CD8$^+$ T Cells Eliminate Liver-Stage *Plasmodium berghei* Parasites without Detectable Bystander Effect

Ian A. Cockburn,[a,b] Sze-Wah Tse,[a]* Fidel Zavala[a]

Johns Hopkins Malaria Research Institute, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA[a]; Department of Pathogens and Immunity, John Curtin School of Medical Research, Australian National University, Canberra, Australia[b]

Immunization with attenuated *Plasmodium* sporozoites or viral vectored vaccines can induce protective CD8$^+$ T cells that can find and eliminate liver-stage malaria parasites. A key question is whether CD8$^+$ T cells must recognize and eliminate each parasite in the liver or whether bystander killing can occur. To test this, we transferred antigen-specific effector CD8$^+$ T cells to mice that were then coinfected with two *Plasmodium berghei* strains, only one of which could be recognized directly by the transferred T cells. We found that the noncognate parasites developed normally in these mice, demonstrating that bystander killing of parasites does not occur during the CD8$^+$ T cell response to malaria parasites. Rather, elimination of infected parasites is likely mediated by direct recognition of infected hepatocytes by antigen-specific CD8$^+$ T cells.

Immunization with irradiated sporozoites induces a CD8$^+$ T cell response that can protect against live parasite challenge (1–3). Despite the well-established role of CD8$^+$ T cells in immunity, the mechanism by which they find and eliminate parasites remains unclear. One key outstanding question is whether CD8$^+$ T cells kill via contact-mediated processes following direct recognition of the infected hepatocyte or whether they may also kill bystander parasites at a distance via the production of inflammatory mediators. This is a critical issue, as bystander killing processes would potentially allow immunosurveillance by a smaller number of CD8$^+$ T cells. If, however, killing takes place only after recognition of each individual infected hepatocyte by cognate CD8$^+$ T cells, a much larger number of cells would be required for protection.

A variety of studies have attempted to determine which are the critical effector molecules for parasite elimination. Collectively, these data conclude that a range of redundant mechanisms operate, including the secretion of tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) (2, 4, 5), both of which may be produced by malaria-specific CD8$^+$ T cells (6). On the other hand, there is only limited evidence for the use of the perforin-granzyme pathway in the elimination of *Plasmodium* parasites by CD8$^+$ T cells (6, 7). The importance of secreted cytokines in parasite elimination has led to the suggestion that these may spread though the liver and mediate the killing of bystander parasites (8, 9). Such bystander killing could potentially occur after the recognition of antigen presented by either an infected hepatocyte or an uninfected antigen-presenting cell. Nonetheless, a variety of lines of evidence support the notion that CD8$^+$ T cell killing occurs following cognate CD8$^+$ T cell-hepatocyte interactions. Recent independent *in vivo* imaging studies observed clustering of CD8$^+$ T cells around infected hepatocytes that led to the eventual killing of the parasites (10, 11). Moreover, several *in vitro* and *in vivo* studies suggest that the infected cells themselves are able to present antigen directly to effector CD8$^+$ T cells (12–15). However, it is possible that in addition to the elimination of cognate parasites, bystander parasites may also be eliminated during this potent CD8$^+$ T cell response.

To test whether bystander killing can occur following sporozoite challenge, we transferred antigen-specific effector CD8$^+$ T cells to mice that were then coinfected with two *Plasmodium berghei* strains, only one of which could be recognized directly by the transferred T cells. Using this strategy, we demonstrated that bystander killing of parasites does not occur during the CD8$^+$ T cell response to malaria parasites. Rather, we found that CD8$^+$ T cell activity is restricted to the immediate microenvironment of the infected cell and thus that protection depends upon each parasite being found and eliminated by cognate CD8$^+$ T cells.

MATERIALS AND METHODS

Mice and parasites. C57BL/6 and B6-Ly5.2 mice were obtained from the National Cancer Institute (Frederick, MD). OT-I mice (16) were obtained from David Sacks (NIAID, Bethesda, MD). *P. berghei*-CS^{5M} was previously generated in our laboratory (15). *P. berghei*-ConF (17) was obtained from Rogerio Amino and Robert Menard (Institut Pasteur, Paris, France), and *P. berghei*-mCherry (18) was kindly provided by Volker Heussler (University of Bern). All experimental procedures on animals were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University.

