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# Immune-Mediated Mechanisms of Parasite Tissue Sequestration during Experimental Cerebral Malaria

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**Cerebral malaria is a severe complication of malaria. Sequestration of parasitized RBCs in brain microvasculature is associated with disease pathogenesis, but our understanding of this process is incomplete. In this study, we examined parasite tissue sequestration in an experimental model of cerebral malaria (ECM). We show that a rapid increase in parasite biomass is strongly associated with the induction of ECM, mediated by IFN-γ and lymphotoxin α, whereas TNF and IL-10 limit this process. Crucially, we discovered that host CD4$^+$ and CD8$^+$ T cells promote parasite accumulation in vital organs, including the brain. Modulation of CD4$^+$ T cell responses by helminth coinfection amplified CD4$^+$ T cell-mediated parasite sequestration, whereas vaccination could generate CD4$^+$ T cells that reduced parasite biomass and prevented ECM. These findings provide novel insights into immune-mediated mechanisms of ECM pathogenesis and highlight the potential of T cells to both prevent and promote infectious diseases.** ***The Journal of Immunology*****, 2010, 185: 3632–3642.**

Cerebral malaria (CM) is a severe complication of blood-stage *Plasmodium falciparum* infection. Severe malaria syndromes, including cerebral malaria, account for ~800,000 deaths worldwide each year, with 89% of mortalities occurring in Africa, 88% being children under the age of 5 y (1). The pathogenesis of CM is poorly understood, but cerebral pathology is associated with the sequestration of mature parasitized RBCs (pRBCs) in the microvasculature of tissues (2, 3). This characteristic feature of *P. falciparum* infection serves as an immune evasion strategy by the parasite that prevents the removal of pRBCs in the spleen, thus facilitating parasite survival (3–5). Although advantageous for the parasite, this strategy concentrates malaria parasites, and the metabolic and inflammatory responses triggered by them, in vital organs such as the brain (6). Parasite sequestration may lead to vascular obstruction, endothelial cell activation, and the production of proinflammatory cytokines (reviewed in Refs. 6, 7). In addition to parasite-induced pathology, a large body of work indicates that host immune responses to parasites also play an important role in CM pathogenesis (8). A robust proinflammatory response mediated by activated T cells and cytokines, as well as recruitment of activated leukocytes to the brain, have been associated with CM (9, 10).

Experimental cerebral malaria (ECM) caused by *Plasmodium berghei* ANKA (PbA) infection in mice displays key features of human CM (reviewed in Refs. 6, 11). ECM-susceptible mice develop a neurologic condition characterized by paralysis, ataxia, convulsions, and coma 6–8 d after PbA infection, resulting in 80–100% mortality at relatively low peripheral blood parasitemia (11). Both CD4$^+$ and CD8$^+$ T cells have been implicated in ECM development (12–15), and the spleen is thought to play a central role in priming PbA-specific T cell responses (16, 17). The recruitment of activated malaria-specific CD8$^+$ T cells to the brain is observed in late-stage ECM (18, 19), and mice depleted of CD8$^+$ T cells are protected from disease (14). Although the precise mechanisms by which CD8$^+$ T cells mediate cerebral pathology is still unclear, it has been suggested that they may damage cerebral microvascular endothelial cells in a perforin-dependent manner (14, 20, 21). Local production of proinflammatory cytokines such as TNF (22), IFN-γ (23), and lymphotoxin α (LTα) (24) is also critical for ECM pathogenesis. CD4$^+$ T cell depletion studies have demonstrated a causal role for this T cell subset in the induction of ECM by a mechanism thought to involve IFN-γ production, which amplifies local and systemic inflammatory cascades (12, 13, 15). The combined effects of CD4$^+$ and CD8$^+$ T cell activation are postulated to lead to blood–brain barrier breakdown with vascular leakage and/or hemorrhaging into the brain (25).

In contrast to ECM, findings from nonlethal mouse models of malaria indicate that CD4$^+$ T cells are required for the development of protective immunity and parasite control (26, 27). IFN-γ plays a central role in limiting parasite growth through the activation of macrophages (28, 29) and their release of parasiticidal mediators and promoting the generation of cytophilic Abs (9, 30–33). Thus, CD4$^+$ T cells are not only critical for the effective control of peripheral blood parasitemia, but they are also key

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Abbreviations used in this paper: CM, cerebral malaria; ECM, experimental cerebral malaria; GIT, gastrointestinal tract; LTα, lymphotoxin α; nRBC, normal RBC; PbA, *Plasmodium berghei* ANKA; PbA*luc*, PbA line (231c11) expressing luciferase; PbG, transgenic PbA parasites expressing only GFP; PbTg, *P. berghei* parasites expressing MHC class II-restricted OVA epitopes fused to GFP under the control of the ef1-α promoter; p.i., postinfection; pRBC, parasitized RBC; SEA, *Schistosoma mansoni* egg Ag; Treg, regulatory T cell.



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mediators of ECM pathology, highlighting the delicate balance that exists between host-mediated control of infection and development of disease. Defining the factors that distinguish pathogenic from protective $CD4^+$ T cell responses is important to help prevent severe malaria.

