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Granzyme B Expression by CD8+ T Cells Is Required for the Development of Experimental Cerebral Malaria

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Parasite burden predicts disease severity in malaria and risk of death in cerebral malaria patients. In murine experimental cerebral malaria (ECM), parasite burden and CD8+ T cells promote disease by mechanisms that are not fully understood. We found that the majority of brain-recruited CD8+ T cells expressed granzyme B (GzmB). Furthermore, gzmB−/− mice harbored reduced parasite numbers in the brain as a consequence of enhanced antiparasitic CD4+ T cell responses and were protected from ECM. We showed in these ECM-resistant mice that adoptively transferred, Ag-specific CD8+ T cells migrated to the brain, but did not induce ECM until a critical Ag threshold was reached. ECM induction was exquisitely dependent on Ag-specific CD8+ T cell-derived perforin and GzmB, but not IFN-γ. In wild-type mice, full activation of brain-recruited CD8+ T cells also depended on a critical number of parasites in this tissue, which in turn, was sustained by these tissue-recruited cells. Thus, an interdependent relationship between parasite burden and CD8+ T cells dictates the onset of perforin/GzmB-mediated ECM. The Journal of Immunology, 2011, 186: 000–000.

Serious malaria syndromes including cerebral malaria (CM) are thought to claim the lives of ~900,000 people, mostly children, each year (1). The cellular and molecular mechanisms that cause this life-threatening condition are unclear. However, one of the best predictors of whether a patient will develop severe malaria (including CM) is not the number of parasites circulating freely in the bloodstream (blood parasitemia), but instead, the total number of parasites in the whole body, including those sequestered in tissues (2, 3). Postmortem studies of brains from CM patients show that sequestration of parasitized RBCs (pRBCs) within the brain microvasculature is a major histological feature of this disease (4–6). Furthermore, treatment with antiparasitic drugs, such as artesunate, kills parasites faster and reduces case fatality in patients with CM (7), compared with slower acting drugs such as quinine. Thus, parasite burden appears to be a crucial factor in determining whether a malaria patient will develop CM and/or die of it. Leukocytes have also been observed in the brains of children who have died with CM (5). The role of these cells in disease pathogenesis remains unknown, although elevated levels of some cytokines produced by leukocytes have previously been associated with CM in some, but not all, studies (8–13).

A number of experimental CM (ECM) models have been developed, including the infection of C57BL/6 mice with Plasmodium berghei ANKA (PhA). This model has been extensively used to study the immune response during ECM (14–17). It has been shown that parasite-specific CD8+ T cells are primed by conventional dendritic cells in the spleen and that these cells are found in the brain at the onset of neurologic disease (17–20). Furthermore, depletion of these cells from clinically ill mice completely protects against disease, showing that CD8+ T cells mediate end-stage immune pathology in ECM (17, 18, 21), although the specific molecules produced by these cells in vivo to cause cerebral disease have not been clearly defined. In a study by Nitcheu et al. (22), CD8+ T cells were harvested from infected, perforin-deficient mice and transferred into rag2−/− mice. These cells failed to induce ECM, whereas wild-type control CD8+ T cells did cause disease. Although of interest, this small preliminary study did not assess whether transferred CD8+ T cells had migrated to the brain. Therefore, the role of CTL in the brain during ECM remains to be determined. In contrast to studies on immune responses during ECM, the role of parasite burden in driving disease in ECM has received relatively little attention (23, 24). We recently reported that parasite burden rapidly increases prior to disease onset in ECM and that CD8+ T cells facilitate parasite tissue accumulation via mechanisms that remain undefined (24). In addition, two recent studies showed that drug-mediated killing of parasites during later stages of infection protected against ECM (23, 25). The interrelationship between the CD8+ T cells and pRBCs in the brain for the development of ECM was recently investigated (23). However, the significance of parasite biomass for the activation/phenotype of the CD8+ T cells in the brain has not yet been elucidated.
Although we understand many of the signals that orchestrate CD8\(^+\) T cell activation in secondary lymphoid organs, we know less about the control of CD8\(^+\) T cell function in peripheral tissue sites, particularly in the brain. The brain is uniquely isolated from its own vasculature by the blood–brain barrier (BBB) (26), and yet CD8\(^+\) T cells in the bloodstream can cross into brain parenchymal tissue during infection (27, 28), cancer (29–31), and autoimmune disease (32, 33). Recent studies report that local antigen stimulation may be required either for CD8\(^+\) T cell retention and motility arrest in brain microvasculature or crossing the BBB (34–36). However, the effect of locally available Ag on CD8\(^+\) T cell function in the brain remains relatively unstudied. Suboptimal CD8\(^+\) T cell function in the brain can be enhanced under some circumstances (30, 37), demonstrating the sensitivity of these cells to modulatory signals in this tissue. However, the requirement of local antigenic stimulation for CD8\(^+\) T cell function in the brain is poorly understood. Using an ECM model, we have studied the relationship between CD8\(^+\) T cell function and parasite burden, both of which are crucial drivers of fatal pathology in mice. We have taken advantage of reduced parasite burdens in the brain of ECM-resistant, granzyme B (GzmB)-deficient mice to identify an exquisite interdependence between tissue-sequestered parasites and CD8\(^+\) T cell function that determines the onset of cerebral disease in mice.

