

# Blood-Stage *Plasmodium berghei* Infection Generates a Potent, Specific CD8<sup>+</sup> T-Cell Response Despite Residence Largely in Cells Lacking MHC I Processing Machinery

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Murine cerebral malaria is a complex disease caused by *Plasmodium berghei* ANKA infection. Several cell types, including CD8<sup>+</sup> T cells, are essential effectors of disease. Although the use of transgenic parasites expressing model antigens has revealed the induction of cytotoxic T lymphocytes (CTL) specific for these model antigens, there is no direct evidence for a response to authentic blood-stage parasite antigens, nor any knowledge of its magnitude. Our studies show that there is a dramatic primary parasite-specific CTL response, akin to viral immunity, reaching approximately 30% of splenic CD8<sup>+</sup> T cells, with many producing interferon- $\gamma$  and tumor necrosis factor- $\alpha$ . These cells express granzyme B and other markers of specific responders, are cytolytic, and respond to a broad array of major histocompatibility complex (MHC) I-restricted epitopes, 5 of which are identified here. Our studies indicate that vigorous CTL responses can be induced to pathogens even when they largely reside in red blood cells, which lack MHC I processing machinery.

Most cases of human malaria are caused by 2 main *Plasmodium* species, *P. falciparum* and *P. vivax*. Like humans, rodents have their own specific *Plasmodium* species, which cause varying forms of malarial disease. One such pathogen, *Plasmodium berghei* ANKA (PbA), causes acute infection in C57BL/6 (B6) mice; this infection is lethal within about a week. Although debated [1], it has been argued that this disease exhibits many

characteristics that parallel severe disease in humans; as such, this disease has been used to study the cellular mechanisms that mediate pathology [2]. For malaria, pathology is entirely associated with the blood stage of the parasite life cycle and, in the case of PbA infection, is immune mediated. Many cell types play negative or positive roles in the progression toward fatal disease. Contributing cell types include CD8<sup>+</sup> and CD4<sup>+</sup> T cells [3–6], CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells [7, 8], natural killer cells [9], natural killer T cells [10], and  $\gamma\delta$  T cells [11]. Among these cell types, CD8<sup>+</sup> T cells play a critical role in the effector phase of PbA-induced cerebral malaria (ECM) [3, 5], though the magnitude and specificity of this response are largely undocumented.

The prominent role of CD8<sup>+</sup> T cells in this malarial disease is somewhat surprising given that *Plasmodium* parasites reside largely within red blood cells (RBCs), which lack the machinery required for major histocompatibility complex (MHC) I presentation. Clearly, this does not exclude infected RBCs from acting as

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a source of antigen for cross-priming [12] but does suggest that any resultant CD8<sup>+</sup> T-cell responses will have little impact upon parasites residing in RBCs. Indeed, it may be that the ability to prime a robust CD8<sup>+</sup> T-cell response that is intrinsically unable to deal with blood-stage infection underpins much of the ensuing immune pathology. Although previous studies have used transgenic *P. berghei* parasites expressing model antigens to dissect antigen presentation [12–14], the magnitude of the CD8<sup>+</sup> T-cell response remains largely undefined. Furthermore, clear demonstration that PbA induces parasite-specific CD8<sup>+</sup> T cells to authentic rather than model antigens is lacking. Here we explore the immune response to PbA infection and identify a robust parasite-specific CD8<sup>+</sup> T-cell response akin to that seen for pathogens such as viruses, which reside in MHC I-expressing cells and are amenable to control by CD8<sup>+</sup> T cells. To facilitate better understanding of the immune response to PbA, we have also identified several natural peptide epitopes in B6 mice and have shown that CTL to these epitopes produce interferon (IFN)- $\gamma$  and induce lysis of target cells during an ensuing PbA infection. Combined, we show that parasite-infected RBCs can prime a potent parasite-specific CD8<sup>+</sup> T-cell response that includes a range of specificities capable of producing fully fledged CTL effector functions.

## MATERIALS AND METHODS

### Mice

B6 and BALB/c mice aged 6–12 weeks were bred and maintained at the Walter and Eliza Hall Institute and the Department of Microbiology and Immunology, University of Melbourne. All procedures were approved by the Melbourne Health Research Animal Ethics Committee.

### Flow Cytometry

Cells were labeled with monoclonal antibodies specific for CD8 (53–6.7), CD11c (N418), Thy1.2 (30-H12), CD45.1 (A20), or CD11a (LFA-1  $\alpha$  chain, M17/4). Dead cells were excluded by propidium iodide staining. Cells were analyzed by flow cytometry on a FACsCalibur or FACsCanto (BD Biosciences), using the Weasel 2 or FlowJo 9.3.1 software packages.

### PbA Infection and Drug Treatment

Mice were infected intravenously with 10<sup>6</sup> PbA parasitized RBCs. After infection, mice were injected intraperitoneally with 0.4 mg chloroquine daily from day 4 to day 8, followed by 600 mg/L chloroquine in drinking water on day 9 and day 10.

