### Title

CD8+ T Cells Specific for a Malaria Cytoplasmic Antigen Form Clusters around Infected Hepatocytes and Are Protective at the Liver Stage of Infection

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CD8+ T cells specific for a malaria cytoplasmic antigen form clusters around infected hepatocytes and are protective at the liver stage of infection

Running title: CD8+ T cell recognition of cytoplasmic malaria antigen

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ABSTRACT

Following *Anopheles* mosquito-mediated introduction into a human host, *Plasmodium* parasites infect hepatocytes and undergo intensive replication. Accumulating evidence indicates that CD8\(^+\) T cells induced by immunization with attenuated *Plasmodium* sporozoites can confer sterile immunity at the liver stage of infection; however, the mechanisms underlying this protection are not clearly understood. To address this, we generated recombinant *Plasmodium berghei* ANKA expressing a fusion protein of an ovalbumin epitope and green fluorescent protein in the cytoplasm of the parasite. We have shown that the ovalbumin epitope is presented by infected liver cells in a transporter associated with antigen processing–dependent manner and becomes a target of specific CD8\(^+\) T cells (OT-I cells), leading to protection at the liver stage of *Plasmodium* infection. We visualized the interaction between OT-I cells and infected hepatocytes by intravital imaging using two-photon microscopy. OT-I cells formed clusters around infected hepatocytes, leading to the elimination of the intra-hepatic parasites and subsequent formation of large clusters of OT-I cells in the liver. Interferon-\(\gamma\) expressed in CD8\(^+\) T cells was dispensable for this protective response. Additionally, we found that polyclonal
ovalbumin-specific memory CD8\(^+\) T cells induced by de novo immunization were able to confer sterile protection, although the threshold frequency of the protection was relatively high. These studies revealed a novel mechanism of specific CD8\(^+\) T cell-mediated protective immunity, and demonstrated that proteins expressed in the cytoplasm of *Plasmodium* parasites can become targets of specific CD8\(^+\) T cells during liver-stage infection.
INTRODUCTION

Plasmodium sporozoites are transmitted by the bites of Anopheles mosquitoes under the skin and are transported via the bloodstream to the liver, where they infect hepatocytes. Immunization with irradiated sporozoites can induce sterile protection at pre-erythrocytic stages of infection in both mice and humans (1-3). Similarly, sterile protective immunity is induced by Plasmodium parasites that have been genetically attenuated by a gene deletion and which arrest at the hepatic stage (4, 5). Recent studies have shown that the infection of mice under chloroquine shield induces a protective immune response at the hepatic stage of infection (6). Immunization by these methods induces multiple different mechanisms of protection involving CD8+ T cells, CD4+ T cells, B cells, and NK cells (7, 8). Among the major effector cells are CD8+ T cells, which recognize malaria antigen in association with major histocompatibility complex (MHC) class I during liver-stage infection (9).

Targets for protective immunity against malaria were identified using antibodies obtained from mice immunized with irradiated sporozoites, including circumsporozoite protein.
(CSP), which was extensively investigated (10, 11). CSP is expressed on the surface of sporozoites and liver-stage malaria parasites and is the most advanced target antigen of liver-stage vaccine development. The major liver-stage effector cells specific for CSP are CD8$^+$ T cells, as shown by the depletion of CD8$^+$ T cells with the antibody abrogating protection and by the resistance to subsequent challenge infection conferred by cloned specific T cells. Further studies using CSP-transgenic mice indicated that additional protective antigens are present, although CSP is the major antigen that can induce protection against pre-erythrocytic forms of malaria in BALB/c mice (12). Additional candidate antigens at the liver stage of infection include sporozoite surface protein 2 (SSP), which was identified using an antibody produced by BALB/c mice after immunization with irradiated sporozoites, and which induces protection that is mediated by CD8$^+$ T cells, CD4$^+$ T cells, and antibodies (13-15). Protective immunity via immunization is much more difficult to establish in C57BL/6 (B6) mice than in BALB/c mice, partly because the H-2$^b$-restricted cytotoxic T lymphocyte (CTL) epitope is not present in CSP (16). However, protection is induced in B6 mice by immunization with attenuated *Plasmodium* parasites or infection under a chloroquine shield. This protective
immunity is also mediated by CD8+ T cells, whose target antigen is not CSP. These latter studies suggest the existence of unknown target antigens recognized by CD8+ T cells in infected hepatocytes, in addition to CSP and SSP2.