Generation of *P. berghei* CS^{5M}-mCherry parasites. *P. berghei* CS^{5M}-mCherry parasites were generated by crossing two previously generated parasite lines: *P. berghei* CS^{5M} (15) and *P. berghei*-mCherry (18). Briefly, mice were coinfected with *P. berghei* CS^{5M} and *P. berghei*-mCherry at a ratio of 10:1. *Anopheles stephensi* mosquitoes were allowed to feed on the mice, and subsequently, sporozoites dissected from these mosquitoes were used to infect naive animals. Parasites expressing the mCherry transgene were sorted from the blood of these mice by use of a FACSAria cell sorter and used to infect mice. Subsequently, the mCherry-positive progeny were cloned and the clones screened for the insertion of the CS^{5M} transgene by PCR as described previously (15).

Preparation of OT-I effector cells. CD45.1$^+$ OT-I CD8$^+$ transgenic cells specific for the SIINFEKL epitope were adoptively transferred into C57BL/6 mice (1×10^6 cells per mouse) before immunization with 5×10^6 PFU per mouse of vaccinia virus-OVA. Seven to 10 days later, the spleens were harvested and lymphocytes were prepared by passage over a Lymphoprep-M gradient (Cedarlane Laboratories). The number of CD8$^+$ CD45.1$^+$ effector cells was then quantified by flow cytometry and the volume normalized such that 5×10^6 effector cells were transferred per mouse.

In vivo **imaging and analysis.** The livers of anesthetized C57BL/6 mice were exposed by surgery as previously described (19). For each mouse, a tile scan of the entire area of the liver was acquired by laser confocal spinning disk microscopy (3i), using a $10\times$ air objective (Zeiss). Tile scans were formed into a montage and thresholded in order to facilitate counting of the different fluorescent parasites. For calculation of the number of parasites/mm^3, we determined that the depth of field of the tile scan was 50 μm based on the objective used.

Flow cytometry. *P. berghei* CS^{5M}-mCherry and *P. berghei*-ConF parasitemias were quantified by flow cytometry. Five microliters of blood was taken from the tails of infected mice and diluted in 995 μl Hanks balanced salt solution (HBSS) prior to analysis by flow cytometry using an LSR-II instrument (BD Biosciences) equipped with a 561-nm green laser which enabled the acquisition of mCherry fluorescence in the phycoerythrin (PE)-Texas Red channel. Green fluorescent protein (GFP) fluorescence was acquired in the fluorescein isothiocyanate (FITC) channel.

Analysis of parasite loads in livers by reverse transcription-PCR (RT-PCR). Quantification of liver-stage parasites was performed as previously described (20). Briefly, 42 h after challenge with 1×10^4 *P. berghei* sporozoites, livers were excised and parasite loads were determined by quantitative PCR analysis of *P. berghei* 18S rRNA by use of SYBR green (Applied Biosystems).

RESULTS

Experimental design and development of *P. berghei* CS^{5M}-mCherry parasites. To determine whether CD8$^+$ T cells can kill bystander parasites as well as cognate parasites, we developed an experimental strategy in which we infected mice with 2 related *P. berghei* parasites expressing different fluorescent markers. One fluorescent strain would express a model CD8$^+$ T cell epitope that could be the target of protective antigen-specific effector cells, while the other fluorescent strain would not carry this epitope and therefore could not be recognized directly by the antigen-specific CD8$^+$ T cells. We reasoned that if bystander killing took place, transfer of activated antigen-specific CD8$^+$ T cells would result in the elimination of both the cognate and noncognate parasites. On the other hand, if CD8$^+$ T cells killed via localized effector mechanisms, the development of the noncognate parasites would be unaffected by the transfer of the activated CD8$^+$ T cells (Fig. 1A).