Until recently, studying the relationship between peripheral blood parasitemia, parasite tissue sequestration, and disease severity has been difficult. Typically, peripheral blood parasitemia is a poor predictor of parasite biomass and disease severity (34), because it only measures circulating immature parasites, not tissue-sequestered mature parasites (35). Although most studies on ECM focus on neurological complications, PbA infection causes multiorgan disease (36), particularly in the lung (37). We and others have employed transgenic, luciferase-expressing PbA to measure parasite biomass in peripheral tissues (38–42). However, the contribution of host-derived factors to parasite sequestration in deep tissue sites during ECM is completely unknown. In this study, we show that PbA sequestration in various organs, including the brain, is associated with the onset of ECM. Furthermore, we demonstrate that T cell-mediated immune mechanisms promote this process. Finally, using two models of immune modulation, we demonstrate that helminth coinfection exacerbates $CD4^+$ T cell-dependent parasite sequestration in deep tissue sites, whereas vaccine-induced $CD4^+$ T cell responses can limit this process and protect from ECM.

## Materials and Methods

### *Mice*

Female C57BL/6 mice (6–8 wk of age) were purchased from the Animal Resources Centre (Canning Vale, Western Australia, Australia) and maintained in the animal facility of the Queensland Institute of Medical Research (Herston, Queensland, Australia) under specific pathogen-free conditions. B6.RAG1$^{-/-}$ (43), B6.SJL.Ptprca × OT-II (44), B cell-deficient B6.μMT (45), B6.IFN-γ$^{-/-}$ (46), B6.LTα$^{-/-}$ (47), and B6.TNF$^{-/-}$ mice (48) were bred and maintained in the Queensland Institute of Medical Research animal facility. All of the animal procedures were approved by the Queensland Institute of Medical Research Animal Ethics Committee. Splenectomized C57BL/6 mice were generated in the Queensland Institute of Medical Research animal facility. Briefly, mice were anesthetized, and an incision was made in the skin and peritoneum of the left side of the body. The splenic vascular pedicle was ligated and heat-cauterized, and the spleen was excised. The peritoneal cavity then was sutured, and the skin was stapled. Sham-splenectomized mice underwent the same procedure, except that the spleen was not removed. Staples were removed 7 d after surgery, and mice were allowed 4 wk to recover prior to PbA infection.

### *Parasites and infections*

A transgenic PbA line (231c1l) expressing luciferase (PbA*luc*) and GFP under the control of the ef1-α promoter (38) was used for all of the experiments unless otherwise stated. For adoptive transfer experiments, transgenic *P. berghei* parasites expressing MHC class II-restricted OVA epitopes fused to GFP under the control of the ef1-α promoter (PbTg) were used (18). Transgenic PbA parasites expressing only GFP (PbG) were used as control parasites. Mice infected with transgenic PbA parasites were provided with drinking water containing pyrimethamine (10 mg/ml) to select for drug-resistant transfectants (49). All of the PbA infections were established from parasites passaged in C57BL/6 mice once. All of the mice, unless otherwise stated, were challenged with $10^5$ PbA pRBCs i.v. Peripheral parasitemia was assessed from Diff-Quick (Lab Aids, Narrabeen, Australia) stained thin blood smears. Mice were examined daily for clinical signs of ECM and scored as described previously (38). Hemoglobin levels were measured using the HemoCue Hb 201 analyzer according to manufacturer's instructions (HemoCue, Angelholm, Sweden). For experimental infection with *Schistosoma mansoni*, mice were infected percutaneously with either 80–100 (high dose) or 30–40 (low dose) *S. mansoni* cercariae as described previously (50). Infections were allowed to establish in C57BL/6 mice over an 8-wk period prior to infection with PbA.

### *Immunizations*

PbA Ag was prepared as described previously (51, 52). Briefly, blood from PbA-infected B6.LTα$^{-/-}$ mice was collected when peripheral blood parasitemia reached 35–40%. Blood was centrifuged, and the pellet was subjected to two rounds of RBC lysis in distilled water. The parasite pellet was then washed twice with PBS and subjected to three freeze–thaw cycles at −70 and 37°C. The parasite lysate was then disrupted further by passing through a syringe three times with a 26-gauge needle. An amount of PbA lysate Ag equivalent to$10^6$ PbA pRBCs in a volume of 50 μl was admixed with an equal volume of aluminum hydroxide (Imject Alum; Pierce, Rockford, IL). Mice were immunized s.c. with 100 μl of this PbA lysate vaccine on the abdomen. For experiments modulating regulatory T cell (Treg) function, mice were administered 0.5 mg anti-CD25 mAb (PC61; American Type Culture Collection, Manassas, VA) i.p. 1 d prior to vaccine priming. Three weeks after primary immunization, the mice were boosted i.p. twice, 2 wk apart, with the same amount of PbA lysate diluted in PBS. Two weeks later, mice were challenged i.v. with $10^5$ PbA*luc* pRBCs.