Materials and Methods

Mice and ethics

Female C57BL/6 mice and congenic CD45.1\(^*\) C57BL/6 mice aged 6–8 wk were purchased from the Australian Resource Centre (Canning Vale, Perth, WA, Australia) and maintained under conventional conditions. C57BL/6 GzmB-cluster deficient mice, lacking GzmB and the minor granzymes GzmC and GzmF (38, 39) and OTI mice were bred and maintained in house. This study was carried out in strict accordance with guidelines from The National Health and Medical Research Council of Australia, as detailed in the document Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th edition, 2004. All animal procedures and protocols were approved (A02-633M) and monitored by the Queensland Institute of Medical Research Animal Ethics Committee.

Parasites and infections

PhA, nonlethal P. yoelii, lethal P. yoelii, and P. chabaudi AS were used in all experiments after one in vivo passage in mice. A transgenic PhA (231c1l) line expressing luciferase (Pha-luc) and GFP under the control of the EF1-\(\alpha\) promoter was used for experiments involving in vivo imaging (40). Transgenic PhA strains expressing model T cell epitopes, Pha-OVA, and control strains were maintained and used as previously reported (20). All mice were infected with 10\(^7\) pRBCs (unless stated otherwise) i.v. via the lateral tail vein. Blood parasitemia was monitored by examination of Diff-Quick–stained (Lab Aids, Narrekeen, NSW, Australia) thin blood smears obtained from tailbleeds. Sodium artesunate (Gulin Pharmaceuticals, Gulin, Guangxi, China) was prepared according to the manufacturer’s instructions, diluted in saline, and administered to mice twice with an interval of 12 h at 5 mg/kg via i.p. injection.

Disease assessment

Mice were monitored twice daily after day 5 postinfection (p.i.) and clinical ECM evaluated. Clinical ECM scores were defined by the presentation of the following signs: ruffled fur, hunching, wobbly gait, limb paralysis, convulsions, and coma. Each sign was given a score of 1. Animals with severe ECM (accumulative scores of 4) were sacrificed by CO\(_2\) asphyxiation according to ethics guidelines and the following time point given a score of 5 to denote death. Brain tissue from Pha-infected mice was examined at day 6 p.i. At this time, C57BL/6 control mice were scoring between 2 and 4 and had significant recruitment of CD8\(^+\) T cells into the brain compared with naive age-matched controls.