To develop fluorescent *P. berghei* parasites expressing a model antigen, we crossed previously generated *P. berghei*-mCherry parasites (18) with *P. berghei* CS^{5M} (15) to create a line of parasites which we designated *P. berghei* CS^{5M}-mCherry (CS^{5M}-mCherry) (see Materials and Methods). *P. berghei* CS^{5M} parasites express the SIINFEKL epitope from ovalbumin in place of the endogenous SYIPSAEKI epitope in the circumsporozoite (CS) protein and are therefore efficiently eliminated by effector OT-I cells which recognize the SIINFEKL epitope. As expected, we found that effector OT-I cells were also able to kill parasites in mice infected with CS^{5M}-mCherry parasites. For control parasites, we used *P. berghei*-ConF (CS^{WT}-GFP), which carries the endogenous CS protein and expresses GFP under the control of the HSP70 promoter (17). Crucially, effector OT-I cells were unable to elimi-
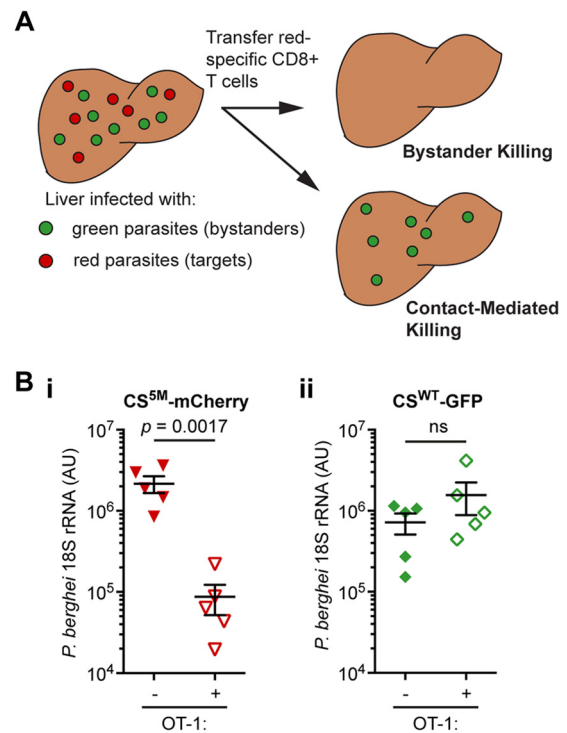

FIG 1 Development of *P. berghei* CS^{5M}-mCherry parasites. (A) General experimental design for bystander killing experiments. (B) RT-PCR quantification of the number of parasites in the liver at 42 h postinfection, with or without transfer of 3×10^6 effector OT-I cells prior to infection. *P. berghei* 18S rRNA was measured 42 h after infection with either 1×10^4 CS^{5M}-mCherry parasites (i) or 1×10^4 CS^{WT}-GFP parasites (ii). Data are based on 5 mice per group and were analyzed by 2-tailed Student's *t* test. AU, arbitrary units.

nate parasites from mice infected with CS^{WT}-GFP parasites alone (Fig. 1B).

Antigen-specific effector CD8$^+$ T cells do not kill bystander liver-stage parasites. To test whether antigen-specific effector cells can kill bystander parasites as well as those expressing their cognate epitope, 5×10^6 effector OT-I T cells were transferred to mice that were subsequently coinfected with equal numbers of CS^{WT}-GFP and CS^{5M}-mCherry parasites. Control mice which had not received effector OT-I T cells were also coinfected with the 2 parasite lines. At 24 to 26 h postinfection, the mice were anesthetized and prepared for intravital imaging as described previously (19). Using spinning disk confocal microscopy, we were able to observe numerous CS^{WT}-GFP and CS^{5M}-mCherry parasites in the livers of control mice. Conversely, in the presence of OT-I cells, only CS^{WT}-GFP parasites could be seen, though some red debris was also observed (Fig. 2A), suggesting that the red (cognate) parasites, but not the green (noncognate) parasites, had been eliminated.

To quantify the numbers of CS^{5M}-mCherry and CS^{WT}-GFP parasites in mice with and without effector OT-I cells, tile scans of the entire exposed area of the liver were taken and the numbers of CS^{5M}-mCherry and CS^{WT}-GFP parasites counted. While CS^{5M} parasites were undetectable in mice that received OT-I cells, the number of CS^{WT}-GFP parasites in the liver was not significantly affected by the ongoing killing of CS^{5M}-mCherry parasites by effector OT-I cells, suggesting that bystander killing was not taking place (Fig. 2B).
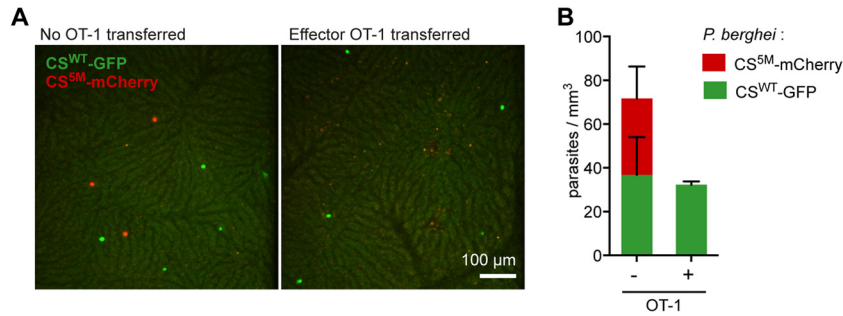
FIG 2 Antigen-specific effector CD8^{+} T cells do not kill bystander liver-stage parasites. (A) Representative confocal images of the livers of mice 24 to 26 h after coinfection with 5×10^5 (each) CS[5M]-mCherry and CS[WT]-GFP parasites. Different groups of mice either received 5×10^6 effector OT-I T cells or did not receive effector cells. (B) Quantification of the numbers of parasites seen in the liver after coinfection with 1×10^5 (each) CS[5M]-mCherry and CS[WT]-GFP parasites, with or without adoptive transfer of 5×10^6 effector OT-I cells. Data are based on tile scans of the entire exposed area of the liver for 3 mice per group and are representative of 2 similar experiments.

