development.

Memory CD8 T cells: Mediators of Sterilizing Immunity to Liver-Stage Malaria

Malaria sporozoites are delivered into the skin by mosquito bite. Subsequently, some sporozoites invade the vasculature and traffic to the liver, where they must invade and develop in hepatocytes (liver-stage) before progressing to infect red blood cells[10, 13] (blood-stage; Box 1). To mediate immunity, a CD8 T cell must use its T cell receptor (TCR) to recognize *Plasmodium*-derived peptides bound to major histocompatibility complex I (MHC-I) molecules on an infected hepatocyte surface[14]. During hepatocyte infection, *Plasmodium* **antigens** must enter the hepatocyte cytosol to be accessible to MHC-I processing and presentation pathways[15]. Of note, several protective antigens, all of which are derived from proteins expressed by **pre-erythrocytic stages** of the parasite, have been identified. For example, mouse models demonstrate that the sporozoite-expressed circumsporozoite protein (CSP) and thrombospondin-related adhesive protein (TRAP), both of which are involved in hepatocyte invasion, are targets of protective CD8 T cells[16, 17]. *P. falciparum* CSP and TRAP are recognized by CD8 T cells in malaria-exposed or vaccinated humans[18–20]. However, some malaria proteins that elicit CD8 T cell responses are not productive targets of vaccine-induced protection[16], presumably because they do not enter the hepatocyte cytosol during infection, while others do not elicit secondary immune responses upon whole parasite booster vaccination[21], likely because subsequent attenuated infections are cleared by the adaptive immune response before such proteins can be expressed. Finally, the liver-stage proteome is estimated to contain thousands of proteins, either unique to liver-stage or shared between extra-hepatic stages, and this estimate vastly outnumbers described liver-stage malaria CD8 T cell epitopes[22, 23]. Thus, the rules governing the relevance of specific antigens for incorporation into subunit vaccines remain undefined.

Mice immunized with RAS by mosquito bite or iatrogenic vaccination contain pre-erythrocytic-stage-specific memory CD8 T cells in various tissues, including the blood, spleen, liver, liver dLN, and skin dLN[14, 24, 25]. Memory CD8 T cells exist in the blood, spleen, and liver in RAS immunized NHP and the blood of immunized humans[4]. Significant phenotypic heterogeneity exists among these memory cells, and the contributions of each type to protective immunity remain incompletely understood. Memory CD8 T cells are broadly classified as circulating effector memory (Tem), central memory (Tcm) and peripheral memory (Tpm), or non-recirculating tissue resident memory (Trm). Circulating memory CD8 T cell subsets are distinguished based on expression of the surface markers CD62L, CCR7, and CX₃CR1[26], while Trm cells express CD69, multiple **integrins** such as CD49a, CD11a, and often CD103, as well as chemokine receptors such as CXCR3 and CXCR6 at homeostasis[27]. Circulating memory CD8 T cells traffic through the blood, **secondary lymphoid organs** (SLOs), and non-lymphoid tissues (NLTs) based on expression of specific functional homing receptors and ligands[28]. In contrast, Trm cells remain largely confined to the originally infected NLT and its associated draining lymph node (dLN)[27, 29]. Upon reinfection, memory CD8 T cells exhibit altered motility dynamics, produce inflammatory **cytokines** and **chemokines** to recruit and activate other immune effector cells, and directly kill infected cells[28, 30].

It has only recently been recognized that liver Trm cells, which are CD69⁺LFA1^{hi}CXCR3⁺CXCR6⁺CD49a^{hi} and remain confined to the liver, are required for sterilizing immunity to liver-stage malaria in mouse models of whole parasite vaccination or “prime and trap/target” immunization[31–33]. Human liver Trm cell numbers correlate with improved control of viral infections of the liver, suggesting a critical role for these populations in human immunity to liver infection[34]. Thus, effective and deliverable vaccines inducing sterilizing liver-stage immunity in humans will likely require incorporation of robust liver Trm cell formation. However, generation of vigorous liver Trm populations in humans by conventional non-i.v.-delivered subunit vaccination approaches presents a substantial challenge, as mice[35] and NHPs[4] receiving intramuscular (i.m.) or intradermal (i.d.) vaccinations exhibit lower frequencies of *Plasmodium* specific CD8 T cells in the liver and lower rates of sterilizing immunity as compared to i.v. immunized controls.