### Preparation of PbA Asexual Mixed Blood-Stage and Schizont-Stage Lysate

Blood (~10% parasitemia) was harvested from PbA-infected B6 mice 4 days after infection, and RBCs were lysed with 0.05% saponin/phosphate buffered saline (PBS) to release parasites. These parasites were then washed in PBS before pelleting in

aliquots of  $2 \times 10^8$  parasites, which were snap-frozen on dry ice and stored at  $-80^\circ\text{C}$  until required. To obtain schizont-stage parasites, asexual mixed blood-stage RBCs were harvested from PbA-infected Wistar rats (~5% parasitemia). Parasites were cultured in RPMI 1640 supplemented with 25% fetal bovine serum at  $37^\circ\text{C}$  under 5% CO<sub>2</sub>, 1% O<sub>2</sub> in nitrogen until the schizont stage of development was reached. Schizonts were purified on a 50% nycodenz gradient as previously described [15], and aliquots of  $2 \times 10^8$  pelleted, PBS-washed parasites were then snap-frozen on dry ice and stored at  $-80^\circ\text{C}$  until required. To prepare parasite lysates, parasites were resuspended in serum-free Dulbecco's modified Eagle's medium medium, frozen, and thawed 3 times and passed through a 30G needle 5 times. Parasite lysates were 2-fold serially diluted and used as an antigen source in the ex vivo intracellular cytokine staining (ICS) assay.

### Dendritic Cell Isolation

Conventional dendritic cells (DCs) were isolated from the spleen of FMS-like tyrosine kinase 3 receptor ligand (Flt3-L)-treated B6 mice as previously described [16]. Flt3-L was given by subcutaneous injection of  $5 \times 10^6$  Flt3-L-expressing B6 melanoma cells 9–10 days prior to harvesting splenic DCs [17].

### Ex Vivo ICS for Granzyme B, Interferon- $\gamma$ , and Tumor Necrosis Factor- $\alpha$

To detect CD8<sup>+</sup> T cells producing IFN- $\gamma$  and tumor necrosis factor (TNF)- $\alpha$ , 10<sup>6</sup> splenocytes from day 7 PbA-infected and chloroquine-treated (day 4–day 6) mice were restimulated for 5 hours at  $37^\circ\text{C}$  in the presence of 10  $\mu\text{g}/\text{mL}$  brefeldin A with 10<sup>6</sup> conventional DCs (from Flt3-L-treated mice) that had been preincubated with serially diluted PbA blood-stage or schizont lysate overnight. For peptide testing, 10<sup>6</sup> splenocytes were restimulated with 3.3–10  $\mu\text{g}$  peptide without DCs. Cells were then labeled with anti-CD8 and anti-Thy1.2 antibodies (Abs), followed by fixation with 1% paraformaldehyde for 20 minutes at room temperature. Intracellular staining with anti-IFN- $\gamma$  and anti-TNF- $\alpha$  Abs was performed in buffer containing 0.33% saponin. Cells were washed twice before analysis by flow cytometry. Then  $1\text{--}2 \times 10^6$  splenocytes were analyzed for intracellular granzyme B expression in a manner similar to IFN- $\gamma$  and TNF- $\alpha$  staining, but omitting in vitro restimulation with antigen.

### In Vivo CTL Assay

In vivo CTL lysis of carboxyfluorescein diacetate, succinimidyl ester (CFSE)-labeled peptide-coated splenocyte targets was performed as previously described [12]. Splenocytes from B6 mice were divided into 2 equal portions. One portion was pulsed with 33–100  $\mu\text{g}/\text{mL}$  peptide (Mimotopes: 1 of LSGRYNDL, WGDEFKEL, EIYIFTNI, LLPHFSIL, or YYYDYDKI) for 1 hour at  $37^\circ\text{C}$  and then labeled with a high concentration (5  $\mu\text{M}$ ) of CFSE (CFSE<sup>hi</sup> population). The other was incubated for 1 hour at  $37^\circ\text{C}$  without peptide and labeled with a low concentration of

CFSE (CFSE<sup>lo</sup> population). Equal numbers of cells were combined, and  $2 \times 10^7$  cells were injected into day 6 PbA-infected and chloroquine-treated mice. In vivo CTL lysis of CFSE-labeled, peptide-coated splenocyte targets was assessed 12–16 hours after the transfer of target cells.

### Prediction and Synthesis of PbA Epitopes

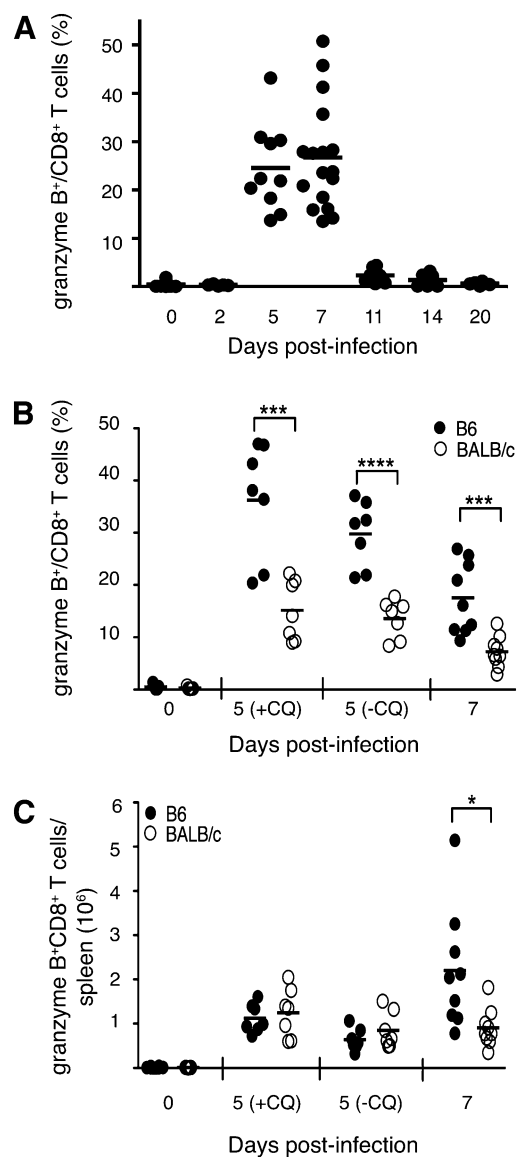
Using the online PlasmoDB resources (<http://plasmodb.org>), 88 PbA blood-stage proteins that have *P. falciparum* orthologs and 365 without *P. falciparum* orthologs were selected for peptide prediction analysis using the online SYFPEITHI MHC prediction algorithm (<http://www.syfpeithi.de>). We selected 125 H-2K<sup>b</sup>-restricted nanomers with a minimum score of 23, comprising 63 peptides with a score >22 from 48 PbA proteins with *P. falciparum* orthologs and 62 peptides with a score >24 from 51 PbA proteins without *P. falciparum* orthologs. These peptides (1–3 mg, >70% purity) were synthesized as Cleaved PepSets by Mimotopes. The peptides were dissolved in 300  $\mu$ L DMSO and stored in aliquots at  $-20^\circ\text{C}$  until required.