Research efforts are in progress to identify novel malaria antigen targets expressed at the liver stage. Genome-wide expression profiling studies have indicated that many malaria proteins are expressed during liver-stage infection (17, 18). However, the criteria that would frame the search for target malaria antigens have not yet been established. Several studies have suggested that the localization of antigen within microbial pathogens is important for the generation of specific T cells and the resulting protection. It is generally thought that secreted antigens are more accessible to antigen presentation pathways and induce strong T cell immune responses (19). For example, intracellular bacteria such as Mycobacterium tuberculosis remain in the phagosome, where they survive and replicate. The secreted form of the antigens expressed in these bacteria can be presented via the MHC I pathway, through a process that appears to be facilitated by an increase in permeability of the endosomal membrane by the microbe (20, 21). In an infection model
using recombinant *Trypanosoma cruzi* expressing an ovalbumin (OVA) epitope, it was shown that host cells were able to present OVA via the MHC I pathway when the antigen was produced in secretory form, but not the cytoplasmic or transmembrane form (22). It has also been proposed that CSP is released from the surface of sporozoites directly into the cytoplasm of host hepatocytes, where it binds to RNA-associated host cell targets (23, 24). Furthermore, CSP is released from the surface of sporozoites when they travel through hepatocytes before reaching the final infected hepatocyte, and appears to be presented by these traversed hepatocytes to specific T cells (25). Therefore, the search for candidate malaria antigens for liver-stage infection is generally focused on molecules expressed on the surface of parasites. However, it is not clear whether intracytoplasmic molecules are able to become targets of the protective immune responses during liver-stage infection.

In this study, we generated recombinant parasites that exhibited cytoplasmic expression of an OVA epitope presented by MHC I. We examined whether this epitope was presented by infected hepatocytes and whether it became a target of specific OT-I CD8+ T
cells leading to protection at the liver stage of infection. We also examined the mechanisms underlying the presentation of this antigen and visualized the interaction of OT-I cells with infected hepatocytes by intravital imaging using two-photon microscopy (TPM). The results of these experiments suggest that CD8\(^+\) T cells can recognize cytoplasmic malaria antigens, form clusters around infected hepatocytes, and protect against parasites.
MATERIALS AND METHODS

Parasites

Recombinant *P. berghei* ANKA (PbA) expressing class II and class I OVA epitopes fused to the N- and C-terminus of a *P. yoelii* hsp70 fragment (PbA-hsOVA), respectively, and *P. berghei* ANKA expressing OVA class I epitope fused to the C-terminus of green fluorescent protein (GFP) (PbA-gfpOVA) were constructed as previously described (26) (Fig. 1A). PbA-hsOVA expresses a recombinant fusion protein containing the N-terminal sequence (aa 1–5) of *P. berghei* hsp70, an OVA323-339 MHC II epitope, a truncated sequence (aa 201–398) of *P. yoelii* hsp70, and an OVA257-264 MHC I epitope. PbA-gfpOVA express a protein containing an OVA257-264 MHC I epitope fused to the C-terminus of GFP. After transfection, mice were infected and were maintained under the presence of the anti-malaria drug pyrimethamin. PbA-gfpOVA were enriched by sorting of GFP-positive erythrocytes using FACSaria (BD Biosciences, San Jose, CA). The stable transfecant was cloned by limiting dilution in mice and was maintained by alternating passage between *Anopheles stephensi* and BALB/c mice. Sporozoites were prepared from salivary glands of *A. stephensi* after 18–24 days of infection with
Animals

OT-I and OT-II transgenic mice expressing the T cell receptor (TCR) specific for OVA257-264/K\textsuperscript{b} and OVA323-339/I\textsuperscript{Ab}, respectively, were provided by Dr. H. Kosaka (Osaka University, Osaka, Japan) (27, 28). TAP\textsuperscript{−/−} mice (B6 background) were provided by Dr. H. Watanabe (Ryukyu University, Okinawa, Japan) (29). B6.SJL and OT-I or OT-II mice were interbred, and the offspring were intercrossed to obtain CD45.1\textsuperscript{+} OT-I or OT-II mice. DsRed transgenic, IFN-γ\textsuperscript{−/−} and perforin\textsuperscript{−/−} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). DsRed transgenic mice and OT-I mice were crossed to produce DsRed/OT-I mice. OT-I and IFN-γ\textsuperscript{−/−} or perforin\textsuperscript{−/−} mice were bred to produce IFN-γ\textsuperscript{−/−} OT-I mice, perforin\textsuperscript{−/−} OT-I mice, and IFN-γ\textsuperscript{−/−} perforin\textsuperscript{−/−} OT-I mice. B6 and BALB/c mice were purchased from SLC (Shizuoka, Japan). Mice were maintained in the Laboratory Animal Center for Animal Research at Nagasaki University and were used at the age of 8–14 wks. To generate bone marrow chimeras, B6 or TAP\textsuperscript{−/−} mice were lethally irradiated (900 rad) and received bone marrow cells (1.0 × 10\textsuperscript{7}; prepared from
TAP$^{\alpha}$ or B6 mice) intravenously on the following day. Mice were left for at least two months before infection to allow for reconstitution of the lymphoid system. The animal experiments reported herein were approved by the Institutional Animal Care and Use Committee of Nagasaki University and were conducted according to the guidelines for Animal Experimentation at Nagasaki University.