Pre-erythrocytic-stage-specific Tcirc cell frequencies correlate with sterilizing liver-stage immunity in mice[16, 17]. Additionally, *in vitro*-activated effector CD8 T cells, which traffic to infected tissue via the circulation, eliminate infected hepatocytes in mice[36, 37]. However, the existence of liver Trm cells was unknown at the time of these studies, and so Tcirc cell contributions to liver-stage malaria immunity remain undefined. Tcirc cells contribute to control of influenza[38] and vaccinia[39] viral infections of the mouse lung and skin, respectively, suggesting that Tcirc may perform similar functions during liver infections. Continued investigation of the protective capacity of Tcirc cell populations may be warranted under the view that (1) Tcirc cells be more easily elicited than resident counterparts using conventional vaccine approaches and (2) vaccines targeting both resident and non-resident memory CD8 T cells might engender more protective and longer-lasting immune responses. However, liver Trm cells will be more prominently discussed here because of their well-defined contributions to sterilizing immunity to liver-stage malaria.

CD8 T cell Priming by Whole Parasite Vaccination: Delivery Route Matters

Vaccine delivery route appears to dictate the quality and longevity of *Plasmodium*-specific memory CD8 T cell responses. This may be partly due to differences in CD8 T cell priming. Naïve CD8 T cells circulate through blood and SLOs until encountering cognate antigen displayed by **antigen-presenting cell** (APC) MHC class II[30]. APCs and CD8 T cells form an immunological synapse, a cell-cell connection that delivers TCR-, costimulatory molecule-, and cytokine-mediated signaling to promote naïve CD8 T cell differentiation into potent cytotoxic effector CD8 T cells[40]. Effector CD8 T cells then undergo robust proliferation, massive changes in gene expression, and traffic to infected tissues, where they contribute to pathogen clearance[41]. Subsequently, effector CD8 T cells undergo substantial numerical contraction, and surviving cells form the initial memory CD8 T cell pool. However, there is no evidence that effector CD8 T cells - which are thought to be primed in various SLOs during and after liver-stage malaria - contribute to control of liver-stage malaria in malaria naïve subjects. In part, this may be due to the short duration of the liver-stage, which lasts two days in mice and seven in humans[24], and the low number of sporozoites delivered by mosquito bite[42] and thus infected hepatocytes. Consequently, CD8 T cell-mediated sterilizing immunity to liver-stage malaria seems to only be achievable after generation of memory CD8 T cells.

Memory CD8 T cell establishment depends on coordinated signaling pathways and expression of surface receptors that are often regulated by transcription factors. Circulating memory CD8 T cell formation has been extensively studied, but knowledge gaps remain[43]. The transcription factor landscape of Trm cells is under intense study, with the general findings that transcription factors such as Hobit, Blimp-1, Runx3, and Klf2 promote expression of tissue retention genes or repress expression of tissue egress genes[27]. Hobit and Blimp1 are required for optimal liver Trm cell development in mice[44]. Interleukin-15 (IL-15), a cytokine constitutively secreted in the liver[45], induces CD8 T cell expression of the transcription factor ETV5, which drives Hobit expression and liver Trm formation[46]. Additionally, LFA-1, an integrin composed of CD11a/CD18 heterodimers that is highly expressed by memory CD8 T cells, appears to be required for mouse liver Trm cell formation, as CD11a-deficient CD8 T cells do not form liver Trm cells in response to RAS vaccination or systemic infection with lymphocytic choriomeningitis virus (LCMV)[32]. Of note, CD69, a canonical marker of tissue residency during homeostasis that is driven by TCR and cytokine signaling during inflammation[27], does not appear to be required for establishment or tissue residency of liver Trm cells after LCMV infection[47]. However, the role of CD69 in liver Trm formation after RAS vaccination is unknown.