## RESULTS

### A Large Proportion of Endogenous CD8<sup>+</sup> T Cells Express Granzyme B During PbA Infection

In an attempt to detect endogenous CD8<sup>+</sup> T-cell responses to PbA, we measured the proportion of granzyme B-expressing CD8<sup>+</sup> T cells during the course of infection (Figure 1A). In these studies, B6 mice were cured of infection by treatment with chloroquine daily from day 4 to day 10 to prevent lethal disease. Temporal examination of the proportion of granzyme B<sup>+</sup> CD8<sup>+</sup> T cells showed that a very large proportion (mean, 25%) of CD8<sup>+</sup> T cells expressed granzyme B within 5 days of infection. This number peaked on days 5–7 and then quickly returned to background levels by day 14, coincident with parasite clearance (data not shown). This finding strongly suggested that an intense PbA-specific CD8<sup>+</sup> T-cell response ensued upon infection, although it was possible that a proportion of the granzyme B-expressing cells were nonspecific, as previously reported [14]. In unpublished studies using transgenic parasites expressing model antigens and corresponding memory transgenic T cells, we found that approximately 25% of nonspecific memory cells could be induced to express granzyme B upon infection. Because only about 30% of splenic CD8<sup>+</sup> T cells are memory phenotype (CD44<sup>high</sup>) in our colony, their contribution to granzyme B-expressing cells is likely to be only about a quarter of this (7.5%). This implied that the majority of granzyme B<sup>+</sup> cells detected during PbA infection (Figure 1A) are likely to be parasite specific.

The above results were generated using mice cured of PbA infection on day 4 by chloroquine treatment. To test whether this treatment might affect the proportion of granzyme B<sup>+</sup> cells, perhaps due to effects on antigen presentation, we compared



**Figure 1.** Granzyme B production by CD8<sup>+</sup> T cells during *Plasmodium berghei* ANKA (PbA) infection. **A**, B6 mice were infected intravenously with  $10^6$  PbA and their spleens harvested. CD8<sup>+</sup> T cells were analyzed for granzyme B expression at different time points. PbA-infected mice were treated with chloroquine intraperitoneally daily from day 4 to day 8 and were given oral chloroquine on days 9 and 10. The data shown for each time point are pooled from 2 to 7 independent experiments (d0,  $\times 7$ ; d2,  $\times 2$ ; d5,  $\times 3$ ; d7,  $\times 7$ ; d11,  $\times 3$ ; d14,  $\times 2$ ; d20,  $\times 2$ ). **B** and **C**, B6 (●) or BALB/c (○) mice were infected intravenously with  $10^6$  PbA. Granzyme B expression by splenic CD8<sup>+</sup> T cells was compared on day 5 and day 7. Spleens analyzed on day 5 were treated or not treated with chloroquine on day 4, whereas spleens analyzed on day 7 were treated daily from days 4 to 6. Data are pooled from 2 independent experiments. Each circle represents an individual mouse; horizontal bars are the arithmetic mean. \* $P = .0146$ ; \*\*\* $P = .0008$ , and \*\*\*\* $P < .0001$ .

responses on day 5 for treated and untreated mice (Figure 1B and C). These responses were comparable. Later comparisons were hampered by the death of untreated mice by ECM. In these experiments, we also examined the response of ECM-resistant

BALB/c mice (Figure 1B and C). Like B6 mice, they generated a substantial proportion of granzyme B<sup>+</sup> cells, though this proportion was significantly less than for B6 mice, as also seen for the total number of granzyme B<sup>+</sup> cells on day 7 (Figure 1B and C).