### *In vivo bioluminescence imaging*

Bioluminescence was detected with the In Vivo Imaging System 100 (Xenogen, Alameda, CA). At selected time points, PbA*luc*-infected mice were anesthetized with isoflurane and injected s.c. with 150 mg/kg D-luciferin (Xenogen) 6 min before imaging. Mice were then killed by $CO_2$ asphyxiation and subjected to intracardial perfusion with 20 ml ice-cold PBS to remove circulating blood and allow only the measurement of pRBCs sequestered in tissue microvasculature. Bioluminescence was calculated as photons per second per square centimeter per steridian using Living Image (Xenogen) and IGOR Pro software (WaveMetrics, Lake Oswego, OR).

### *Histology*

Lung and brain tissue were removed from mice with or without cardiac perfusion and processed for wax embedding and preparing tissue sections for H&E staining, as described previously (24).

### *Immunofluorescence and confocal microscopy*

After cardiac perfusion, lungs and brains were removed from mice and fixed by immersion in 4% (w/v) paraformaldehyde for 1–1.5 h. Organs were then incubated in a 30% (w/v) sucrose solution overnight and frozen in Tissue-Tek OCT (ProSciTech, Thuringowa, Queensland, Australia) on dry ice. Seven-micrometer sections were cut and stored in the dark at −80°C until required. Slides were brought to room temperature, and membranes were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, Castle Hill, New South Wales, Australia) for 10 min at room temperature in a humidifying chamber. Slides were washed three times for 5 min in PBS. Slides were blocked with 5% donkey serum for 30 min at room temperature and then washed once with PBS. The following primary and secondary Abs were used for staining: anti–VCAM-1 (rat) (Biolegend, San Diego, CA), anti-GFP (rabbit), anti-rat Alexa Fluor 594 (donkey), and anti-rabbit Alexa 488 (donkey) (Invitrogen, Mount Waverley, Victoria, Australia). After 1 h of incubation with primary Abs, slides were washed three times for 5 min with PBS. Sections were then incubated with secondary Abs, washed, and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Confocal microscopy was performed using the TCS SP2 Confocal System (Leica Microsystems, Deerfield, IL) equipped with ArKr/HeNe lasers and Leica Confocal Software (version 2.61; Leica Microsystems).

### *Proliferation assays*

$CD4^+$ T cells were purified from splenocytes taken from PbA-infected mice by MACS (>90% purity) (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+$ T cells resuspended in RPMI 1640 medium, 10% (v/v) FCS, and antibiotics were seeded in 96-well round-bottom plates ($5 \times 10^5$ cells per milliliter). Irradiated (25 Gy) naive syngeneic splenocytes were added ($1 \times 10^7$ cells per milliliter) as a source of APCs. Cells were stimulated with PbA pRBCs ($5 \times 10^6$ cells per milliliter), *S. mansoni* egg Ag (SEA) (15 μg/ml), or Con A (2.5 μg/ml). Medium alone and an equivalent number of normal RBCs (nRBCs) served as negative controls. Cells were incubated at 37°C in 5% (v/v) $CO_2$ for 3 d. Supernatants were collected and stored at −70°C until assayed for cytokine levels. Proliferation was measured by [$^3$H]thymidine incorporation during the final 16–18 h of a 4-d culture.

### *Measurement of serum and culture supernatant cytokine levels*

Serum cytokine levels were measured using BD Cytometric Bead Array Flex Sets and a FACSArray plate reader (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions.

### *In vivo Ab-mediated cell depletion and cytokine blockade*

Mice were administered 0.5 mg anti-CD4 mAb (clone YTS191.1) or control rat IgG (Sigma-Aldrich) i.p. 1 d prior to PbA*luc* infection and 4 d post-infection (p.i.). $CD8^+$ T cell depletion was performed by administering

0.5 mg anti-CD8β mAb (clone 53-5.8) i.p. on the day of PbA*luc* infection and every second day thereafter. Control animals received the same amount of rat IgG. For IL-10 blockade, a single injection of anti–IL-10R mAb (1 mg; clone 1B1.3a) or control rat IgG was administered i.p. 1 d prior to PbA*luc* infection. TNF and LTα blockade was performed by administering TNFR2-Ig (Enbrel; Amgen, Thousand Oaks, CA), a fusion protein that has been shown previously to inhibit the functional activity of both murine TNF and soluble LTα homotrimers (53). A total of 0.2 mg TNFR2-Ig was administered i.p. on the day of PbA*luc* infection and every second day thereafter. Control mice received control human IgG (Intragam; Commonwealth Serum Laboratories, Parkville, Victoria, Australia).

### *Adoptive transfer of OT-II cells*

$CD4^+$ T cells were purified from the spleens of $CD45.1^+$ transgenic OT-II mice by MACS purification (>90% purity) and were labeled with CFSE (Molecular Probes, Eugene, OR) at a final concentration of 2 μM as described previously (54, 55). Briefly, purified OVA-specific $CD4^+$ OT-II cells resuspended in PBS were incubated for 7 min at room temperature with CFSE and then washed twice in PBS. Recipient $CD45.2^+$ mice were injected i.v. with $1 \times 10^6$ CFSE-labeled $CD4^+$ OT-II cells and challenged i.v. 2 h later with$1 \times 10^6$ PbTg or PbG cells. Transferred $CD4^+$ OT-II cells were identified by flow cytometry using CD45.1, TCRβ, and $CD4^+$ mAb staining. The proliferation of transferred cells was calculated based on CFSE dilution by these cells.