Adoptive transfer and PbA infection

To prepare activated OT-I cells, pooled cells from the spleen and inguinal lymph nodes of OT-I mice were prepared and cultured in the presence of OVA$_{257-264}$ peptide (2 $\mu$g/ml) for 3 days. OT-II cells were purified from spleen and inguinal lymph node cells of OT-II mice using anti-CD4 IMag (BD Biosciences). Dendritic cells were prepared from B6 splenocytes using CD11c-microbeads and AutoMACS (Myltenyi Biotec, Bergisch Gladbach, Germany). OT-II (6 $\times$ 10$^6$/ml) and dendritic cells (1 $\times$ 10$^5$/ml) were co-cultured in the presence of OVA$_{323-339}$ peptide (3 $\mu$g/ml) for 5 days. Mice received OT-I (1–100 $\times$ 10$^5$) or OT-II (3 $\times$ 10$^7$) cells through the tail vein, and were challenged with 300–500 infectious sporozoites 2 days later. The proportion of OT-I (CD45.1) cells
in the total CD8+ T cell population was determined by staining peripheral blood lymphocytes (PBLs) with APC-anti-CD8 and PECy7-anti-CD45.1 mAbs. For the experiments involving *de novo* priming of CD8+ T cells (Fig. 6) and parasite burden in the liver (Fig. 3), mice were challenged with 1,000 and 5,000 sporozoites, respectively. Mice were monitored for parasitemia daily (starting 4 days after infection) by microscopic examination of standard blood films. Parasite burden was determined by real-time PCR using liver RNA and is expressed as a ratio of the cDNA of *Plasmodium* 18S rRNA to cDNA of mouse G3PDH, as described previously (30).

**Confocal and two-photon microscopy**

PbA-gfpOVA sporozoites were obtained from the salivary glands of infected *A. stephensi* mosquitoes. To prepare PbA-infected hepatocytes, HepG2 cells (1 × 10^4) were cultured in HepG2 medium (500 µL; DMEM containing 10% fetal calf serum, 1% penicillin/streptomycin, and 1% non-essential amino acids) using Fluorodish (World Precision Instruments, Sarasota, FL) for 3 days as described previously (31). PbA-gfpOVA sporozoites (1 × 10^4) were added to the culture and incubated for 3 h,
followed by the addition of invasion medium (500 µL; HepG2 medium supplemented with 3 mg/ml of glucose). The medium was replaced 12 h later, and the culture was maintained for a total of 24 h in the invasion medium, after which cells were stained. PbA-gfpOVA-infected red blood cells (RBCs) were collected from the tail vein of the infected mice. Sporozoites, infected HepG2 cells, and RBCs were incubated in the presence of Bodipy-TR-C5-ceratide (5 µM, Invitrogen, Carlsbad, CA) for 15 min at 37°C, washed 3 times with PBS, and stained with DRAQ5 (1.25 µM, Biostatus, Leicestershire, UK) for 30 min at 37°C. Images were acquired with an inverted TCS SP5 MP confocal microscope with a 63× glycerol immersion lens (Leica Microsystems, Wetzlar, Germany).

For intravital imaging, spleen cells and lymph node cells from DsRed/OT-I mice were cultured in the presence of OVA257-264 for 3 days. Activated DsRed/OT-I cells (3–10 × 10⁶) were adoptively transferred into B6 mice. Two days later, the mice were infected (or not infected, for controls) with PbA-gfpOVA sporozoites (1 × 10⁴). At 40–48 h post-infection, mice were anesthetized with isoflurane. The abdomen was then shaved
and a midline incision was made through the dermis and peritoneum and the liver was carefully exteriorized. Mice were placed on a platform with a centrally located hole, where a cover glass was attached. An O-ring with a 9.8 mm inner diameter was placed on the cover glass to prevent movement of the liver during imaging. Images were acquired with an inverted TCS SP5 TPM microscope equipped with an OPO laser (Leica Microsystems) and with a 25 × 0.95NA water immersion objective. During observation with fluorescence microscopy (DMI6000B, Leica Microsystems), the numbers of GFP^+ infected hepatocytes and OT-I clusters were determined by counting manually within the field inside the O-ring (~75 mm^2). The number of OT-I cells in each cluster was determined using Imaris software (Bitplane, Zurich, Switzerland) after acquiring a 3 dimensional image of each cluster with TPM.

Generation of OVA-specific memory CD8^+ T cells

Specific memory CD8^+ T cells were induced in mice as described previously (32) with slight modifications. B6 mice were immunized intravenously with bone marrow-derived dendritic cells (2.5 × 10^5) pulsed with OVA_{257-264} peptide (1 mM). Seven to 9 days later,
these mice were boosted by infection with *Listeria monocytogenes* expressing OVA (LM-OVA; 1–10 × 10^6 CFU) (33). After 2 months, PBLs from these mice were stained with FITC-anti-CD8 mAb and PE-OVA_{257-264}/H-2K^b tetramer (MBL, Nagoya, Japan), and the proportion of OVA-specific CD8^+ T cells was determined using FACS Canto II (BD Biosciences).