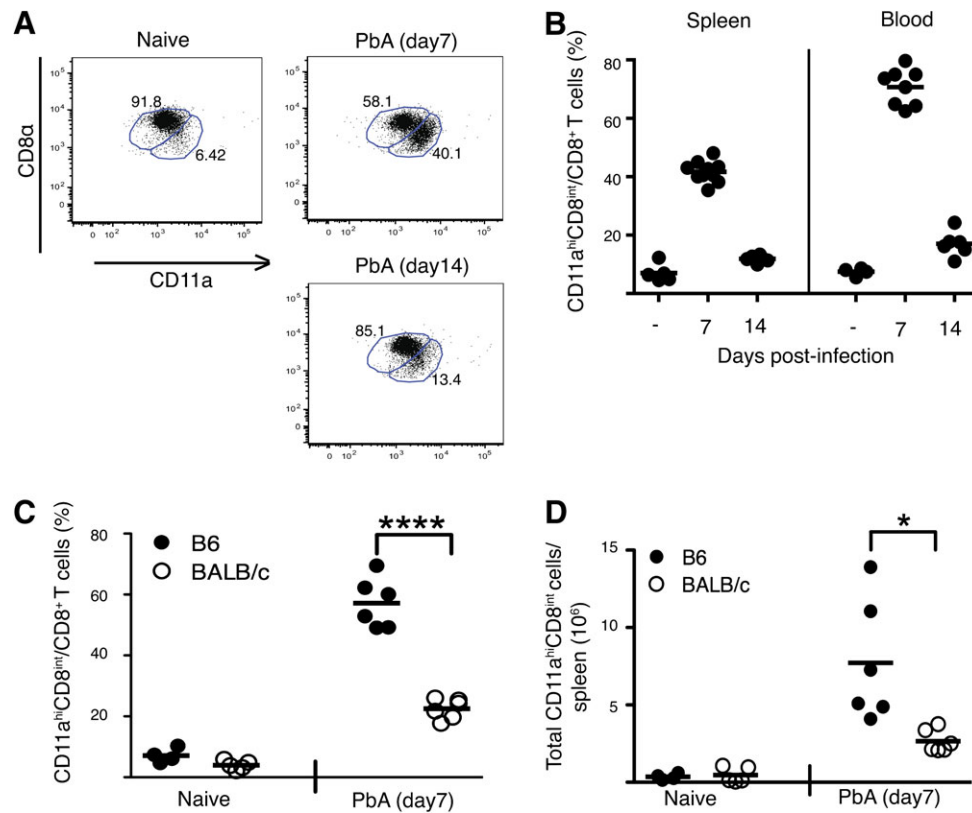
### A Large Proportion of CD8<sup>+</sup> T Cells Downregulate CD8 $\alpha$ and Upregulate CD11a

An alternative approach to detecting expansion of specific CD8<sup>+</sup> T cells was recently reported for bacterial and viral infections [18] and vaccination with radiation-attenuated *P. berghei* and *Plasmodium yoelii* sporozoites [19]. These authors showed that responding specific cells, upon antigen encounter, permanently change their CD8 and CD11a levels to CD8<sup>intermediate(int)</sup>CD11a<sup>high</sup>. To determine the extent of specific CD8<sup>+</sup> T-cell expansion to PbA, B6 mice were infected and the proportion of CD8<sup>int</sup>CD11a<sup>high</sup> cells was then assessed on days 7 and 14 (Figure 2A and B). Like granzyme B expression, this approach also revealed a very large proportion (about 40%) of splenic CD8<sup>+</sup> T cells with this phenotype on day 7, with even higher proportions in the blood. Given the proportion of these

cells in naive mice was approximately 7%, this implied about 30% of the splenic CD8<sup>+</sup> T cells were parasite specific at the acute phase of the response. By day 14, proportions in the spleen and blood had dropped to means of 12% and 17%, respectively. Assessment of ECM-resistant BALB/c mice for induction of CTL by this method revealed a substantial proportion (Figure 2C) and number (Figure 2D) of CD8<sup>int</sup>CD11a<sup>high</sup> cells, but again significantly less than in B6 mice.

### Detection of a CD8<sup>+</sup> T-Cell Response to Authentic PbA Antigens

Although the above data with granzyme B, CD8 $\alpha$ , and CD11a staining provided evidence for a strong parasite-specific CD8<sup>+</sup> T-cell response to PbA during blood-stage infection, these approaches did not directly demonstrate antigen specificity. This led us to develop an ICS assay for detection of PbA-specific CD8<sup>+</sup> T cells based on our knowledge that splenic CD8<sup>+</sup> DCs are efficient at cross-presenting antigens to CD8<sup>+</sup> T cells [20]. Mice were infected with PbA; on day 7, spleens were harvested and splenocytes cultured with purified DCs that had been pre-cultured overnight with different doses of lysate from either asynchronous PbA blood-stage parasites or purified schizonts.