### *Flow cytometry*

For FACS analysis, the following Abs were used: allophycocyanin-conjugated anti-TCRβ-chain, PE-Cy5–conjugated anti-CD4, and PE-conjugated anti-CD45.1. FACS was performed using a FACSCalibur or FACSCanto II (BD Biosciences) instrument and analyzed using FlowJo software (Tree Star, Ashland, OR).

### *Statistical analysis*

Differences in the survival of treatment groups were analyzed using the Kaplan-Meier log-rank test with Prism version 4.03 for Windows (GraphPad Software, San Diego, CA). Differences in parasitemia, cytokine levels, and bioluminescence were determined using the Mann-Whitney *U* test using Prism. For all of the statistical tests, $p < 0.05$ was considered significant.

## Results

### *ECM induction is associated with a dramatic increase in total body PbA biomass*

Sequestration of *P. falciparum*-infected erythrocytes in the microvasculature of vital organs is associated with severe malaria disease (10, 56). To establish if this important feature of human malaria pathology could be modeled following PbA infection, we first compared peripheral blood parasitemia with total parasite biomass in the same mice over the course of PbA infection. To visualize parasite distribution throughout the bodies of live mice, ECM-susceptible C57BL/6 mice were infected with PbA*luc* (38). Peripheral blood parasitemia and bioluminescence (total body parasite biomass) were measured daily from day 3 p.i. (patency) to the time of severe ECM symptoms (day 6 p.i.). A linear increase in parasitemia during the course of PbA infection was observed (Fig. 1*A*), with mice succumbing with cerebral symptoms 6 d p.i. when blood parasitemia was <10%. In contrast, parasite biomass measured over the same time period showed an exponential increase (Fig. 1*B*, 1*C*). Significantly, parasite biomass increased to a much greater extent (10-fold) than blood parasitemia (2-fold) from the onset of cerebral symptoms (day 5 p.i.) to time of sacrifice (day 6 p.i.), indicating that parasites were accumulating in tissue microvasculature and that this was associated with the onset of ECM. These findings indicate that total body parasite burden, not peripheral blood parasitemia, is most predictive of disease induction and severity.

### *Organ-specific sequestration of PbA pRBCs*

We next examined where parasites accumulated in PbA-infected mice when they developed ECM. A comparison of organ distribution of parasites was made with and without intracardial perfusion.

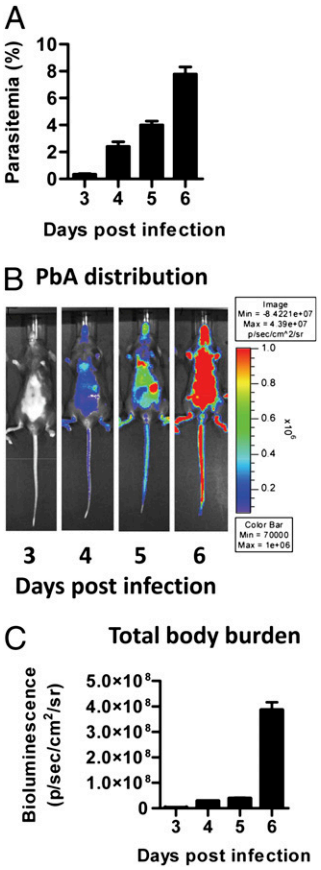


**FIGURE 1.** Increased PbA accumulation in tissues coincides with the development of ECM. Mice were infected with PbA*luc*. The course of peripheral blood parasitemia was determined by microscopic examination of blood smears (*A*). At the times indicated, mice were injected with luciferin, and whole-body pseudocolor images (*B*) and total body bioluminescence (*C*) were recorded for 1 min, 6 min after luciferin injection. Images are one representative of five mice per group. Data are from one representative experiment of two performed. Each bar represents the mean ± SEM.

Perfusion was performed immediately after sacrifice to remove circulating blood, allowing the measurement of only pRBCs trapped in tissue microvasculature (hereafter referred to as sequestration). In the absence of perfusion, PbA pRBCs were found in all of the organs studied, with the majority residing in heart, lung, spleen, liver, kidney, and gastrointestinal tract (GIT) (Fig. 2*A*). After perfusion, the majority of PbA pRBCs were observed in the lung, spleen, liver, kidney, and GIT (Fig. 2*A*, 2*B*). Interestingly, the brain represented only a small proportion of parasite tissue accumulation, highlighting the multiorgan distribution of parasites in ECM. Interestingly, the bioluminescence observed in the GIT was confined to the mesenteric lymph nodes following perfusion, indicating that the majority of parasites in the GIT were freely associated with circulating blood and not trapped in the microvasculature of this tissue. Together, these results show that at the time of ECM PbA pRBCs accumulate in most vital organs, including in the brain.