**Statistical Analysis**

Data are expressed as means ± standard deviation (SD). Statistical analysis was performed using the Mann-Whitney *U* test for the comparison of two experimental groups, and the data were analyzed using GraphPad Prism software. Differences with a *p* value of < 0.05 were considered significant.
RESULTS

Cytoplasmic expression of OVA-GFP fusion proteins in recombinant PbA

To investigate the mechanisms of protection against liver-stage malaria, we generated two recombinant PbA constructs (Fig. 1A). The first construct expresses a fusion protein of the OVA257-264 epitope fused to the C-terminus of GFP (PbA-gfpOVA); the second expresses a fusion protein of the OVA323-339 MHC II epitope, a portion of *P. yoelii* hsp70, and the OVA257-264 MHC I epitope (PbA-hsOVA). The sequence of *P. yoelii* hsp70 was used because an antigen fused to this portion of hsp70 was shown to promote priming of specific T cell responses (34, 35). Since the fusion protein constructs did not contain a signal sequence, its expression was expected to be limited to the cytoplasm of the parasite.

To confirm the localization of the expressed protein, confocal microscopy was used to examine the expression of the fusion protein in sporozoites and infected cells after staining with membrane marker bodipy-TR-C5-ceramide and nuclear marker DRAQ5 (36) (Fig. 1B). The GFP-fused protein was localized in the cytoplasm of PbA-gfpOVA sporozoites. At 24 h post-infection with sporozoites, GFP protein was detected within the parasitophorous membrane of the infected HepG2 cells, but was not observed in the host.
cytoplasm. We also examined the expression of GFP in the infected RBCs, and observed that GFP was also localized within the parasitophorous membrane in these cells.

OT-I cell-mediated protection against liver-stage infection with PbA

We examined whether CD8$^+$ T cells from OT-I mice are protective against liver-stage infection with PbA-hsOVA and PbA-gfpOVA. OT-I cells were activated prior to transfer, since previous studies indicated that the activation of specific CD8$^+$ T cells was required for protection against sporozoite infection at the liver stage (37). B6 mice were inoculated with different doses of preactivated OT-I cells and then infected with PbA-hsOVA or wild-type PbA sporozoites, and the levels of parasitemia were monitored daily (Fig. 2A). Transferred OT-I cells were identified as CD45.1$^+CD8^{low}$ T cells (38). Mice that received $1 \times 10^7$ OT-I cells were completely protected from challenge infection with PbA-hsOVA but not with PbA, indicating that the protective effect was specific to the OVA-expressing parasites. We also observed that the protection was OT-I dose dependent, and that mice receiving less than $1 \times 10^6$ OT-I cells developed parasitemia (Fig. 2A). OT-I cells constituted 42.1% and 3.4% of the CD8$^+$ T cell population in PBL
from mice receiving $1 \times 10^7$ and $1 \times 10^6$ OT-I cells, respectively, indicating that high
levels of OT-I cells were required for sterile protection at the liver stage of infection. Similarly, sterile protection was observed when mice receiving OT-I cells were infected
with PbA-gfpOVA sporozoites (Fig. 2B). We also examined whether CD4$^+$ T cells from
OT-II mice were protective against the liver-stage infection with PbA-hsOVA (Fig 2C).
Although parasitemia appeared 5 days after infection in both mice transferred and not
transferred with OT-II, the levels of parasitemia were lower in the OT-II-transferred mice,
suggesting that OT-II cells have protective roles against infection with PbA-hsOVA. However, sterile immunity was never achieved at the liver stage by inoculation with
OT-II cells, although the proportion of OT-II cells in the CD4$^+$ T cell population was as
high as 43.8%.

To confirm that the observed decrease in parasitemia was due to the inhibition of parasite
growth at the liver stage, parasite burden in the liver was examined by real-time PCR of
parasite ribosomal RNA (Fig. 3A). OT-I cells were found to significantly inhibit the
parasite burden in the liver of mice infected with PbA-hsOVA (90.1% reduction), but not
with PbA, indicating that the protection was specific to the OVA-expressing PbA. We
next wanted to examine whether the OVA antigen-presenting pathway utilizes the
classical MHC class I pathway. To this end, B6 and TAP\(^{-/-}\) mice were inoculated with
OT-I cells, infected with PbA-hsOVA, and examined for parasite burden in the liver. OT-I
cells significantly inhibited the parasite burden in B6 mice (99.8% reduction), but not in
TAP\(^{-/-}\) mice after challenge infection with PbA-hsOVA sporozoites, indicating that the
antigen presentation pathway did utilize the classical TAP-dependent pathway (Fig. 3B).
Furthermore, we generated bone marrow chimeras between B6 and TAP\(^{-/-}\) mice to
examine whether TAP expressed in hematopoietic cells or hepatocytes is critical for the
protection. After inoculation with OT-I and infection with PbA-hsOVA, the parasite
burden in the liver was significantly reduced in bone marrow chimeras when B6 mice
were used as recipients. The reductions were 98.2% in the B6 → B6 chimera compared
to B6 → TAP\(^{-/-}\), and 98.1% in the TAP\(^{-/-}\) → B6 chimera compared to TAP\(^{-/-}\) → TAP\(^{-/-}\),
indicating that TAP expression in the radioresistant host is critical for the protection
against challenge infection with PbA-hsOVA (Fig. 3C). These results strongly suggest
that hepatocytes infected with PbA-hsOVA sporozoites process and present the OVA
epitope via the classical MHC class I pathway, which is consistent with a previous study using *P. berghei* expressing a mutant CS protein containing an OVA epitope (39).