Development of bystander parasites to the asexual blood stage is complete. In the previous experiment, we were unable to detect any bystander killing of CS[WT]-GFP parasites at 24 to 26 h postinfection. However, it is possible that the continuing presence of cytokines such as IFN-γ and TNF-α produced during the effector phase of the CD8^{+} T cell response may prevent the complete development of bystander CS[WT]-GFP parasites. To test this, we transferred effector OT-I cells to mice that then received CS[5M]-mCherry and CS[WT]-GFP sporozoites at a ratio of 4:1. A ratio of 4:1 CS[5M]-mCherry to CS[WT]-GFP parasites was used to increase the

likelihood of observing bystander killing. Control groups of mice received either CS[5M]-mCherry and CS[WT]-GFP parasites without any activated OT-I cells or effector OT-I cells and CS[WT]-GFP parasites alone.

In mice that did not receive OT-I cells, both asexual CS[5M]-mCherry and CS[WT]-GFP parasites could readily be detected by flow cytometry on day 4 postinfection (Fig. 3A, panel i, and B). Conversely, in coinfected mice that received OT-I cells, only CS[WT]-GFP parasites could be seen from day 4 onwards (Fig. 3A, panel ii, and B). Importantly, the number of CS[WT]-GFP parasites
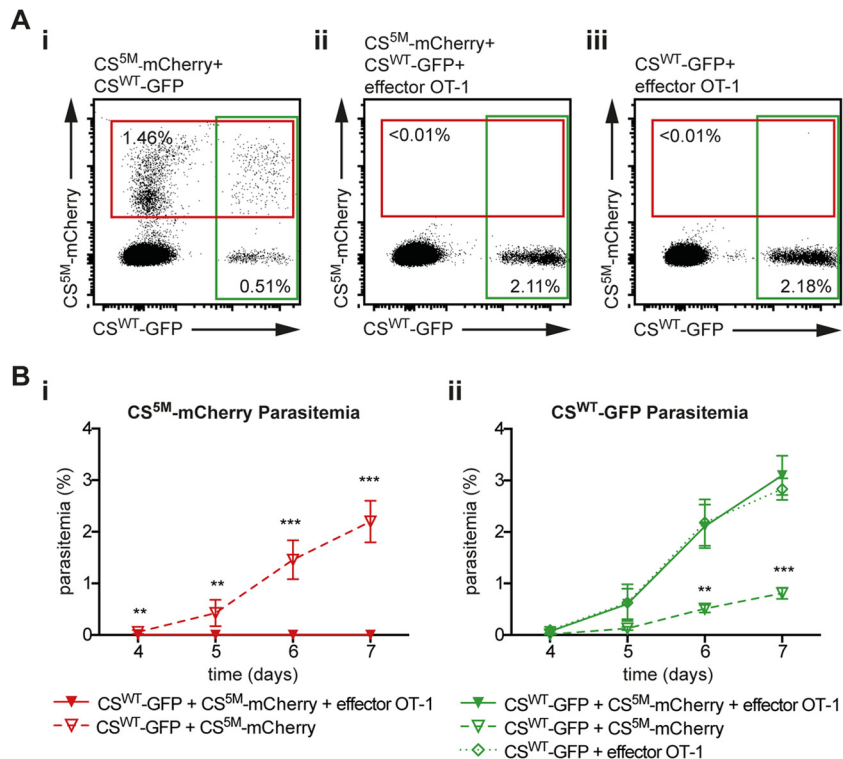

FIG 3 Complete liver-stage development of bystander parasites. (A) Representative flow cytometry plots of blood-stage CS[WT]-GFP and CS[5M]-mCherry parasites at day 6 post-sporozoite immunization under various conditions, i.e., coinfection of mice with 4×10^4 CS[5M]-mCherry and 1×10^4 CS[WT]-GFP parasites (i), coinfection of mice with 4×10^4 CS[5M]-mCherry and 1×10^4 CS[WT]-GFP parasites following adoptive transfer of 5×10^6 effector OT-I T cells (ii), and infection of mice with 1×10^4 CS[WT]-GFP parasites following adoptive transfer of 5×10^6 effector OT-I cells (iii). (B) Quantification of CS[5M]-mCherry (i) and CS[WT]-GFP (ii) daily parasitemias in the three groups of mice shown in panel A. Parasitemia was analyzed by Student's t test, using the group that received both parasite strains and effector OT-I cells as the reference (**, $P < 0.01$; ***, $P < 0.001$). Data are based on 5 to 7 mice per group from one of two similar experiments.