### *PbA pRBCs adhere to tissue vasculature in mice with ECM*

Given that PbA infection causes both brain and lung pathology (36, 37), we examined the interaction between pRBCs and tissue vasculature in brain and lung tissue sections taken from mice with ECM (Fig. 2*C*). In the absence of perfusion, RBCs were observed in lung venules of naive mice and mice with ECM. However, venules were clear of RBCs in lungs taken from perfused mice

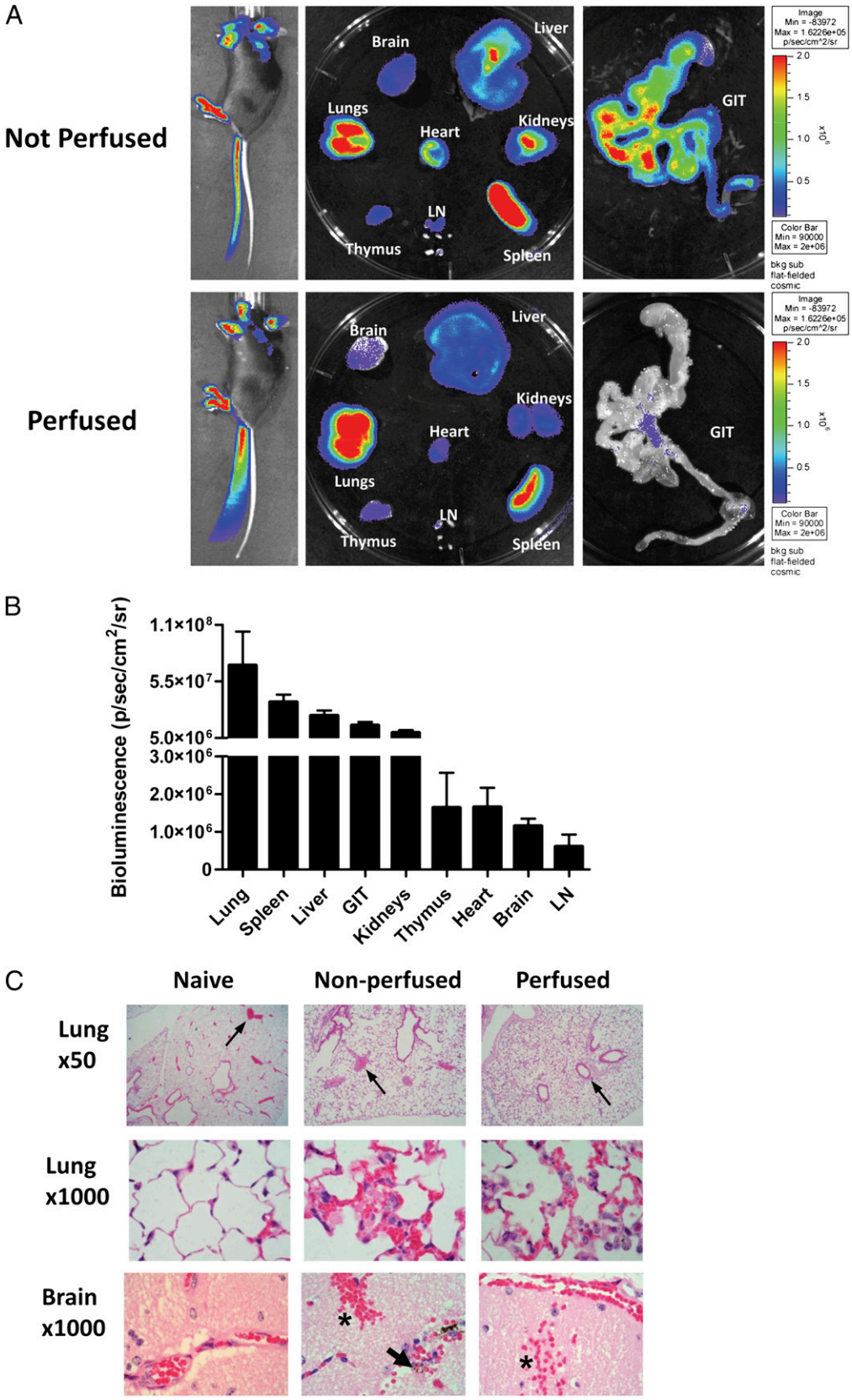


**FIGURE 2.** Organ-specific accumulation of PbA parasites. Bioluminescence imaging of organs from nonperfused (*A*, *upper panel*) or perfused (*A*, *lower panel*, *B*) mice following infection with PbA*luc*. Mice ($n = 5$) were infected with $10^5$ PbA*luc* pRBCs, and organs were imaged when severe ECM symptoms were observed (day 6 p.i.; *B*). Histological examination of H&E-stained lung and brain tissue sections from naive mice and mice with ECM with or without perfusion via the heart, as indicated (*C*). Thin arrows indicate lung venules in sections taken at low magnification (×50). Higher magnification (×1000) photographs show infected and uninfected RBCs in lung and brain tissue. Thick arrow (nonperfused brain; *lower middle panel*) indicates infected RBCs distinguishable from uninfected RBCs by the presence of hemozoin. Asterisks indicate cerebral hemorrhages. Data are from one representative experiment of two performed. Each bar represents the mean ± SEM.