**In vivo imaging of the interaction between OT-I cells and infected hepatocytes**

After observing the protective effect of OT-I cells, we aimed to directly visualize the interaction of infected hepatocytes with the effector OT-I cells using TPM. For this purpose, mice were inoculated (or not inoculated, for controls) with pre-activated DsRed/OT-I cells and infected with PbA-gfpOVA sporozoites. Two days later, the livers of the infected mice were surgically exposed and imaging was performed. In mice infected with PbA-gfpOVA sporozoites, GFP+ cells were clearly visible after 24 h and the quantity of GFP continued to increase for 24–48 h after infection (data not shown). We observed a defined surface area (75 mm²) of the liver using TPM at 40–48 h after sporozoite infection. When a low dose (3 × 10^6) of OT-I cells was inoculated into the mice, we observed numerous OT-I clusters formed around GFP+ cells (Fig. 4A). The number of OT-I cells in each of these clusters was relatively small (mean 34.2, range 10–71) (Fig. 4D GFP+). Using time-lapse imaging, we were able to observe the
disappearance of GFP$^+$ cells while in contact with OT-I cells, suggesting that the OT-I cells are directly involved in the elimination of intra-hepatic parasites (Fig. 4A right, supplementary Fig. 1). When the number of inoculated OT-I cells was increased to the dose sufficient for sterile protection ($1 \times 10^7$), fewer GFP$^+$ cells remained in the liver (Fig. 4B, C left panel), and the number of OT-I clusters increased (Fig. 4B, C right panel). The number of OT-I clusters in the liver of the OT-I-inoculated, PbA-infected mice was similar to the number of GFP$^+$ cells in the PbA-infected mice without OT-I-inoculation, suggesting that the clusters were formed following elimination of infected hepatocytes by OT-I cells (compare the left and right panels of Fig. 4E). Additionally, we determined that the number of OT-I cells in clusters containing GFP$^+$ cells (mean 28.4, range 14–48) was much lower than in clusters that did not contain GFP$^+$ cells (mean 293.8, range 15–1,415) in mice inoculated with $1 \times 10^7$ OT-I cells (Fig 4D). The OT-I clusters were barely detectable in OT-I-inoculated mice without PbA infection and, if present, were formed by small numbers of OT-I cells (Fig. 4E right panel, F).

**Effector function of OT-I cells**
The clustering of OT-I cells around infected hepatocytes suggests that the effector mechanisms of CD8\(^+\) T cells in liver-stage malaria might be different from the classical CTL killing mechanisms. Thus, we evaluated the effector function of CD8\(^+\) T cells during protection at the liver stage of infection with PbA-hsOVA or PbA-gfpOVA. CD8\(^+\) T cells were prepared from OT-I, IFN-\(\gamma\)^{-/-} OT-I, perforin^{-/-} OT-I, or IFN-\(\gamma\)^{-/-} perforin^{-/-} OT-I mice, activated \textit{in vitro}, and transferred into B6 mice, which were infected with sporozoites of PbA-hsOVA or PbA-gfpOVA and examined for parasitemia (Fig. 5). After infection with PbA-hsOVA, no parasitemia was detected in mice receiving IFN-\(\gamma\)^{-/-} OT-I, perforin^{-/-} OT-I, or IFN-\(\gamma\)^{-/-} perforin^{-/-} OT-I cells, indicating that the expression of IFN-\(\gamma\) and perforin in CD8\(^+\) T cells was dispensable for the protection against liver-stage infection (Fig. 5A). When the mice were infected with PbA-gfpOVA, a delayed onset of parasitemia was detected in 2/5 infected mice receiving transferred IFN-\(\gamma\)^{-/-} perforin^{-/-} OT-I cells, and 1/5 mice receiving perforin^{-/-} OT-I cells (Fig 5B). These results suggest that IFN-\(\gamma\) and perforin are partially involved in the protective effects of OT-I cells, although these molecules are not essential for protection. The difference in the results of infection with PbA-hsOVA and PbA-gfpOVA may be due to the differences in the
efficiency of antigen presentation; the OVA epitope may be more efficiently presented to
OT-I cells for PbA-hsOVA infection than for PbA-gfpOVA infection.