The timing and location of inflammatory cues and cognate antigen influence memory CD8 T cell establishment. Sporozoites injected into mouse skin have several fates: remain in the skin, travel to the skin dLN, or enter the bloodstream to infect hepatocytes (Figure 1A). Some skin-migratory sporozoites enter the skin dLN and are captured by CD8α⁺CD11c⁺ dendritic cells (DC), a resident APC population. CD8α⁺CD11c⁺ DCs subsequently present sporozoite-derived antigens to prime naïve CD8 T cells[48]. CD8 T cells primed in the skin dLN migrate to the spleen and liver, and these cells appear contribute to protective liver-residing memory CD8 T cell populations[14]. Despite evidence for important roles of skin

dLN in generating liver-stage protection, RAS administered in the skin do not generate sterilizing immunity to liver-stage malaria in humans[4]. Sporozoites seem to infect hepatocytes to equal degrees after i.v. and subcutaneous (s.c.) vaccination, but s.c. delivery may engender immunoregulatory responses in the skin dLN and fewer liver-localized memory CD8 T cells[35].

A prominent role for the liver and liver dLNs in priming liver-stage protective CD8 T cell responses is increasingly supported. Protective epitopes such as TRAP are shed during hepatocyte invasion[49] and thus should be accessible to liver APC populations. Late-arresting genetically attenuated *Plasmodium* parasites (GAP) vaccination primes CD8 T cell responses with broader specificity than those primed after vaccination with early liver-stage arresting parasites, suggesting that unique epitopes are expressed during liver-stage parasites development[50, 51]. During mouse liver-stage malaria, monocyte-derived CD11c⁺CSFR1⁺F4/80⁺ APCs infiltrate the liver, capture *Plasmodium* from infected hepatocytes, and migrate to the liver dLN to prime naïve CD8 T cells that eventually form protective CD8 Tem and Trm cells[25] (Figure 1B). The liver dLN is the primary site of liver-stage specific CD8 T cell priming after infected mosquito bite. Mice without CD11c⁺CSFR1⁺F4/80⁺ APCs fail to develop immunity to liver-stage malaria after RAS vaccination[25]. Taken together, studies show that the skin and liver dLNs are sites of protective pre-erythrocytic-stage specific CD8 T cell priming after whole parasite vaccination. Elucidating mechanisms of memory CD8 T cell generation after s.c., i.m., and i.v. immunization will inform effective human vaccine development.

Data supporting the role of liver- and liver dLN-primed CD8 T cells in liver-stage immunity have inspired research into novel methods of generating liver Trm cells. A leading hypothesis in the field is that vaccines will generate long-lasting protection with fewer immunizations via 1) an initial vaccine that primes memory CD8 T cells against a protective epitope, and 2) a second booster vaccine that “traps/targets” circulating CD8 T cells to form liver Trm cells by using a hepatotropic vector expressing cognate antigen. Using this “prime-and-trap/target” strategy, large numbers of protective liver Trm cells can be generated in mice by priming and boosting with various combinations of adjuvants, hepatotropic viral vectors, and whole parasite vaccines[31, 52, 53]. Despite this, translation of these approaches to humans remains challenging, as clinical trials demonstrate detectable circulating CD8 T cell responses but low levels of liver-stage protection in malaria-naïve humans immunized with a prime-boost strategy of modified adenovirus and vaccinia virus expressing protective liver-stage epitopes[5, 9]. However, this is likely due to i.m. administration of the vaccine, as mouse studies demonstrate that “prime and target methods generate superior protective memory CD8 T cell responses and immunity when delivered i.v. [53]. Further exploration of the requirements for inducing optimal liver-stage protective memory CD8 T cell responses in humans are necessary.