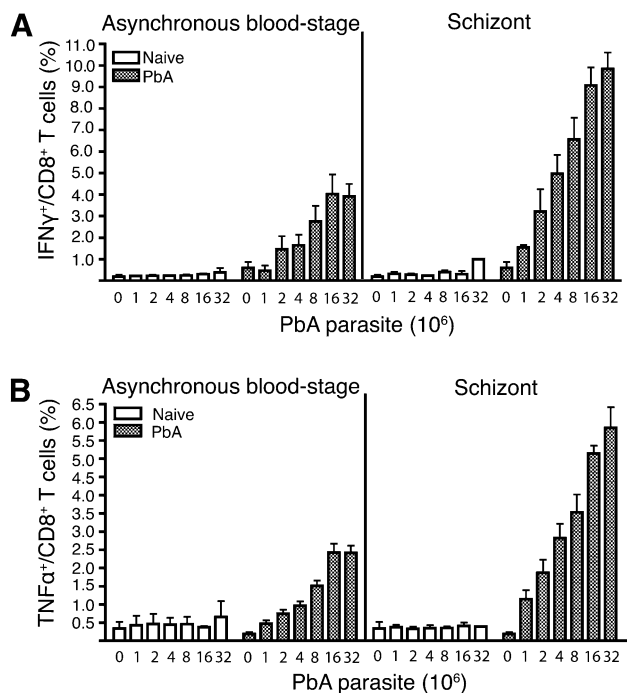


**Figure 2.** CD8<sup>+</sup> T cells downregulate CD8 $\alpha$  and upregulate CD11a during PbA infection. On day 7 or day 14 after infection of B6 mice with *Plasmodium berghei* ANKA (PbA), CD8<sup>+</sup> T cells from the spleen and blood were examined for the expression of CD8 $\alpha$  and CD11a. **A**, Representative plots showing the expression of CD11a and CD8 $\alpha$  on CD8<sup>+</sup> T cells from naive and infected B6 mice in the spleen. **B**, The percentage of CD8<sup>intermediate</sup>CD11a<sup>high</sup> CD8<sup>+</sup> T cells in the spleen and the blood of B6 mice is shown. Each dot represents an individual mouse. The data are pooled from 2 to 3 independent experiments. **C** and **D**, B6 (●) or BALB/c (○) mice were infected with PbA and their spleens were analyzed 7 days later. The percentage or total number of CD8<sup>intermediate</sup>CD11a<sup>high</sup> CD8<sup>+</sup> T cells is shown. Data shown are pooled from 2 independent experiments. \**P* = .0114; \*\*\*\**P* < .0001.

Two antigen sources were used to provide the best opportunity to detect antigen specificity. The DCs used were induced *in vivo* by Flt-3 ligand and contained 30%–40% CD8<sup>+</sup> DCs. After 5 hours of incubation in the presence of brefeldin A and DC-bearing antigen, CD8<sup>+</sup> T cells were stained for intracellular IFN- $\gamma$  and TNF- $\alpha$  (Figure 3). Although very few CD8<sup>+</sup> T cells from naive mice produced either of these cytokines, up to 10% of these cells from infected mice made IFN- $\gamma$  and up to 6% made TNF- $\alpha$ . Because the cytokine responses to titrated schizont antigen did not plateau, it is likely that these proportions are underestimates. Unfortunately, in this assay system the amount of antigen could not be further increased. In any case, these data indicated that on day 7 at least 10%, but probably a much greater percentage, of CD8<sup>+</sup> T cells were parasite specific.

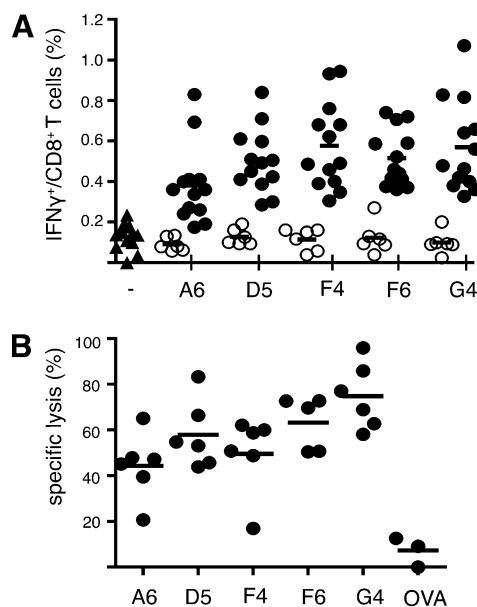
### Identification of MHC I-Restricted Epitopes From PbA Blood-Stage Antigens

We have shown that there is a dramatic expansion of CD8<sup>+</sup> T cells in response to PbA infection that probably exceeds 30% of splenic CD8<sup>+</sup> T cells, of which at least a third express IFN- $\gamma$ . However, whether any of these specific CD8<sup>+</sup> T cells were cytotoxic was not directly demonstrated. To address this point, we



**Figure 3.** CD8<sup>+</sup> T cells specific for *Plasmodium berghei* ANKA (PbA) antigens are activated during infection. Splenocytes from naive B6 mice (open bars) or B6 mice infected 7 days earlier with PbA (hatched bars) were restimulated for 5 hours in the presence of brefeldin A, at a 1:1 ratio with dendritic cells that were previously incubated overnight with different amounts of blood-stage PbA lysate (either asynchronous or schizont stages). Cells were then stained intracellularly with (A) interferon (IFN)- $\gamma$  or (B) tumor necrosis factor (TNF)- $\alpha$ . Data shown are pooled from 3 experiments, with 1 or 2 mice per experiment.

undertook an epitope discovery process using the PlasmoDB database and the online SYFPEITHI MHC prediction algorithm. We selected test peptides from a pool of proteins biased to the schizont stage—a stage known to have efficiently stimulated CD8<sup>+</sup> T cells in the assays shown in Figure 3. A total of 453 proteins were examined for K<sup>b</sup>-restricted epitopes using the SYFPEITHI prediction algorithm, and 125 peptides with a score >22 were chosen from 99 proteins. These peptides were tested in an intracellular IFN- $\gamma$  assay using splenocytes from mice infected for 7 days with PbA (data not shown). From this analysis, we identified 5 peptides (termed A6, D5, F4, F6, and G4) that stimulated weak but consistent responses (Figure 4A). Each peptide was derived from a different schizont-stage protein, of which G4 came from an activator protein 2 (AP-2) transcription factor, F4 and F6 came from proteins required for DNA replication, and D5 was derived from a cell cycle-regulated enzyme that catalyzes the *de novo* synthesis of deoxyribonucleotides by