Abs and other reagents

Allophycocyanin or Pacific Blue conjugated anti–TCR\(\beta\)-chain, PE-Cy5– or PE-conjugated anti-CD4, PE-Cy5–conjugated anti-CD8, PE– or FITC–conjugated anti-CD45.1, and allophycocyanin- or Pacific Blue-conjugated anti–IFN-\(\gamma\) were purchased from Biolegend (San Diego, CA) or BD Biosciences (Franklin Lakes, NJ). PE-conjugated anti-human GzmB, with mouse cross-reactivity, was purchased from Invitrogen (Mount Waverley, VIC, Australia). Anti-CD4 (YTS191.1), anti-CD8\(^8\) (53-5.8), and isotype control mAbs were purified from culture supernatants by protein G column purification (Amersham Biosciences, Uppsala, Sweden) followed by endotoxin removal ( Mustang Membranes; PallLife Sciences, East Hills, NY). Purified control rat IgG was also used in some experiments and purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). CD4\(^+\) T cell or CD8\(^+\) T cell depletion (>95% efficiency in the spleen in this study) was achieved by administration of 0.5 mg YTS191.1 or 53-5.8 mAb i.p., followed by 0.2 mg i.p. doses every 3 d. SHINEFEL and FILKSINE peptides were produced in-house at Queensland Institute of Medical Research, with identity and purity confirmed by mass spectrometry. Peptides were dissolved in saline and 100 \(\mu\)g administered to mice via i.v. injection.

Preparation of tissue mononuclear cells

Spleen cells were isolated by passing tissue through a 100-\(\mu\)m sieve in RPMI 1640 tissue-culture medium supplemented with 2% (v/v) FCS (Wash Buffer). RBCs were lysed using hypotonic red cell lysis buffer according to the manufacturer’s instructions (Sigma-Aldrich) and washed once more with Wash Buffer. In all experiments involving analysis of cell populations in the brain, mice were perfused via the heart with 20 ml PBS to remove blood and nonadherent cells from the brain microvasculature. Brain mononuclear cells were isolated by digesting tissue in collagenase type 4 (1 mg/ml; Worthington Biochemical, Lakewood, NJ) and DNase I (0.5 mg/ml; Worthington Biochemical) at room temperature for 40 min, before passing through a 100-\(\mu\)m sieve and washing twice with Wash Buffer. The cell pellet was resuspended in 33% (v/v) Percoll in PBS and centrifuged at 693 \(\times\) g for 12 min at room temperature. Supernatant containing debris was removed, and the leukocyte pellet was washed once in Wash Buffer. RBCs lysed as described above, and washed and resuspended in RPMI 1640 medium supplemented with 5% (v/v) FCS.

FIGURE 1. Robust expression of GzmB by CD8\(^+\) T cells during ECM.

C57BL/6 mice were infected with PhA, and at time points indicated CD8\(^+\) T cells (gated) from spleen (A) or brain (B) (on day 6) were assessed directly ex vivo for GzmB and IFN-\(\gamma\) production by intracellular staining. Quadrants in FACS plots indicate isotype control Ab staining levels. Graph in A shows the percentage of splenic CD8\(^+\) T cells expressing GzmB over the course of infection for five mice per time point. Data presented are representative of more than five independent experiments performed. C. C57BL/6 mice were infected with PhA, nonlethal P. yoelii, lethal P. yoelii (10\(^7\) pRBC), or P. chabaudi AS, and the GzmB index was calculated when mice displayed parasitemias between 4.6 and 5.4% (on day 5 p.i. for all infections). Data are representative of two independent experiments performed with three mice per group.
Flow cytometric analysis

For the staining of cell-surface Ags, cells were incubated with fluorochrome-conjugated mAbs on ice for 20 min. Intracellular cytokine staining for IFN-γ and GzmB was performed using a BD Fixation/Permeabilization kit (BD Biosciences) according to the manufacturer’s instructions after cells had been incubated in 5 μg/ml brefeldin A (Sigma-Aldrich) in RPMI 1640 medium supplemented with 5% (v/v) FCS for 3 h at 37°C/5% CO₂. In some experimental analyses, a GzmB index was calculated by multiplying the number of GzmB⁺ cells by their geometric mean fluorescence intensity of GzmB expression. Data were acquired on an FACSCanto II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR). Cell populations in the spleen and brain were defined as follows: CD4⁺ T cells (CD4⁺TCRβ⁺) and CD8⁺ T cells (CD8⁺TCRβ⁺). Cytokines in tissue-culture supernatants and serum samples were quantified using the cytometric bead array flexsets (BD Biosciences) on an FACSArray equipped with BD Flexset analysis software (BD Biosciences).