Memory CD8 T cell Maintenance: A Battle Against Attrition

Liver-stage malaria vaccination efforts frequently suffer from inability to induce long-lasting sterilizing immunity, probably a consequence of insufficient generation of and/or subsequent numerical attrition of protective memory CD8 T cells. Memory CD8 T cells are maintained

via homeostatic proliferation in response to environmental cues such as the pro-survival cytokine IL-15, which is required to maintain liver Trm cell numbers[54]. CSP antigen persists in RAS-immunized mice up to six weeks post vaccination, promoting optimal memory CD8 T cell generation in the spleen[14]. However, this may have consequences for liver Trm cell function, as persistent antigen after influenza virus lung infection drives functional exhaustion of lung Trm cells, as measured by increased expression of programmed cell death protein-1 (PD-1) and lymphocyte-activation gene 3 (LAG3)[55]. Exhausted liver Trm cells are found after whole parasite vaccination, a process that appears to be mediated in part by non-CD8 T cell type I IFN signaling[56]. Understanding how the cytokine milieu, antigen persistence, and other factors regulate liver Trm longevity may offer ways to extend vaccine-induced immunity.

Regulation of liver Trm cell function and number is likely important because these cells possess high cytotoxic potential and mediate liver damage in mice when present at aberrantly high numbers[46]. In specific-pathogen free mice, the half-life of a liver Trm cell generated from RAS vaccination or from *in vitro* activated effector CD8 T cells is estimated to be 28 days and 36 days, respectively[54]. In contrast, Tcirc cells generated after LCMV infection remain numerically stable for the life of the mouse[57], and smallpox virus-specific memory CD8 T cells are detectable in human blood decades after infection[58]. Trm cells use the purinergic receptor P2RX7 to sense extracellular nucleotides[59]. Extracellular nucleotides are released during sterile inflammation and tissue infection, and high nucleotide concentrations can trigger liver Trm cell death[59]. TCR signaling protects against P2RX7-mediated apoptosis, suggesting that non-malarial liver or systemic infections, which may be frequent in malaria endemic areas, could erode the malaria-specific liver Trm cell compartment of an immunized human. Indeed, numerical attrition of protective CSP-specific memory CD8 T cells occurs in RAS-immunized mice sequentially exposed to viral pathogens[60]. Of note, booster vaccination rescues this numerical attrition and restores loss of liver-stage immunity[60]. Additionally, lung Trm cells experiencing multiple antigen exposures in the context of vaccination are more numerically stable than those Trm cells experiencing only one vaccination, suggesting that repeated antigen exposure may promote Trm cell longevity[61]. Thus, numerical attrition of liver Trm cells may explain the need for repeated whole parasite booster vaccination to induce sterilizing immunity, which predominantly generates liver Trm cells in mice and NHP[4, 31].

An emerging role of Tcirc cells is as a “reservoir” to generate additional protective Trm cells after vaccination. This hypothesis is supported by mouse studies showing that circulating memory CD8 T cells generate skin Trm cells after viral infection[39, 62] and Tcirc cells convert to mucosal Trm cells *in situ* upon cognate antigen challenge[63]. Trm cells also robustly proliferate during recall responses in the skin[62] and FRT[63], suggesting that secondary memory Trm cells can originate from circulating and/or resident progenitors. Thus, generation of diverse memory CD8 T cell populations through vaccination might provide more resilient immunity.

Detecting Liver-Stage Malaria: Surveillance Strategies in Homeostasis and Inflammation

Mouse liver Trm cells exhibit a crawling motility in the vascular liver sinusoids *in situ*, a pattern observed in other tissues such as the female reproductive tract (FRT)[63], while Teirc cells rapidly flow through the liver sinusoids[31, 32]. It is estimated that approximately 2.5 million liver Trm cells are required to screen 99% of hepatocytes for infection during a 48-hour window of mouse liver-stage malaria[31], supporting the hypothesis that a large number of protective liver Trm cells are required to prevent progression to blood-stage. Such numerical requirements would likely be different in humans, whose liver mass is thousands of times greater than those of mice, but human T cells also have 7 days to find and eliminate infected hepatocytes before progression to blood-stage malaria. Increased understanding of mechanisms of liver Trm and other CD8 T cell subset liver surveillance during homeostasis and inflammation is important to understand how to promote robust detection of liver-stage infection.