(see thin arrows in low-magnification panels), again showing the effectiveness of perfusion at removing the majority of circulating RBCs from the vasculature. Close examination of lung alveoli at high magnification showed the presence of RBCs in intimate association with parenchyma cells lining alveoli, regardless of whether tissue had been perfused. Similarly, both uninfected and infected RBCs (revealed by the presence of hemozoin; see thick arrow in *lower middle panel*) were observed in close association with vascular endothelial cells in brain tissue taken from mice with ECM, regardless of whether they had been perfused. In addition, uninfected and infected RBCs were also found in cerebral hemorrhages (indicated by an asterisk in panels) and occasionally associated with leukocytes in the vascular lumen (data not shown). We also were able to identify GFP$^+$ PbA pRBCs in close association with VCAM$^+$ endothelial cells in perfused lung and brain tissue (Supplemental Fig. 1). Together, these observations indicate that tissue-sequestered pRBCs were the source of the bioluminescent signal detected from live parasites in tissues from perfused mice with ECM.

### *CD4$^+$ and CD8$^+$ T cells mediate PbA tissue sequestration*

The spleen is thought to be a site for the development of protective and pathological immune responses following PbA infection, but the requirement of this organ for ECM onset caused by PbA has not been demonstrated formally (4). To evaluate the importance of the spleen in PbA tissue sequestration and ECM induction, sham-operated and splenectomized C57BL/6 mice were challenged with PbA*luc* and monitored. Splenectomized mice failed to develop ECM and had significantly reduced total parasite biomass compared with that of sham-operated mice, which succumbed to ECM by day 7 p.i. (Fig. 3*A*). Thus, the spleen contributes to PbA tissue sequestration and the development of ECM.

The host immune response to PbA plays a major role in disease pathogenesis (8). Therefore, we next investigated the contribution of T and B cells to PbA tissue sequestration. T and B cell-deficient B6.RAG1$^{-/-}$ mice failed to develop ECM, as previously reported for B6.RAG2$^{-/-}$ mice (14), and had significantly lower parasite burdens compared with those of ECM-susceptible C57BL/6 controls (Fig. 3*B*). In contrast, B cell-deficient B6.μMT mice had significantly higher parasite burdens compared with those of control C57BL/6 mice at the time of ECM, and all of the mice succumbed to ECM by day 8 p.i. (Fig. 3*C*). These data indicate that B cells do not contribute to PbA tissue sequestration but may help to limit this process. Depletion of either CD4$^+$ or CD8$^+$ T cells prevented the development of ECM, as expected (12, 13, 57), and, critically, resulted in significant reductions in parasite biomass (Fig. 3*D*, 3*E*). Thus, both CD4$^+$ and CD8$^+$ T cells contribute to PbA tissue sequestration during ECM.

### *PbA tissue sequestration is mediated by IFN-γ and LTα*

Studies in mouse and human malaria indicate an important role for cytokines in disease pathogenesis (7, 11). Therefore, we next investigated whether key inflammatory cytokines known to play a role in ECM development contributed to PbA tissue sequestration. C57BL/6 mice deficient in IFN-γ, TNF, or LTα were infected with PbA*luc*, and parasite biomass was measured at the time control C57BL/6 mice developed ECM (Fig. 4). As expected, both B6.LTα$^{-/-}$ and B6.IFN-γ$^{-/-}$ mice failed to develop ECM and showed prolonged survival compared with that of C57BL/6 controls (23, 24). Importantly, total PbA biomass was significantly reduced in mice lacking LTα or IFN-γ compared with that of ECM-susceptible controls (Fig. 4*A*, 4*B*). These results indicate that both LTα and IFN-γ play key roles in PbA tissue sequestration. In contrast, B6.TNF$^{-/-}$ mice exhibited a small delay in ECM onset, but with