In vivo bioluminescence imaging

PbA-luc pRBCs were visualized by imaging whole bodies or dissected organs with an I-CCD photon-counting video camera and in vivo imaging system (IVIS 100; Xenogen, Alameda, CA). Mice were anesthetized with fluothane and injected i.p. with 0.1 ml 5 mg/ml α-luciferin firefly potassium salt (Xenogen). Five minutes afterward, images were captured on the IVIS 100 according to the manufacturer’s instructions (Xenogen). Parasites were visualized in the brain after removal from mice that had been perfused with 15 ml saline via the heart. Bioluminescence generated by PbA-luc in mice or brain tissue was measured according to the manufacturer’s instructions. The unit of measurement was photons/second/cm²/steradian.

Statistical analysis

Differences in survival of treatment groups were analyzed using the Kaplan–Meier log-rank test. Differences in parasitemia, cytokine levels, cell numbers, and bioluminescence were analyzed using the nonparametric Mann–Whitney U test. For all statistical tests, p < 0.05 was considered significant.

Results

Robust expression of GzmB by CD8⁺ T cells during ECM, but not other rodent models of malaria

We first analyzed GzmB expression in CD8⁺ T cells during PbA infection and noted very little expression in the spleen over the first 4 d of infection (Fig. 1A). However, on day 6 p.i., when mice displayed ECM symptoms, we observed dramatic upregulation of GzmB by CD8⁺ T cells in the spleen, but not in lymph nodes (data not shown), with approximately one third of all splenic CD8⁺ T cells expressing GzmB (Fig. 1A). A substantial proportion of these cells also expressed IFN-γ in the spleen (Fig. 1A), a cytokine that is critically required for ECM induction (15, 41). CD8⁺ T cells recruited to the brain during ECM all expressed GzmB, with a large proportion again expressing IFN-γ (Fig. 1B). Because the frequency of GzmB-positive CD8⁺ T cells was so substantial during PbA infection, we examined GzmB expression in other rodent malaria models to determine if this was a common feature of experimental malaria infection. At day 5 p.i., when blood parasitemia was similar between infections (~5% pRBCs), GzmB expression by CD8⁺ T cells was significantly higher in PbA infections than in P. chabaudi or P. yoelii infections (Fig. 1C). Thus, CD8⁺ T cells dramatically increase GzmB expression during PbA infection, more so than in other, non-ECM models of malaria.

Previous studies have reported that GzmB-producing regulatory T (Treg) cells may be important immune regulators (reviewed in Ref. 42). Therefore, we next examined whether Treg cells produced GzmB in response to PbA infection. The number of GzmB-
positive Treg cells in the spleens of C57BL/6 mice increased significantly \( (p < 0.001) \) by day 4 p.i. (Supplemental Fig. 1). Hence, Treg cells are also a significant source of GzmB following PbA challenge.

**GzmB-deficient mice do not develop ECM**

GzmB cluster-deficient C57BL/6 mice \((gzmB^{-/-})\); lacking expression of GzmB and the minor GzmC and GzmF) and wild-type controls were infected with PbA and monitored for ECM symptoms. Wild-type mice typically displayed neurologic symptoms around day 6 p.i. and succumbed to infection between days 6 and 8 p.i., whereas \( gzmB^{-/-} \) mice rarely displayed physical signs of disease and were completely resistant to ECM (Fig. 2A). Blood parasitemias on day 6 p.i. were not significantly different between the two groups (Fig. 2B). However, by using a transgenic PbA-luc strain, we were able to measure live parasite biomass in mice, which takes into account not only pRBCs in circulating blood, but also those accumulating in tissue microvasculature, as we have previously described (40, 43). By measuring parasite-derived bioluminescence emanating from PbA-luc–infected wild-type and \( gzmB^{-/-} \) mice, we observed significantly lower total parasite burden in \( gzmB^{-/-} \) mice (Fig. 2C). Furthermore, after circulating blood had been removed by saline perfusion, fewer parasites were detected in the brains of \( gzmB^{-/-} \) mice compared with wild-type mice (Fig. 2D). Of note, we also observed substantially reduced blood parasitemia and parasite burden in perforin-deficient, ECM-resistant mice (Supplemental Fig. 2A, 2B). Together, these data suggest that GzmB and perforin both play a critical role in determining susceptibility to ECM by promoting parasite accumulation in tissue.