Homeostasis

CD8 T cells express an array of surface chemokine receptors and integrins that mediate localization to various tissues, each of which express distinct homing ligands and soluble factors, during homeostasis and inflammation[64]. Liver Trm cells share surface markers with other Trm subsets, including CD69, which limits lymphocyte egress to dLNs through downregulation of the sphingosine-1-phosphate receptor (S1P1R), and CD44, which binds to hyaluronic acid and extracellular matrix proteins to promote cell structure maintenance, migratory capacity in peripheral tissue, and transmigration during immune challenge[65]. However, the unique hepatic microenvironment (Figure 1C), consisting of sinusoids lined by liver sinusoidal endothelial cells (LSECs)[66], dictates unique local CD8 T cell patterns of chemokine receptor and integrin expression. For example, in contrast to skin and lung Trm cells, mouse liver Trm cells do not express high levels of CD103, a receptor that localizes cells to epithelial cell connections[31, 67]. This may be due to low levels of E-cadherins, the ligand for CD103, in the liver. However, some human liver Trm cells express CD103[34], suggesting species-specific roles of CD103 in liver Trm cell function.

LSECs express CXCL16, which mediates effector CD8 T cell homing to the liver via interaction with CXCR6 in mouse models of inflammation[68], and liver Trm cells express CXCR6 during homeostasis[31]. Liver Trm cells highly express CD49a[44], a component of the VLA-1 integrin that binds to collagen in the basement membrane of epithelial cell layers. CD49a appears to be involved in promoting establishment and survival of Trm cells[65]. Interestingly, hepatic sinusoids express relatively low levels of ICAM-1[69], the ligand for LFA-1, suggesting that CD8 T cell interactions of moderate strength may promote optimal liver Trm patrolling phenotype. The diverse array of homing receptors and integrins expressed by liver Trm cells suggests that complex, incompletely understood networks of motility factors drive liver Trm dynamics.

Inflammation

Tissue infection or sterile inflammation result in endothelial cell upregulation of selectins and integrin ligands that mediate intravascular effector and memory CD8 T cell rolling, adhesion, and extravasation at the postcapillary venule[70]. Such endothelial cell alterations can be mediated in mice by IFN γ from Trm cells in the skin and FRT responding to local infection, a process named “sensing and alarm function”[63, 67, 71]. Although liver Trm cells are known to produce high levels of IFN γ in response to *in vitro* TCR stimulation[31], it is unknown if liver Trm-derived IFN γ mediates lymphocyte recruitment during liver-stage malaria.

Lymphocyte infiltration of the liver appears to differ greatly from canonical tissue recruitment, as effector CD8 T cell adhesion occurs in both the sinusoids and post-capillary venules and is not preceded by rolling[72]. Selectins, mediators of lymphocyte tethering and rolling, are not involved in lymphocyte adhesion at liver sinusoids[69]. Although some integrins, such as LFA-1, dictate liver Trm cell dynamics *in situ*[32], they appear to be redundant or uninvolved in effector cell recruitment to the inflamed liver[69]. Effector CD8 T cells secrete interferon gamma (IFN γ) during viral infection of the liver, and IFN γ mediates local expression of the CXCR3 ligands CXCL9, CXCL10, and CXCL11 to promote effector cell recruitment[73, 74]. During liver-stage infection of malaria-naïve mice, *Cxcl9* and *Cxcl10* are upregulated in the liver and IFNAR signaling mediates innate immune cell recruitment[75]. However, CXCR3 ligand blockade only partially reduces effector CD8 T cell recruitment to the liver[74], suggesting that multiple recruitment mechanisms exist. These issues have yet to be addressed in CD8 T cell immunity to liver-stage malaria.