**Figure 4.** Cytotoxic T lymphocytes (CTLs) specific for 5 *Plasmodium berghei* blood-stage epitopes are induced during *P. berghei* ANKA (PbA) infection. A, Splenocytes from day 7 PbA-infected (●) or naive mice (○) were restimulated with peptides (A6, D5, F4, F6, or G4) for 5 hours in the presence of brefeldin A and stained intracellularly for interferon (IFN)- $\gamma$ . Splenocytes from PbA-infected mice restimulated without peptides (▲). Data shown are pooled from 5 experiments. B, To measure CTL activity *in vivo*, splenocytes were coated with PbA-specific peptides (A6, D5, F4, F6, or G4) or ovalbumin peptide (SIINFEKL) as negative control. Peptide-coated targets were transferred intravenously into B6 mice infected 7 days earlier with PbA. Spleens were harvested and analyzed for percent-specific lysis 12–16 hours later. Data are pooled from 3 experiments. Peptide name, epitope sequence, and PlasmoDB *P. berghei* no. (*Plasmodium falciparum* no.) are as follows: A6, LSGRYNDL, PB000367.00.0 (PF13\_0125); D5, WGDFEKL, PB001267.00.0 (PF14\_Q352); F4, EIYIFTNI, PB000785.02.0 (PF10235w); F6, LLPHSIL, PB001115.01.0 (PFB0895c); and G4, YYYDYDKI, PB000863.01.0 (PFD0985w). The 5 peptides shown here were identified in initial studies in which 125 peptides were tested.

the reduction of the corresponding ribonucleotides. Although no putative function has been assigned to the protein from which A6 was derived, this protein might be secreted because it contains a signal sequence. These epitopes were then used to coat CFSE-labeled target cells in an in vivo cytotoxicity assay as previously described [12]. Figure 4B shows that lytic CTL specific for each of these 5 epitopes could be detected in vivo.

## DISCUSSION

CD8<sup>+</sup> T cells have been implicated as effectors of ECM pathogenesis. It has been proposed that antigen-specific CTL primed in the periphery by DCs migrate to the brain to mediate immune pathology, although the target cells in the brain have yet to be identified [4, 12]. The extent of CTL priming has been shown to dictate the outcome of many diseases caused by microbial infection. For example, virus-specific CTL priming during herpes simplex virus 1 skin infection correlates with clearance of this virus [21]. Humans with the HLA-Bw53 allele, which associates with binding to a liver-stage antigen-1 epitope, have a linkage to protection from severe plasmodial disease [22, 23]. Although the magnitude of CTL priming is likely to be a major factor that contributes to disease progression or outcome, very little is known about the magnitude of CTL responses specific for naturally occurring *Plasmodium* epitopes during blood-stage plasmodial infection, which is the stage when ECM occurs, mainly because relevant epitopes have not been identified. To investigate the extent of CTL priming, we took 3 approaches for estimating antigen-specific CTL responses: (1) granzyme B expression by CTL, (2) cytokine production by CTL in response to restimulation with parasite antigen, and (3) changes in surface molecule expression known to mark antigen-specific cells. We showed that in B6 mice, which are highly susceptible to ECM, extensive CTL priming was induced by PbA infection. Interestingly, ECM-resistant BALB/c mice also showed evidence for substantial CTL responses, though this was generally lower than that seen in B6 mice. Whether it is the lower extent of CTL induction or any of multiple other contributing factors that protect BALB/c mice from ECM is currently unclear. By undertaking an epitope discovery approach for B6 mice, we have identified 5 PbA epitopes, which in turn allowed us to directly demonstrate lytic activity of antigen-specific CTL in the periphery.

Accumulating evidence has suggested the requirement for antigen-specific CTL in ECM pathogenesis. The transfer of activated CD8<sup>+</sup> T cells from mice infected with blood-stage PbA into ECM-resistant RAG-deficient mice induced lethal disease in these mice [24]. The existence of authentic CTL epitopes is supported by studies reporting selective expansion of CD8<sup>+</sup> T cells bearing the V $\beta$  8.1,2 TCR in the brain and peripheral blood during ECM [5, 25]. We recently demonstrated that CD8<sup>+</sup> T cells specific for model epitopes expressed by transgenic