**Enhanced CD4\(^+\) T cell responses in \( gzmB^{-/-} \) mice control parasite biomass during ECM**

We next examined the impact of GzmB deficiency on the host immune response during PbA infection. Analysis of serum cytokines revealed that \( gzmB^{-/-} \) mice produced significantly higher levels of IFN-\( \gamma \), TNF, IL-6, and MCP-1 than wild-type mice by day 6 p.i. (Supplemental Fig. 3). When splenic T cell responses were examined, we found substantially higher numbers of IFN-\( \gamma \)–producing CD4\(^+\) T cells and CD8\(^+\) T cells in \( gzmB^{-/-} \) mice compared with wild-type mice (Fig. 3A, 3C), as well as in perforin-deficient mice compared with wild-type mice (Supplemental Fig. 2C). Importantly, anti-CD4 and anti-CD8\( \beta \) Ab depletion studies in \( gzmB^{-/-} \) mice demonstrated that CD4\(^+\) T cells, but not CD8\(^+\) T cells, mediated improved control of parasite biomass (Fig. 3B, 3D). Taken together, these data show that GzmB deficiency facilitates the generation of CD4\(^+\) T cell responses during PbA infection that are better able to control parasite growth.

**CD8\(^+\) T cells migrate to the brain but cannot induce ECM in \( gzmB^{-/-} \) mice**

Because the number of activated splenic CD8\(^+\) T cells was greater in \( gzmB^{-/-} \) mice than in wild-type mice at day 6 p.i., we examined whether these cells were being retained in the spleen, thus preventing their recruitment to the brain. This was not the case because CD8\(^+\) T cell recruitment to the brain proceeded equally well in wild-type and \( gzmB^{-/-} \) mice (Fig. 4A). However, these data provided no information on the Ag specificity of CD8\(^+\) T cells entering the brain at the onset of ECM. To address this issue and determine whether GzmB production by wild-type,
parasite-specific CD8+ T cells could be sufficient for ECM induction, we employed a transgenic PbA strain (PbA-OVA) expressing the MHC class I-restricted epitope SIINFEKL from chicken egg OVA, which is recognized by OVA-specific CD8+ T (OTI) cells (20). Congenically marked OTI cells were transferred into wild-type and gzmb−/− mice, which were then infected with PbA-OVA or PbA. OTI cells were readily detected in the spleens of mice infected with PbA-OVA, but not PbA, 6 d later (data not shown). Importantly, OTI cells were recruited to the brains of wild-type and gzmb−/− mice infected with PbA-OVA, but not to the brains of mice infected with PbA (Fig. 4B). These data indicate that Ag-specific CD8+ T cell activation and recruitment to the brain is a GzmB-independent process. Crucially, gzmb−/− mice exhibiting clear expansion and recruitment of Ag-specific, wild-type OTI cells to the brain never displayed ECM symptoms, indicating that brain-recruited CD8+ T cells alone are insufficient for the induction of CNS immune pathology.

Parasite-specific CD8+ T cells recruited to the brain are not fully activated in gzmb−/− mice

To determine why OTI cells did not induce ECM in gzmb−/− mice, we next examined their activation status in the spleens and brains of wild-type and gzmb−/− mice. The number of splenic OTI cells producing GzmB or IFN-γ in PbA-OVA–infected gzmb−/− recipients was significantly greater than in C57BL/6 controls on day 6 p.i., when control animals had developed ECM symptoms (Fig. 5A). At the same time, there was no difference in the total number of OTI cells (Fig. 4B) or the number of these cells producing GzmB in the brains of PbA-OVA–infected gzmb−/− and C57BL/6 recipients (Fig. 5B). However, there was a significant reduction in the number of IFN-γ–producing OTI cells in the brains of PbA-OVA–infected gzmb−/− recipients compared with C57BL/6 recipients (Fig. 5B). Thus, despite there being no defect in the ability of OTI cells to migrate to the brain following PbA infection, fewer of these cells produced IFN-γ. The expression of IFN-γ has been considered a reliable marker of OTI CTL activity, despite OTI cells not needing to produce IFN-γ to kill target cells (44). Thus, these data suggest that brain-recruited, parasite-specific CD8+ T cells require additional stimulation in this tissue to become fully activated.