in coinfected mice that received OT-I cells was statistically indistinguishable (by unpaired Student's *t* test) from the number in control mice that received just CS^WT-GFP and effector OT-I cells, i.e., in the absence of an ongoing cognate CD8^+ T cell response (Fig. 3A, panel iii, and B). Interestingly, the number of blood-stage CS^WT-GFP parasites was smaller in the presence of a blood-stage CS^5M-mCherry infection, presumably due to competition between the two parasite lines (Fig. 3B). Together, these data exclude the possibility of cytokines killing by a delayed action on liver-stage parasites and further support the finding that *Plasmodium*-specific T cells do not induce bystander killing.

DISCUSSION

In these experiments, we found that CD8^+ T cells can eliminate parasites only by locally acting effector functions following cognate interactions with antigen. The most parsimonious explanation for our data would be that CD8^+ T cells recognize a cognate antigen presented by the infected hepatocyte itself, resulting in killing via a contact-mediated process, e.g., directed secretion of IFN-γ or the activity of perforin. However, we cannot formally exclude the possibility that other cells in the microenvironment—perhaps traversed hepatocytes or Kupffer cells—present antigen, provided that they are in close proximity to parasite-infected cells (12).

Importantly, these experiments were designed to maximize the possibility of detecting bystander killing. We used large numbers of parasites in all experiments (1×10^4 to 1×10^5), i.e., several orders of magnitude larger than the number of parasites injected by an infected mosquito, which is in the range of 1×10^1 to 1×10^3 (21–23). Moreover, the use of mice, which have small livers, further increased the density of parasite infection. We also used large numbers of T cells (5×10^6), giving a dose that was clearly capable of eliminating all cognate parasites, as seen in the experiments in which infection was allowed to proceed to the blood stage. Finally, we primed the T cells by using a recombinant vaccinia virus, a regimen that has previously been shown to produce a large proportion of polyfunctional effector T cells secreting a range of cytokines and having strong cytolytic activity (24).

Our data are perhaps surprising in the light of previous findings that have suggested that IFN-γ and TNF-α are among the main effector molecules involved in the elimination of *P. berghei* parasites (2, 4–6). Release of these cytokines into the liver might reasonably be expected to induce bystander killing. In particular, TNF-α has been reported to be secreted multidirectionally following the ligation of CD8^+ T cells, suggesting that it might act on bystander cells in addition to cognate targets (25). IFN-γ secretion has generally been assumed to be synaptic and highly localized, though multidirectional secretion has also been reported (25–27). In a recent study, IFN-γ produced by CD4^+ T cells was able to induce bystander killing of *Leishmania major* parasites in the skin (28). However, the radius over which bystander killing could occur was calculated at ~80 μm, whereas from the imaging experiments shown in Fig. 2, we would estimate the average separation of bystander parasites to be ~300 μm when 1×10^5 parasites are used for infection. Thus, it may be that infected hepatocytes are simply not close enough to allow bystander killing to occur. Surprisingly, given that killing occurs locally, previous studies have shown little or no role for perforin and granzyme in the killing of *P. berghei* parasites as used in this study (6, 7). On the other hand, perforin has been shown to play a role in CD8^+ T cell killing of *P.*

yoelii parasites *in vitro* (29), though with conflicting results *in vivo* (6, 30).

An important implication of these data is that elimination of parasites from the liver by CD8^+ T cells is dependent upon each and every parasite being found and eliminated by at least one cognate CD8^+ T cell. However, the interaction of a cognate CD8^+ T cell with antigen can initiate the recruitment of further CD8^+ T cells to the region surrounding the infected hepatocyte, which may increase the likelihood of other parasites being eliminated (10). Nonetheless, given the large size of the liver and the low frequency of infected hepatocytes in natural infection, it is perhaps not surprising that high frequencies of CD8^+ T cells are required for sterile protection (31, 32). Overall, these data further support the notion that robust immunization strategies, perhaps including the use of prime-boost regimens, will be required for the induction of protective CD8^+ T cell responses against liver-stage malaria parasites (33, 34).

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