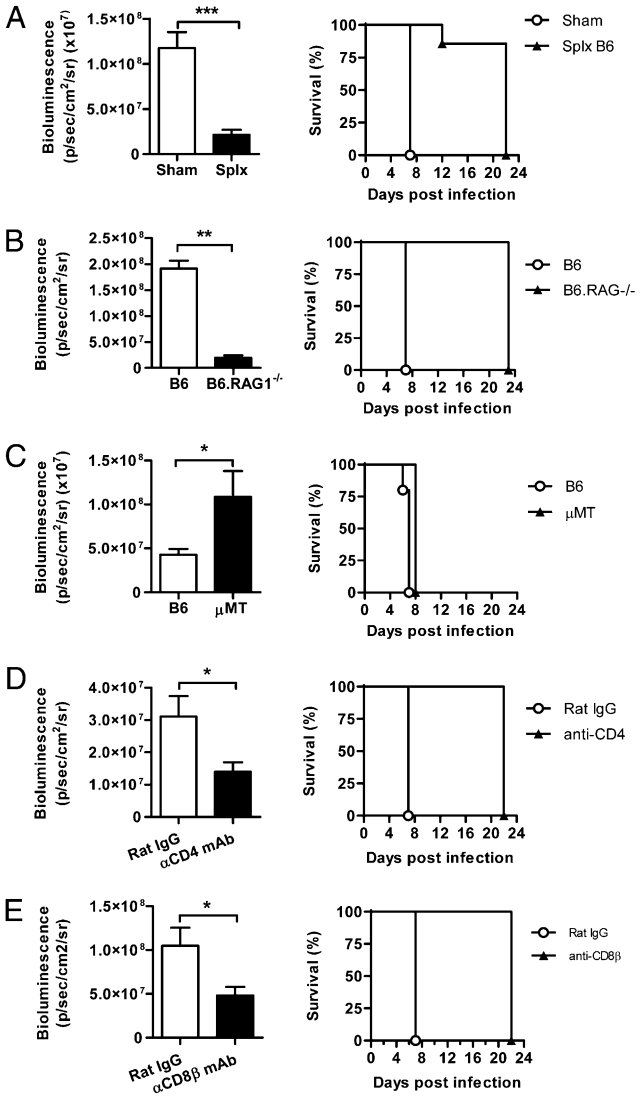


**FIGURE 3.** CD4$^+$ and CD8$^+$ T cells contribute to PbA tissue sequestration during ECM. Whole-body bioluminescence (*left panel*) and survival (*right panel*) of splenectomized (*A*), RAG1$^{-/-}$ (*B*), μMT (*C*), CD4$^+$ T cell-depleted (*D*), and CD8$^+$ T cell-depleted mice (*E*) relative to those of immunocompetent control mice following infection with PbA*luc* are shown. Bioluminescence imaging was performed when control mice developed ECM (day 7 p.i.). Each bar represents the mean ± SEM of five to six mice. Data are from one representative experiment of at least two performed. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

significantly higher parasite biomass than that of C57BL/6 controls (Fig. 4*C*), showing that TNF plays a role in limiting parasite growth and/or tissue sequestration.

Given the opposing roles for TNF and LTα in mediating PbA tissue sequestration and their shared receptor usage, we investigated the effect of blockade of both cytokines simultaneously with a soluble human TNFR2 fusion protein (Enbrel). Mice receiving combined TNF and LTα blockade throughout the course of PbA infection had comparable PbA biomass to that of control mice receiving human Ig and succumbed to ECM (Fig. 4*D*). Thus, blockade of both of these cytokines by Enbrel had a neutral effect on PbA tissue sequestration, indicating that despite blockade of soluble LTα, TNF activity was required to protect mice from ECM, reinforcing the important antiparasitic role played by TNF in malaria and the impact of parasite burden on ECM susceptibility.

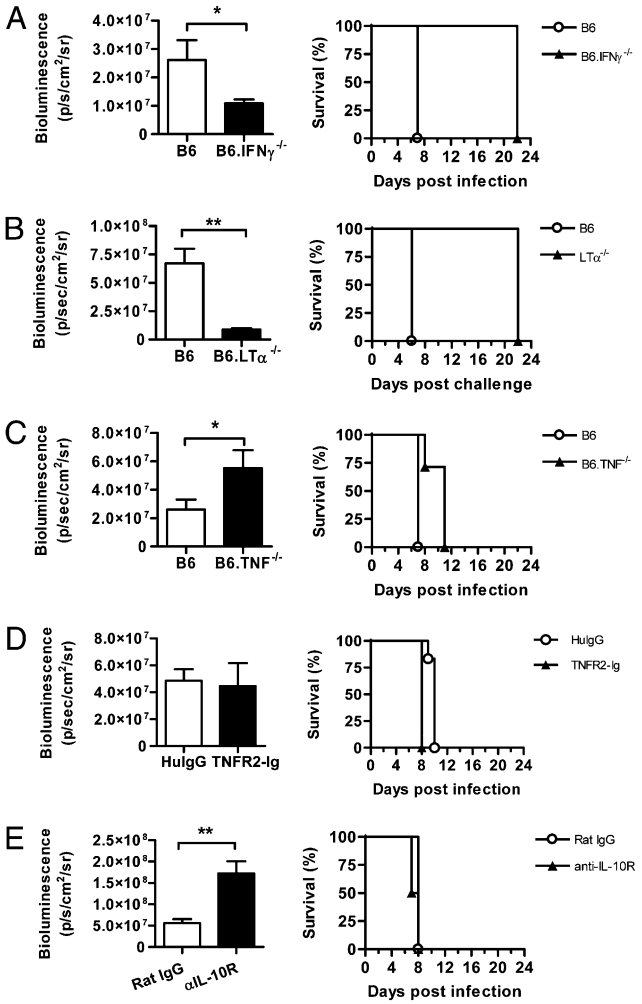


**FIGURE 4.** LTα and IFN-γ mediate PbA tissue sequestration during ECM. Whole-body bioluminescence (*left panel*) and survival (*right panel*) of B6.IFN-$\gamma^{-/-}$ mice (*A*), B6.LTα$^{-/-}$ mice (*B*), B6.TNF$^{-/-}$ mice (*C*), C57BL/6 mice in which both LTα and TNF were blocked throughout the course of infection by administration of TNFR2-Ig (*D*), and mice receiving IL-10R blockade (*E*). Mice were infected with PbA*luc*, and bioluminescence imaging was performed when control mice developed ECM (days 6–10 p.i.). Each bar represents the mean ± SEM of five to six mice. Data are from one representative experiment of at least two performed. $*p < 0.05$; $**p < 0.01$

IL-10 can play an important role in protection against ECM (38). To assess if IL-10 might regulate PbA tissue sequestration, we administered an anti–IL-10R blocking mAb prior to PbA infection and measured parasite biomass at the time of ECM (Fig. 4*E*). Mice receiving IL-10R blockade succumbed to ECM at the same time as control mice. However, these mice had significantly higher parasite biomass relative to that of control animals, indicating a key role for IL-10 in the prevention of PbA tissue sequestration. Together, these studies identify specific proinflammatory and regulatory cytokines that either promote or prevent PbA tissue sequestration.