Parasite-specific CD8+ T cells induce ECM in gzmb−/− recipients when Ag load is elevated via production of perforin and GzmB, but not IFN-γ

Given the reduced parasite biomass in the brains of PbA-infected gzmb−/− mice, relative to wild-type mice (Fig. 2D), we next examined whether increased antigenic stimulation of OTI cells could induce ECM in gzmb−/− recipients. We administered the OTI peptide SIINFEKL or the negative control scrambled peptide FILKSIINE to gzmb−/− recipients of OTI cells that had been infected 6 d previously with PbA-OVA. As expected, the negative control peptide did not trigger IFN-γ production by OTI T cells in the brain (Fig. 6A). In contrast, SIINFEKL peptide stimulation induced substantial IFN-γ expression by OTI cells in the brains of infected gzmb−/− mice (Fig. 6A). This occurred without a change in the overall number of OTI cells in the brain (data not shown). Furthermore, infected gzmb−/− mice that received OTI cells and the irrelevant FILKSIINE peptide (Fig. 6B) or SIINFEKL peptide alone with no cells (data not shown) displayed no symptoms of ECM. Strikingly, PbA-OVA–infected gzmb−/− mice that had received OTI cells and SIINFEKL peptide rapidly developed severe neurologic symptoms with similar kinetics to control C57BL/6 mice infected with PbA-OVA (Fig. 6B). We earlier reported enhanced antiparasitic CD4+ T cell responses in the absence of GzmB and showed that depletion of these cells resulted in increased parasite burden, without inducing ECM (Fig. 3A, 3B). Therefore, as a second approach to increasing parasite burden in PbA-OVA–infected, OTI-recipient, gzmb−/− mice, we depleted CD4+ T cells in these animals from day 5 p.i. Two days after the commencement of Ab treatment, blood parasitemia was ~2-fold higher in mice depleted of CD4+ T cells compared with controls (Supplemental Fig. 4A). As expected, OTI-recipient gzmb−/− mice receiving a control Ab failed to develop disease. However, OTI-recipient gzmb−/− mice depleted of CD4+ T cells developed clinical signs of ECM from day 7 p.i. onwards, and 50% of these animals succumbed to disease by day 9 p.i. (Supplemental Fig. 4B). Therefore, increasing parasitemia by removing antiparasitic CD4+ T cells in OTI-recipient gzmb−/− mice significantly (p < 0.05) increased their susceptibility to ECM. Together, these data show that wild-type, parasite-specific CD8+ T cells can induce ECM in gzmb−/− mice, but only when a critical threshold of parasite Ag is exceeded.

To determine the critical effector molecules that must be expressed by parasite-specific CD8+ T cells to induce ECM, we next transferred OTI CD8+ T cells that were deficient in perforin, GzmB, or IFN-γ into gzmb−/− recipients and then infected these mice with PbA-OVA. We previously confirmed that these gene-deficient OTI cells expand and are recruited to the brain during PbA-OVA infection to the same extent as wild-type OTI cells (data not shown). When these mice were treated with SIINFEKL peptide, it was evident that recipients of IFN-γ–deficient OTI cells developed severe pathology with very similar kinetics to those that had received wild-type OTI cells (Fig. 6C, 6D), with both groups of mice displaying 100% susceptibility to disease. In stark contrast, symptoms were much milder in mice that had received perforin deficient or GzmB-deficient OTI cells, with >70% of mice in both groups failing to develop fulminant ECM (Fig. 6C,

**FIGURE 5.** Parasite-specific CD8+ T cells recruited to the brain are not fully activated in gzmb−/− mice. C57BL/6 wild-type and gzmb−/− mice (n = 5 to 6) were adoptively transferred with 10,000 congenically marked (CD45.1+) CD8+ T cells from OTI mice and infected the same day with PbA-OVA. On day 6 p.i., the numbers of transferred (CD45.1+) OTI CD8+ T cells in the spleen (A) and brain (B) were measured directly ex vivo for GzmB production and IFN-γ production, as indicated. These data are representative of three independent experiments performed. ***p < 0.005, **p < 0.001.
6D). These data show that parasite-specific CD8+ T cells cause severe cerebral pathology through the production of perforin and GzmB, and although IFN-γ production is a reasonable marker of CTL activation, its production by these cells is not critical for the induction of ECM.