Platelets bind to LSEC-associated extracellular hyaluronan in a CD44-dependent manner in mouse models of hepatitis B virus (HBV) infection[76]. Effector CD8 T cells subsequently dock on platelet clusters in an antigen-independent manner, crawl along the sinusoidal endothelium until encountering hepatocytes presenting cognate antigen, and arrest. Cytotoxic effector CD8 T cells then extend processes through porous LSEC fenestrations to interact with hepatocyte MHC-I, triggering cytokine production and hepatocyte killing in a diapedesis-independent manner[76]. It is unknown whether memory CD8 T cells use similar mechanisms to infiltrate the liver.

In vitro-activated CSP-specific effector CD8 T cells cluster around hepatocytes infected with green fluorescent protein (GFP)-expressing liver-stage parasites[36]. Cluster formation depends on pre-erythrocytic-stage specific CD8 T cells and G protein-coupled receptor signaling, but specific and non-specific CD8 T cells are equally represented in clusters. Infected hepatocytes surrounded by robust CD8 T cell clusters appear to die with associated parasite destruction as observed by loss of GFP expression, whereas infected hepatocytes affiliated with one or fewer T cells survive. These data suggest that, at least for effector CD8 T cells, a single malaria-specific T cell may be insufficient to clear an infected hepatocyte. Whether similar constraints exist for infected hepatocyte elimination by resident or circulating memory CD8 T cells remains unknown.

Thus, delineating the mechanisms that distinct memory CD8 T cells subsets use to patrol the liver and localize to sites of infection is a promising area for investigation with direct relevance to generation of effective vaccines against liver-stage malaria.

Sterilizing Liver-Stage Immunity: All or Nothing

Memory CD8 T cell-mediated immunity to liver-stage malaria faces a daunting challenge: eliminate all infected hepatocytes within two (mouse) or seven (human) days or risk progression to blood-stage. Natural malaria infection likely relies on evasion of the immune system to succeed, as an infected mosquito bite only delivers tens to hundreds of sporozoites into the skin[42] and a miniscule fraction of hepatocytes are infected[77]. Such a low parasite burden may trigger little inflammation, resulting in a “needle in a haystack” scenario for CD8 T cell elimination of all infected hepatocytes. Consequently, robust methods of detecting and killing infected hepatocytes are critical to ensure sterilizing liver-stage immunity. Localized innate immune responses probably assist memory CD8 T cells in identifying infected hepatocytes, as CXCL9, CXCL10, IFN β , and IFN γ are produced during liver-stage infection of mice, mediating induction of a liver-wide state of resistance to subsequent sporozoite infection[75]. Additionally, a fraction of liver-stage infected hepatocytes rapidly die *in vitro*, and hepatocyte death limits the liver parasite burden present immediately prior to progression to blood-stage infection *in vivo*[78]. However, these innate immune responses and effector CD8 T cells do not completely clear liver-stage malaria, thus, liver-stage specific memory CD8 T cells are required for sterilizing immunity[79].

Cytotoxic T lymphocytes (CTLs), including effector CD8 T cells and memory CD8 T cells, establish cell-cell contact via TCR binding to peptide-MHC-I on the target cell surface, forming an immunological synapse. Immunological synapse formation triggers the CTL to activate cell killing mechanisms, including secretion of soluble cytotoxic molecules such as perforin and granzymes, surface expression of the Fas ligand (FasL) death domain, and secretion of inflammatory cytokines including IFN γ and tumor necrosis factor α (TNF α) [79]. These molecules trigger target cell apoptosis through methods such as cell membrane pore formation and caspase activation.