Finally, we examined whether OVA-specific polyclonal memory CD8\(^+\) T cells were protective against infection with PbA-hsOVA sporozoites following a previously described protocol (40). Mice were primed with OVA\(_{257-264}\)-pulsed dendritic cells and boosted with LM-OVA infection. Two months later, we examined the proportion of OVA-specific CD8\(^+\) T cells in PBL by staining with OVA/K\(^b\) tetramer. These mice were infected with PbA-hsOVA sporozoites, and the levels of parasitemia in peripheral blood were determined 8 days after infection (Fig. 6). Comparison of the number of tetramer-positive cells with the occurrence of parasitemia showed that mice bearing OVA-specific CD8\(^+\) T cells at levels more than 9.31% of total CD8\(^+\) T cells were completely protected from the sporozoite challenge, while those bearing specific CD8\(^+\) T cells in the range of 1.1–8.8% included both protected and unprotected mice.
DISCUSSION

In this study, we established a novel system to investigate the cellular and molecular mechanisms underlying the protective immune response against liver-stage infection with malaria parasites using a model malaria antigen, OVA. Unlike the CSP model, which utilizes BALB/c mice, our model can be applied in B6 mice. Cockburn et al. generated a model in which CS protein containing an OVA epitope was expressed on the surface of sporozoites, and used B6 mice for the study of protective immunity at the liver stage of infection (39). Our model is distinct from this model in that the antigen is expressed in the cytoplasm of malaria parasites, and can become a target of specific CD8$^+$ T cells during the liver stage of *Plasmodium* infection, leading to sterile protection. Protection was achieved by both the inoculation of activated OT-I cells and by the induction of polyclonal OVA-specific memory CD8$^+$ T cells. Since protection by OT-I cells was dependent on TAP molecule expression in non-hematopoietic host cells, consistent with the previous study (39), it is reasonable to speculate that OVA expressed in the cytoplasm of the parasite is somehow transported into the cytoplasm of hepatocytes for antigen processing. However, we did not detect any leakage of GFP into the
cytoplasm of the infected hepatocytes by confocal imaging. A possible explanation for
this is that cytoplasmic malaria antigens are processed to smaller peptides prior to transfer
into the host cells. Alternatively, the amount of the protein transported to the cytoplasm
may have been too low for visualization by our methods. Whatever the molecular
mechanisms, these results imply that malaria proteins expressed in the cytoplasm of
malaria parasites can be targets of protective immune responses, and should not be
excluded from the pool of candidate malaria vaccine targets.

In our experimental model, we employed intravital imaging to visualize the interaction
between PbA-infected hepatocytes and specific CD8+ T cells. In the absence of
inoculation with OT-I cells, infected hepatocytes were observed as isolated GFP+ cells, as
shown previously by others (41-43). When we used a lower number of OT-I cells for
inoculation (3 × 10^6), clustering of OT-I cells around the infected hepatocytes was
observed, suggesting that OT-I cells recognize the MHC/OVA epitope expressed on the
surface of hepatocytes, and make direct contacts with them. Using time-lapse imaging,
we were able to observe the disappearance of GFP+ intra-hepatic parasites during their
interaction with OT-I cells, implying that the OT-I clusters are directly involved in the elimination of the parasites in the liver. When the number of inoculated OT-I cells was increased to a level sufficient for sterile immunity \((1 \times 10^7)\), the number of GFP\(^+\) cells was dramatically reduced. Furthermore, we observed OT-I clusters that did not contain GFP\(^+\) hepatocytes, and some OT-I clusters were large (containing more than 1,000 OT-I cells), suggesting that the accumulation of OT-I cells in the cluster continued after the elimination of GFP\(^+\) hepatocytes. After the submission of this manuscript, Cockburn et al. (44) published an imaging study of CSP-specific CD8\(^+\) T cells eliminating liver-stage malaria parasites, and showed that CD8\(^+\) T cells form clusters around infected hepatocytes, similar to our study. Thus, cluster formation is not limited to our model system, but occurs in *Plasmodium*-specific CD8\(^+\) T cells eliminating malaria parasites during liver-stage infection.