**Parasites biomass and CD8+ T cell responses promote ECM in wild-type mice in an interdependent manner**

The experiments performed in gzmb<sup>−/−</sup> mice indicate that CD8<sup>+</sup> T cells require a critical Ag threshold in peripheral tissue to provide sufficient stimulation for these cells to cause immune pathology. To further examine the relationship between brain-recruited CD8<sup>+</sup> T cells and parasites accumulated in the brain, wild-type mice that had been infected 6 d previously with PbA, and which were displaying clear ECM symptoms, were treated with sodium artesunate to rapidly kill parasites and thereby reduce Ag load. Similar to other drug intervention studies in the ECM model (23, 25), all drug-treated mice displayed markedly reduced parasite biomass 24 h later (Fig. 7D). In support of findings from previous studies (23, 25), we found that parasite biomass was substantially reduced in mice depleted of CD8<sup>+</sup> T cells compared with control mice, indicating that CD8<sup>+</sup> T cells play a key role in maintaining parasite tissue sequestration during ECM. Taken together, these data reveal a striking interdependence between parasite tissue accumulation and CD8<sup>+</sup> T cell responses. Clearly, both factors drive pathology in murine cerebral malaria, and moreover, each factor depends on the other to do so.

**Discussion**

By studying the impact of GzmB deficiency in ECM, we have discovered that parasite-specific CD8<sup>+</sup> T cells in the brain can only cause severe neurologic symptoms if they receive sufficient antigenic stimulation. Previous studies have shown that CD8<sup>+</sup> T cells can mediate fatal cerebral pathology during blood-stage malaria infection in mice, although in vivo evidence that they employ cytotoxic mechanisms to do so is still lacking (16, 17). Furthermore, the role of parasite biomass in driving CD8<sup>+</sup> T cell immune pathology has until now been completely unappreciated. Our studies provide a direct causal link between parasite load and CD8<sup>+</sup> T cell-mediated pathology in ECM and, moreover, show that these factors are critically dependent on one another. These findings are in concordance with two recent ECM studies showing that parasite burden in the brain during the later stages of infection is crucial for the onset of cerebral pathology (23, 25). Also, by using an adoptive cell transfer system and externally controlling the level of antigenic stimulation, we provide clear in vivo evidence that Ag-specific CD8<sup>+</sup> T cell-derived perforin and GzmB, but not IFN-γ, drive cytotoxic T cell-mediated cerebral pathology.
other cells, such as CD4+ T cells or NK cells, are important.

These data are representative of two independent experiments.

Depleted of CD8+ T cells using anti-CD8 antibody, these animals developed disease rapidly after receiving additional antigenic stimulation. This seems unlikely because these animals developed these reports, we also detected GzmB production by splenic Treg cells during CD4+ T cell responses are a feature of several chronic brain infections and cancers (30, 37), and therefore, our data suggest that increasing MHC class I-restricted Ag presentation, or indeed Ag load, in the brain may facilitate improved antimicrobial or antitumor responses. However, data from our model also indicate that immune-mediated disease pathology is a possible consequence of such strategies and, specifically, that molecules like GzmB may even contribute to the accumulation of parasites in tissue sites such as the brain.

The identity of cells that can mediate MHC class I-restricted cross-presentation of Ag to CD8+ T cells in the brain remain unknown. MHC-restricted stimulation of T cells in nonlymphoid organs is a well-established phenomenon for virus-specific memory CD8+ T cells and conventional and regulatory CD4+ T cells (46, 47). Many cells exist in inflamed brain tissue that are capable of presenting Ag via MHC class I, including endothelial cells, astrocytes, microglia, dendritic cells, macrophages, and inflammatory monocytes, but the cells that can cross-present PbAg in this tissue remain unknown. One study suggested that brain endothelial cells can trigger MHC class I-dependent migration of CD8+ T cells across the BBB, although a requirement for APC stimulation within the brain parenchyma was not investigated (36).