Sterilizing immunity to malaria in mice relies on *Plasmodium* strain-specific combinations of memory CD8 T cell-derived IFN γ , TNF α , and perforin, but does not require granzymes and FAS-FASL interactions[80] (Figure 1D). Liver Trm and Tem cells express IFN γ , TNF α , granzyme B, and the degranulation marker CD107a upon TCR stimulation *in vitro*, although Trm cells express higher levels of these cytotoxic molecules[31]. Liver-stage infected hepatocytes are resistant to FAS- and TNF α -mediated killing *in vitro*[78, 81], suggesting that parasite-mediated mechanisms of immune-evasion may dictate stringent requirements for sterilizing immunity. Determining the kinetics of *in vivo* cytotoxic effector molecule and inflammatory cytokine and chemokine production by distinct memory CD8 T cell subsets will provide a clearer understanding of the composition of optimally protective memory CD8 T cell pools.

Concluding Remarks

For reasons incompletely understood, long-lived CD8 T cell-mediated immunity against liver-stage malaria is difficult to engender in humans. Recent advances inform us that liver Trm cells are critical for sterilizing immunity to liver-stage malaria[31–33, 56]. Liver Trm generation in mice can be achieved by “prime and trap/target” strategies that utilize hepatotropic vectors and adjuvants[31, 52]. However, many knowledge gaps remain (see **Outstanding Questions**), including requirements for optimal liver Trm cell generation and maintenance, memory CD8 T cell mechanisms of liver immunosurveillance, precise contributions of distinct circulating memory CD8 T cell subsets to liver-stage immunity, and *in situ* dynamics and effector functions of memory CD8 T cells during liver-stage infection. Finally, there are few studies of human liver Trm cells[34, 82, 83], none of which examine liver-stage malaria, and translational research to bridge animal and human models is warranted. Addressing these issues may allow for creation of vaccines that induce more durable and protective immunity with fewer booster doses and more deliverable administration routes.

Limitations on evaluating antigen-specific responses and tissue accessibility restrict study of the immunology of the human liver, although minimally invasive liver tissue sampling techniques are being explored[82]. Because of this, resolving many important knowledge gaps will require use of NHP, which permit tissue access and infection with human malaria strains, and mice, which permit tissue access, transgenic approaches, and evaluation of MHC-I restricted antigen-specific CD8 T cell responses. Continual basic and translational scientific efforts are required to inform existing and novel vaccine clinical trials.

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Glossary

Antigen

a molecule or processed molecular fragment that is recognized by a B-cell receptor or T cell receptor.

Antigen-presenting cell (APC)

processes and presents antigen via MHC-I (all nucleated cells) or MHC-I and MHC-II (professional APCs). Professional APCs include dendritic cells (DCs).

CD8 T cell

a lymphocyte that expresses a T cell receptor clone specific for one epitope and the molecule CD8 α , which bind to peptide-MHC-I on antigen-presenting cells.

Chemokine

a cytokine that specifically regulates cell chemotaxis.

Cytokine

a protein secreted by immune cells that binds to receptors to mediate immune signaling pathways.

Integrin

a protein that mediates cell binding to extracellular matrix components. Binding of an integrin may also regulate intracellular signaling pathways.

Pre-erythrocytic stage

a malaria parasite stage that precedes the blood-stage, i.e., sporozoite and liver-stages.

Secondary lymphoid organs (SLOs)

sites where naïve CD8 T cells encounter antigen presented by APCs, differentiate into effector CD8 T cells, and exit the SLOs. Includes spleen and draining lymph nodes (dLNs)

Sporozoite

liver-stage infectious form of *Plasmodium* delivered to host by infected mosquito bite.