parasites are responsible for ECM immune pathology associated with blood-stage *P. berghei* infection. However, no clear evidence has yet been presented that CTLs activated during PbA infection are actually specific for this parasite, rather than simply activated nonspecifically, perhaps due to the cytokine milieu. By monitoring granzyme B expression and the generation of CD8<sup>int</sup>CD11a<sup>high</sup> cells, our studies reveal dramatic expansion of CTL during the course of this murine malarial disease, suggesting that approximately 30% of CD8<sup>+</sup> T cells are directed to PbA at the peak of expansion. Interestingly, brain-sequestered CD8<sup>+</sup> T cells were consistently found to express high levels of granzyme B and CD11a [14, 26, 27], suggesting that antigen-specific CD8<sup>+</sup> T cells primed in the periphery are recruited to the brain, most likely in a chemokine-dependent manner, such as IP-10, as previously reported [28]. Because the treatment with monoclonal Abs against CD11a prevented ECM [29], CD11a might play an important role in the trafficking of antigen-specific cells to the site of inflammation where a corresponding ligand, such as ICAM-1, is expressed. Using an antigen presentation assay designed to efficiently cross-present PbA antigens to CD8<sup>+</sup> T cells, we directly demonstrated that at least 10% of the repertoire specifically upregulate IFN- $\gamma$  in response to schizont antigen exposure, consistent with a role for IFN- $\gamma$  in ECM pathogenesis [6]. Because *Plasmodium* parasites infect RBCs, which generally do not express MHC-I antigen processing machinery, the priming of antigen-specific cells is most likely dependent on cross-presentation by CD8<sup>+</sup> DCs, as demonstrated in previous studies [12, 30], though a contribution by infected reticulocytes to CTL expansion, once initiated by DCs, has not been excluded. Finally, some CTL specificities were precisely defined using an epitope discovery approach that identified 5 K<sup>b</sup>-restricted PbA epitopes. Each of the 5 epitopes analyzed is derived from a different schizont-stage protein. The identification of these epitopes has allowed us to directly demonstrate K<sup>b</sup>-restricted parasite antigen-specific lytic activity during the course of infection. Unfortunately, to date we have not been able to test for specificity within the brain-infiltrating CD8<sup>+</sup> T-cell population. A large proportion of brain-infiltrating cells produce IFN- $\gamma$  ex vivo without further exposure to antigen, masking the small proportion of cells specific for our identified epitopes. Furthermore, attempts to generate tetramers to our defined epitopes have yet to be successful.

Recently, proliferative responses of CD8<sup>+</sup> T cells specific for the hypoxanthine guanine xanthine phosphoribosyl transferase protein were detected in patients during acute *P. falciparum* infection [31]. Furthermore, a novel epitope from human merozoite surface protein 1 (MSP-1) was identified by mass spectrometry, and CTL specific to this epitope were generated [32]. This evidence supports the notion that priming of parasite-specific CD8<sup>+</sup> T cells may also occur in humans. Whether these cells contribute to severe disease in humans, however, is not known. Interestingly, the 5 epitopes identified in our study are

derived from proteins that are conserved among different species of *Plasmodium*, including the human-specific species *P. falciparum* and *P. vivax* (PlasmoDB). This raises the possibility that these proteins might also serve as a source of antigen in humans, though differences in MHC restriction elements obviously affect which epitopes might be presented. The protein sources for epitopes D5, F4, and F6 are known to be involved in transcription or cell cycle [33]. They are also expressed in other stages of the parasite life cycle; for example, protein for F6 is also expressed in gametocytes and proteins for D5 and F4 are also expressed in ookinete and gametocytes (PlasmoDB). These features suggest that the protein sources for D5, F4, and F6 may function as housekeeping proteins.

Our study emphasizes that viruses are not the only pathogens that can induce robust CTL responses. It also implies that the weaponry of the immune system is not always directed appropriately for control of an invading pathogen because the malaria parasite replicates in RBCs, which lacks MHC I antigen-processing machinery. Whether generation of CTL is an unavoidable consequence of immunity or is a fail-safe approach to ensure reactivity in case parasites reside in MHC I-expressing cells is not known; however, this question may be answered with better knowledge of how responses are directed.