The effector mechanisms of CD8\(^+\) T cell-mediated elimination of intra-hepatic parasites are complex. An earlier study suggested that perforin- or Fas-mediated killing is not the main pathway of parasite elimination during the hepatic stage of the infection (45).
Additionally, a recent study using CSP-specific transgenic T cells suggested that IFN-γ is not essential for the protection of mice against infection with *P. yoelii* sporozoites (46). However, IFN-γ and TNF-α have been reported to be important for the protection against liver-stage infection with *P. berghei* as well as *P. yoelii*, while perforin is important for protection against infection with *P. yoelii* but not *P. berghei* (47, 48). In our study, IFN-γ expressed in CD8+ T cells was dispensable for in the elimination of infected hepatocytes during infection with PbA-gfpOVA, whereas perforin was partially involved in this process. Therefore, unlike the elimination of virus-infected or transformed cells (49), perforin/granzyme-mediated killing is not the essential pathway for the elimination of malaria parasites in the liver. Effector CD8+ T cells were shown herein to form clusters around infected hepatocytes, leading to the elimination of the intra-hepatic parasites. These features suggest that a novel mechanism might be involved in the protective immune responses of CD8+ T cells against intra-hepatic parasites. It is intriguing to speculate that other hepatic immune cells such as dendritic cells, Kupffer cells and liver sinusoidal endothelial cells (43) are involved in parasite elimination.
Schmidt et al. showed that the proportion of CSP-specific memory CD8⁺ T cells correlated with sterilizing immunity at the liver stage, with protective effects observed when more than 1% of CD8⁺ T cells in PBL were CSP-specific (40). In our model, the threshold frequency of OVA-specific memory CD8⁺ T cells was much higher and required more than 8% OVA-specific CD8⁺ T cells to achieve sterile immunity in 100% of mice. The probability of sterile immunity was reduced to 28.6% (8/27) when OVA-specific CD8⁺ T cells constituted 1.1–8.8% of PBL. Therefore, the threshold frequency of memory CD8⁺ T cells required for the sterile immunity in our OVA system was higher than that in the CSP system. The localization of antigen expression may influence the efficacy and timing of antigen presentation by hepatocytes. CSP is expressed on the surface of the parasite; thus, it may be readily accessible to the cytoplasm of the infected hepatocytes soon after infection. Further, CSP might be transferred to sinusoidal endothelial cells when sporozoites migrate through hepatic sinusoids prior to infection, and these cells cross-present CSP to specific CD8⁺ T cells in a manner similar to hepatocyte-infecting viruses (50). In contrast, proteins expressed in the cytoplasm of parasites might be transferred to host cells relatively late after infection,
and thus may have a narrower window for sterile protection. Alternatively, the outcome of the individual studies may be affected by differences in the mouse strain used (BALB/c for the CSP study and B6 in our OVA study) or the levels of antigen expressed. A recent transcriptome approach revealed that approximately 2,000 genes are active during liver-stage infection (14). It is possible that many of these proteins are expressed in the cytoplasm of parasites and that combined polyclonal CD8+ T cell responses against different sets of these antigens might achieve sterile protection against malaria parasites in the liver.

Our study showed that malaria proteins expressed in the cytoplasm of parasites can be targets of the protective immune responses by CD8+ T cells. We also visualized the interaction between the infected hepatocytes and specific effector CD8+ T cells, which led to the elimination of the parasites in the liver, and revealed a novel aspect of the effector mechanisms of protective immunity in liver-stage infection. These findings enhance our understanding of the cellular and molecular mechanisms underlying the protective immune responses during the liver stage of malaria infection, and identify novel
candidates for malaria vaccine targets.
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**FIGURE LEGENDS**

**Figure 1**

**The expression of a model antigen in the cytoplasm of recombinant PbA.**

(A) Schematic representation of the transgenic PbA constructs used in this study. (B) PbA-gfpOVA sporozoites, HepG2 cells infected with PbA-gfpOVA sporozoites *in vitro*, and infected RBCs (iRBC) were stained with Bodipy-TR-C5-ceratide and DRAQ5, which mark membrane structure and nuclei, respectively. Images were obtained using confocal microscopy. Arrowheads indicate the margin of the RBC, and the scale bars indicate 5 µm.

**Figure 2**

**OT-I cells protect against infection with sporozoites of OVA-expressing PbA.**

B6 mice were inoculated with activated OT-I CD8+ T cells (0–10^7) (A, B) and infected with sporozoites (300/mouse) of PbA-hsOVA, wild-type PbA (A), or PbA-gfpOVA (B) 2 days later. Alternatively, after transfer with OT-II CD4+ T cells (3 × 10^7 or 0), mice were infected with PbA-hsOVA sporozoites (500/mouse) (C). Representative flow cytometry
profiles of PBLs from mice transferred with OT-I cells (1×10^7) (A, B) or OT-II cells (3×10^7) (C) are shown. The proportion of OT-I or OT-II cells within the total CD8^+ or CD4^+ T cell populations are indicated. Note that the levels of CD8 expression on activated T cells are reduced as reported previously (38). The number in the upper left of each graph indicates the number of transferred cells; the number in parentheses indicates the proportion of OT-I cells in the total CD8^+ T cell population in PBL at the time of infection.

Parasitemia was monitored daily starting 4 days after infection. Values significantly different (p < 0.05) from mice not receiving T cells are indicated (*). The experiments were performed twice (B, C) or 3 times (A); representative data are shown.

Figure 3

TAP-dependent presentation of MHC I epitope by infected host cells.