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Box 1.**Life Cycle of Malaria Parasites**

Plasmodium sporozoites are deposited in the host dermis while an infected mosquito takes a blood meal. From there, sporozoites access the bloodstream, traffic to the liver, and infect hepatocytes. Inside hepatocytes, sporozoites within a parasitophorous vacuole replicate and differentiate over the course of two (mice) or seven (human) days, forming schizonts containing large numbers of merozoites. Merosomes, aggregates of merozoites, are released as hepatocytes die. Upon entering the blood stream, merosomes degrade and release merozoites, which infect red blood cells (RBCs) to initiate blood-stage infection. Within RBCs, merozoites differentiate into asexual trophozoites. Infected RBCs support trophozoite maturation into merozoites, which culminates in RBC lysis and release of additional merozoites to sustain blood-stage infection. A fraction of merozoites differentiate into gametocytes, the parasite sexual stage. Mosquitoes ingesting gametocyte-containing RBCs support *Plasmodium* sexual reproduction and subsequently can deliver sporozoites to new hosts via bite. The *Plasmodium* species *vivax* and *ovale* can form a dormant infection in the liver, leading to later recrudescence of blood-stage infection that does not rely on a mosquito vector.

Prevention of liver-stage progression to blood-stage through vaccination is appealing because (1) the clinically silent liver-stage is a bottleneck that the parasite must pass through, (2) blood-stage malaria is associated with significant clinical morbidity and mortality, and (3) blood-stage infection allows new mosquitoes to acquire the parasite and propagate the disease.

Highlights

- Novel discoveries about the role of memory CD8 T cells in mediating immunity to liver-stage malaria are informing methods of resolving historical issues of malaria vaccine efficacy and implementation.
- Liver resident memory (Trm) cells are essential to mediate sterilizing immunity against liver-stage malaria after whole-parasite immunization in mouse models. Circulating memory CD8 T cell contributions to protection remain undefined.
- Mechanisms of liver Trm cell generation, maintenance, and protective function are active areas of investigation.
- The unique hepatic microenvironment appears to dictate phenotypic characteristics and motility dynamics of local liver Trm cells at homeostasis.

Outstanding Questions

- What environmental cues and cellular signaling pathways promote optimal generation and maintenance of liver Trm cells?
- What are the mechanisms by which liver Trm cells control liver-stage malaria; including roles for cytotoxic molecule production, cytokine-mediated tissue changes, and coordination of responses by other lymphocyte and monocyte populations?
- Do circulating memory CD8 T cells contribute to control of liver-stage malaria?
- How do discoveries in the mouse and non-human primate models translate to the human liver, a relatively inaccessible tissue?
- What are the mechanisms by which humans residing in malaria endemic areas form suboptimal immunity after vaccination as compared to counterparts in non-endemic areas?

Figure 1, Key Figure. Current Understanding of Memory CD8 T Cell Establishment After Whole Parasite Vaccination and Subsequent Protective Recall Responses in Mice.

A. Attenuated sporozoites enter hepatocytes and form an infection that arrests without progression of any parasites to the blood-stage[24]. Type I and II interferon mediate suppression of the liver-stage[75]. Intracellular contents from dying hepatocytes, including liver-stage malaria antigens, are captured by CD11c⁺CSF1R⁺F4/80⁺ antigen presenting cells (APCs), which subsequently traffic to the liver draining lymph node (dLN)[25]. **B.** APCs present liver-stage antigens to naïve CD8 T cells via MHC-I along with costimulatory signaling molecules and cytokines. Naïve CD8 T cells differentiate to effector and finally memory CD8 T cells, segregating into resident (Trm) and circulating (Tcirk) memory populations[25]. **C.** Liver Trm cells crawl along the liver sinusoidal endothelial cells (LSEC) at the steady state, while Tcirk cells rapidly flow through the sinusoids[31, 32]. Kupffer cells, liver resident macrophages, and stellate, quiescent cells that mediate hepatic fibrosis in inflammatory conditions, occupy liver anatomical niches[66]. LSECs produce IL-15[45], a cytokine required for liver Trm development and maintenance[54]. **D.** Vaccine-elicited liver Trm[31, 32, 56] and potentially Tcirk cells specific for sporozoite- and liver-stage-derived antigens that are accessible to hepatocyte MHC-I processing control virulent liver-stage

malaria. Memory CD8 T cell-derived IFN γ , TNF α , and perforin are important for liver-stage control depending on the infecting strain of *Plasmodium*[80].

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