Few studies have investigated the role of APC during the symptomatic stages of ECM, although late depletion of actively phagocytic cells using chlorocholate liposomes did not prevent ECM (48). Recently, we showed that CD4+ T cells control parasite numbers poorly during ECM and, instead, can mediate parasite tissue accumulation via mechanisms requiring IFN-γ and lymphotoxin α (24). Our data suggest that GzmB deficiency results in the generation of bona fide antiparasitic CD4+ T cells that may differ qualitatively from wild-type CD4+ T cell responses. However, when whole-genome transcriptional analysis of CD4+ T cells from infected mice was conducted, we found no major changes in gene expression between wild-type mice and gzmB−/− mice at the peak of the CD4+ T cell response (National Center for Biotechnology Information Gene Expression Omnibus: GSE24903; http://www.ncbi.nlm.nih.gov/gds). Therefore, it appears that extrinsic factors may act upon primed CD4+ T cells to cause them to be pathogenic in one situation (wild-type mice), and antiparasitic in another (gzmB−/− mice). One possibility is that retention of T cells in the spleen facilitates antiparasitic activity, whereas migration out of the spleen conditions peripheral tissues to sequester parasites, an idea also proposed by others (43). The exact mechanisms by which CD4+ T cells and CD8+ T cells promote parasite tissue accumulation in the brain is currently unknown, but may be linked to their local production of inflammatory cytokines, such as IFN-γ, lymphotoxin α, and TNF, which increase expression of endothelial adhesion molecules that can potentially mediate binding of leukocytes, pRBCs, or both (49, 50).

Others have shown that GzmB can be produced by human (51) and mouse (52, 53) Treg cells and that via expression of this molecule, murine Treg cells can suppress the functions of T cells (54, 55), NK cells (55), and B cells (53) in vivo. Consistent with these reports, we also detected GzmB production by splenic Treg cells during PbA infection (Supplemental Fig. 1). Although the main finding from this work is that GzmB production by parasite-specific CD8+ T cells plays a critical role in ECM induction, we cannot exclude a role for Treg cell-derived GzmB in some of our findings. For example, the improved antiparasitic CD4+ T cell responses in PbA-infected gzmB−/− mice may have resulted from reduced Treg cell function, although this seems unlikely given our
recent report that Treg cells do not play a major role in regulating CD4+ T cells during Plasmodium infection (56).

In human CM, although the major histopathological finding is sequestration of RBCs within the brain microvasculature (5, 57), leukocytes have been reported, particularly in the brains of children who have died of CM (5), although these cells await more detailed phenotyping. Given the low frequency of CD8+ T cells in mouse brain tissue during ECM (18), careful CD8+ T cell immunohistological studies are needed in human pediatric CM autopsies. In addition, when the low frequency of CD8+ T cells in human neonates who have died of CM (5), although these cells await more detailed phenotyping, our data demonstrate the potential for sequestered parasite biomass to be exacerbated by deleterious T cell responses.

In human cerebral malaria (7) and ECM (23), rapid reduction in CD8+ T cells during ECM, this is clearly not possible in autoimmune and other T cell-mediated CNS pathologies. However, we also report that MHC class I interactions with CD8+ T cells that have accessed the brain could be a potential strategy for impairing their function. Conversely, our data also indicate that MHC class I stimulation of brain-recruited CD8+ T cells specific for brain tumor Ags or pathogens may be an attractive strategy for improving CD8+ T cell-mediated cancer therapy and clearance of certain pathogens in this organ. Thus, our data provide evidence that modulation of CD8+ T cell function is a potential strategy to improve therapy against a wide range of diseases that occur in the brain. Given the proposed roles for CD8+ T cells in mediating or protecting against numerous brain-related diseases, such as viral encephalitis (58–60), toxoplasmosis (61, 62), multiple sclerosis (63–65), brain tumors (29, 31, 66), and cerebral listeriosis (67–69), our data also indicate that CD8+ T cell maturation of tumor-specific effector CD8+ T cells.

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Disclosures

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References