B6 or TAP^−/− mice were inoculated (or not inoculated, for controls) with activated OT-I CD8^+ T cells and infected with sporozoites (5×10^3) of PbA-hsOVA or PbA (A, B). The numbers in parentheses indicate the proportion of OT-I cells in the total CD8^+ T cell population in PBL at the time of infection (A). Bone marrow (BM) chimeras were generated between B6 and TAP^−/− mice (as described in the Materials and Methods section), inoculated with OT-I cells, and infected with PbA-hsOVA sporozoites (C). Two
days after infection, RNA was extracted from the liver of the infected mice, and parasite burden was determined by real-time PCR. The experiments were performed twice (A) or 3 times (B, C); representative data are shown. ns, not significant; *, $p < 0.05$; **, $p < 0.01$.

**Figure 4**

**Clustering of OT-I cells around GFP$^+$ infected hepatocytes during liver-stage infection with PbA-gfpOVA**

B6 mice were transferred with activated DsRed/OT-I CD8$^+$ T cells at a dose of $3 \times 10^6$ (A, C, D, F) or $1 \times 10^7$ (B, C-F), and were infected with PbA-gfpOVA sporozoites ($1 \times 10^4$). Some mice did not receive DsRed/OT-I or were not infected with PbA-gfpOVA as controls (E, F). Forty-eight hours after infection, the liver was imaged with TPM. The 2 dimensional projections of 3 dimensional imaging volumes are shown, and the scale bars indicate 10 µm (A, B). A still image of GFP$^+$ cell disappearance while in contact with OT-I cells is shown (A, right image; time-lapse image in supplementary Fig. 1). The numbers of GFP$^+$ cells and T-cell clusters were counted within a surface area of 75mm$^2$ using fluorescence microscopy (C, E). GFP$^+$ cells and T-cell clusters were imaged in 3 dimensions using TPM, and the number of OT-I cells within each cluster was determined.
using Imaris software (D, F). The number of OT-I cells were determined separately for clusters containing and not containing GFP+ cells (D). Bars indicate average. *, *p < 0.05; **, *p < 0.01; ***, *p < 0.0001.

Figure 5

Perforin and IFN-γ expressed in CD8+ T cells are dispensable for sterile immunity at the liver stage of infection. Activated CD8+ T cells from perforin−/−IFN-γ−/− OT-I, IFN-γ−/− OT-I, perforin−/− OT-I, wild-type OT-I mice, or no cells (-) were adoptively transferred into B6 mice, which were then infected with sporozoites (300/mouse) of PbA-hsOVA (A) or PbA-gfpOVA (B), and the levels of parasitemia were monitored. Values significantly different (*p < 0.05) from mice not receiving OT-I cells are indicated (*). In each graph, the number in parentheses indicates the proportion of OT-I cells in the total CD8+ T cell population in PBL at the time of infection. The experiments were performed 3 times; representative data are shown.
OVA-specific memory CD8⁺ T cells were protective against infection with PbA-hsOVA. B6 mice were immunized with OVA₂₅⁷₋₂₆₄ as described in the Materials and Methods section. Two months later, the proportion of OVA-specific memory CD8⁺ T cells was determined by staining PBL with anti-CD8 mAb and OVA₂₅⁷₋₂₆₄/H-2Kᵇ tetramer. Representative flow cytometry profiles of PBLs from naive and immunized mice are shown (A). Each bar in the graph shows the proportion of OVA-specific memory CD8⁺ T cells in total CD8⁺ T cells for an individual mouse (left axis) (B). The data are arranged from left to right in order of high to low specific CD8⁺ T cell ratios. These mice were challenged by intravenous injection of PbA-hsOVA sporozoites (1,000/mouse). Parasitemia was assessed 8 days after challenge; each dot shows the level of parasitemia in an individual mouse (right axis). Data from 37 mice are summarized in (C). * < 0.05%. The experiments were performed 3 times; pooled data are shown.
**Figure 4**

(A) Images showing GFP+ cells and OT-I clusters.

(B) Additional images illustrating the same phenomena as in (A).

(C) Graphs depicting the number of GFP+ cells and OT-I clusters.

(D) Bar graphs showing the number of OT-I cells in each cluster.

(E) Additional graphs for a different experimental condition.

(F) Further analysis of OT-I cell clusters under varying conditions.
Figure 5

(A) PbA-hsOVA
Perforin⁺ IFN-γ⁺ OT-I (48.5 ± 7.6 %)
IFN-γ⁺ OT-I (47.9 ± 3.1 %)
Perforin⁺ OT-I (49.5 ± 8.0 %)
OT-I (26.3 ± 8.9 %)
(-)

(B) PbA-gfpOVA
Perforin⁺ IFN-γ⁺ OT-I (53.8 ± 5.1 %)
IFN-γ⁺ OT-I (42.8 ± 3.0 %)
Perforin⁺ OT-I (41.3 ± 7.9 %)
OT-I (60.7 ± 8.8 %)
(-)

Days after infection
Figure 6

(A) Naive vs. Immunized

(B) Graph showing % OVA257-264 specific CD8+ T cells vs. Parasitemia for Mice immunized with OVA257-264

(C) Table showing % OVA257-264 specific CD8+ T cells vs. Challenged vs. Protected vs. % Protected

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