## Notes

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## References

- White NJ, Turner GD, Medana IM, Dondorp AM, Day NP. The murine cerebral malaria phenomenon. *Trends Parasitol* **2010**; 26:11–5.
- Hunt NH, Grau GE, Engwerda C, et al. Murine cerebral malaria: the whole story. *Trends Parasitol* **2010**; 26:272–4.
- Hermsen C, van de Wiel T, Mommers E, Sauerwein R, Eling W. Depletion of CD4+ or CD8+ T-cells prevents *Plasmodium berghei* induced cerebral malaria in end-stage disease. *Parasitology* **1997**; 114:7–12.
- Renia L, Potter SM, Mauduit M, et al. Pathogenic T cells in cerebral malaria. *Int J Parasitol* **2006**; 36:547–54.
- Belnoue E, Kayibanda M, Vigario AM, et al. On the pathogenic role of brain-sequestered alphabeta CD8+ T cells in experimental cerebral malaria. *J Immunol* **2002**; 169:6369–75.
- Yanez DM, Manning DD, Cooley AJ, Weidanz WP, van der Heyde HC. Participation of lymphocyte subpopulations in the pathogenesis of experimental murine cerebral malaria. *J Immunol* **1996**; 157:1620–4.
- Amante FH, Stanley AC, Randall LM, et al. A role for natural regulatory T cells in the pathogenesis of experimental cerebral malaria. *Am J Pathol* **2007**; 171:548–59.
- Nie CQ, Bernard NJ, Schofield L, Hansen DS. CD4+ CD25+ regulatory T cells suppress CD4+ T-cell function and inhibit the development of *Plasmodium berghei*-specific TH1 responses involved in cerebral malaria pathogenesis. *Infect Immun* **2007**; 75:2275–82.
- Hansen DS, D’Ombrain MC, Schofield L. The role of leukocytes bearing natural killer complex receptors and killer immunoglobulin-like receptors in the immunology of malaria. *Curr Opin Immunol* **2007**; 19:416–23.
- Hansen DS, Siomos MA, Buckingham L, Scalzo AA, Schofield L. Regulation of murine cerebral malaria pathogenesis by CD1d-restricted NKT cells and the natural killer complex. *Immunity* **2003**; 18:391–402.
- D’Ombrain MC, Hansen DS, Simpson KM, Schofield L. Gammadelta-T cells expressing NK receptors predominate over NK cells and conventional T cells in the innate IFN-gamma response to *Plasmodium falciparum* malaria. *Eur J Immunol* **2007**; 37:1864–73.
- Lundie RJ, de Koning-Ward TF, Davey GM, et al. Blood-stage *Plasmodium* infection induces CD8+ T lymphocytes to parasite-expressed antigens, largely regulated by CD8alpha+ dendritic cells. *Proc Natl Acad Sci U S A* **2008**; 105:14509–14.
- Lundie RJ, Young LJ, Davey GM, et al. Blood-stage *Plasmodium berghei* infection leads to short-lived parasite-associated antigen presentation by dendritic cells. *Eur J Immunol* **2010**; 40:1674–81.
- Miyakoda M, Kimura D, Yuda M, et al. Malaria-specific and non-specific activation of CD8+ T cells during blood stage of *Plasmodium berghei* infection. *J Immunol* **2008**; 181:1420–8.
- Janse CJ, Ramesar J, Waters AP. High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite *Plasmodium berghei*. *Nat Protoc* **2006**; 1:346–56.
- Vremec D, Pooley J, Hochrein H, Wu L, Shortman K. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J Immunol* **2000**; 164:2978–86.
- Mach N, Gillessen S, Wilson SB, Sheehan C, Mihm M, Dranoff G. Differences in dendritic cells stimulated in vivo by tumors engineered to secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand. *Cancer Res* **2000**; 60:3239–46.
- Rai D, Pham NL, Harty JT, Badovinac VP. Tracking the total CD8 T cell response to infection reveals substantial discordance in magnitude and kinetics between inbred and outbred hosts. *J Immunol* **2009**; 183:7672–81.
- Schmidt NW, Butler NS, Badovinac VP, Harty JT. Extreme CD8 T cell requirements for anti-malarial liver-stage immunity following immunization with radiation attenuated sporozoites. *PLoS Pathog* **2010**; 6:e1000998.
- Schnorrer P, Behrens GM, Wilson NS, et al. The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. *Proc Natl Acad Sci U S A* **2006**; 103:10729–34.
- van Lint A, Ayers M, Brooks AG, Coles RM, Heath WR, Carbone FR. Herpes simplex virus-specific CD8+ T cells can clear established lytic infections from skin and nerves and can partially limit the early spread of virus after cutaneous inoculation. *J Immunol* **2004**; 172:392–7.
- Hill AV, Allsopp CE, Kwiatkowski D, et al. Common West African HLA antigens are associated with protection from severe malaria. *Nature* **1991**; 352:595–600.
- Hill AV, Elvin J, Willis AC, et al. Molecular analysis of the association of HLA-B53 and resistance to severe malaria. *Nature* **1992**; 360:434–9.
- Nitcheu J, Bonduelle O, Combadiere C, et al. Perforin-dependent brain-infiltrating cytotoxic CD8+ T lymphocytes mediate experimental cerebral malaria pathogenesis. *J Immunol* **2003**; 170:2221–8.
- Boubou MI, Collette A, Voegtle D, Mazier D, Cazenave PA, Pied S. T cell response in malaria pathogenesis: selective increase in T cells carrying the TCR V(beta)8 during experimental cerebral malaria. *Int Immunol* **1999**; 11:1553–62.

26. Bagot S, Nogueira F, Collette A, et al. Comparative study of brain CD8+ T cells induced by sporozoites and those induced by blood-stage *Plasmodium berghei* ANKA involved in the development of cerebral malaria. *Infect Immun* **2004**; 72:2817–26.
27. Potter S, Chan-Ling T, Ball HJ, et al. Perforin mediated apoptosis of cerebral microvascular endothelial cells during experimental cerebral malaria. *Int J Parasitol* **2006**; 36:485–96.
28. Nie CQ, Bernard NJ, Norman MU, et al. IP-10-mediated T cell homing promotes cerebral inflammation over splenic immunity to malaria infection. *PLoS Pathog* **2009**; 5:e1000369.
29. Grau GE, Pointaire P, Piguet PF, et al. Late administration of monoclonal antibody to leukocyte function-antigen 1 abrogates incipient murine cerebral malaria. *Eur J Immunol* **1991**; 21:2265–7.
30. deWalick S, Amante FH, McSweeney KA, et al. Cutting edge: conventional dendritic cells are the critical APC required for the induction of experimental cerebral malaria. *J Immunol* **2007**; 178: 6033–7.
31. Woodberry T, Pinzon-Charry A, Piera KA, et al. Human T cell recognition of the blood stage antigen *Plasmodium* hypoxanthine guanine xanthine phosphoribosyl transferase (HGXPRT) in acute malaria. *Malar J* **2009**; 8:122.
32. Carralot JP, Lemmel C, Stevanovic S, Pascolo S. Mass spectrometric identification of an HLA-A\*0201 epitope from *Plasmodium falciparum* MSP-1. *Int Immunol* **2008**; 20:1451–6.
33. Florens L, Washburn MP, Raine JD, et al. A proteomic view of the *Plasmodium falciparum* life cycle. *Nature* **2002**; 419:520–6.