### *Modulation of malaria-specific CD4$^+$ T cell response by* S. mansoni *coinfection and the impact on PbA tissue sequestration*

Our results above indicate that the host T cell response during PbA infection is unable to control parasite growth but instead promotes PbA tissue sequestration and contributes to ECM onset. Therefore, we next examined whether this pathogenic T cell response could be modulated to control early parasite growth and prevent PbA tissue sequestration and ECM development. *S. mansoni* infection modulates host CD4$^+$ T cell immune responses to third-party Ags by generating a potent CD4$^+$ T cell response that includes the production of several regulatory cytokines (Supplemental Fig. 2) (58, 59). *S. mansoni*-infected mice and age-matched naive controls were infected with PbA*luc* and monitored for peripheral blood parasitemia, PbA biomass, and ECM onset. Coinfected mice had higher peripheral blood parasitemia than mice infected with PbA alone from days 5–7 p.i. (Fig. 5*A*) and succumbed to ECM around the same time as these animals (Fig. 5*B*). Significantly, coinfected mice also had a 2- to 3-fold higher total body, but not brain, PbA biomass than mice infected with PbA alone (Fig. 5*C*, 5*D*).

We also evaluated the impact of coinfection on malaria-specific CD4$^+$ T cell responses. CD4$^+$ T cell recall responses to PbA blood stage Ags in *S. mansoni*-coinfected mice were significantly reduced, whereas a strong *S. mansoni*-specific CD4$^+$ T cell response against SEA was observed (Fig. 6*A*). To further study malaria-specific CD4$^+$ T cell responses, we used a transgenic OVA-expressing PbA line (PbTg) to measure the expansion of OVA-specific CD4$^+$ (OT-II) T cells in mice with and without *S. mansoni* coinfection. Proliferation of transferred CFSE-labeled OT-II cells was measured 4 d after PbTg challenge in the spleen (Fig. 6*B*). Control *S. mansoni*-infected mice injected with OT-II cells in the absence of PbTg challenge showed no OT-II cell proliferation (Fig. 6*B*), as was the case in mice infected with non-OVA–expressing PbA (PbG; data not shown). Robust OT-II cell expansion was observed in mice infected with OVA-transgenic PbA (Fig. 6*B–D*). In contrast, the absolute number and expansion of OT-II cells in the spleens of coinfected mice were significantly reduced (Fig. 6*B–D*). Thus, CD4$^+$ T cell responses generated against PbA-expressed Ags could be detected in *S. mansoni*-coinfected mice, but these responses were significantly impaired. After depletion of CD4$^+$ T cells, the majority of coinfected mice failed to develop ECM (Fig. 6*E*), and protection was associated with significantly decreased total body and brain PbA biomass (Fig. 6*F*, 6*G*). Thus, *S. mansoni* coinfection did not prevent ECM, despite modulating malaria-specific CD4$^+$ T cells responses,

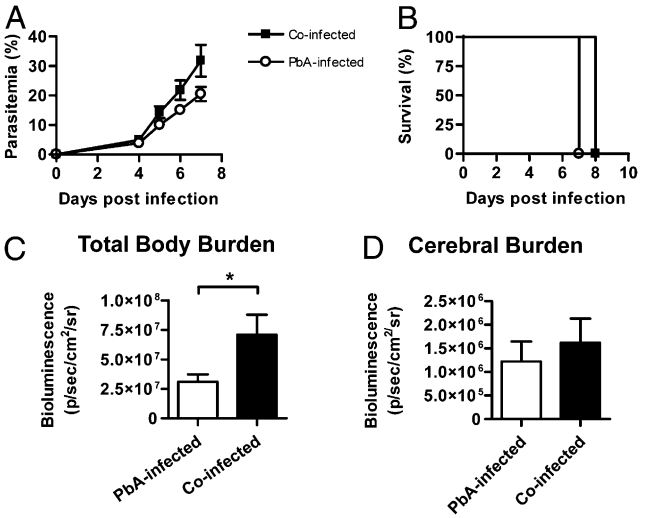


**FIGURE 5.** *S. mansoni* coinfection promotes increased PbA tissue sequestration and fails to prevent ECM. Peripheral blood parasitemia, as determined by microscopic examination of blood smears (*A*) and survival (*B*) of *S. mansoni*-infected mice following infection with PbA*luc*. Bioluminescence imaging was performed when PbA-infected control mice developed ECM (day 7 p.i.). Total body (*C*) and brain (*D*) bioluminescence are shown. Each bar represents the mean ± SEM of four to five mice. A Data are from one representative experiment of two performed. $*p < 0.05$.

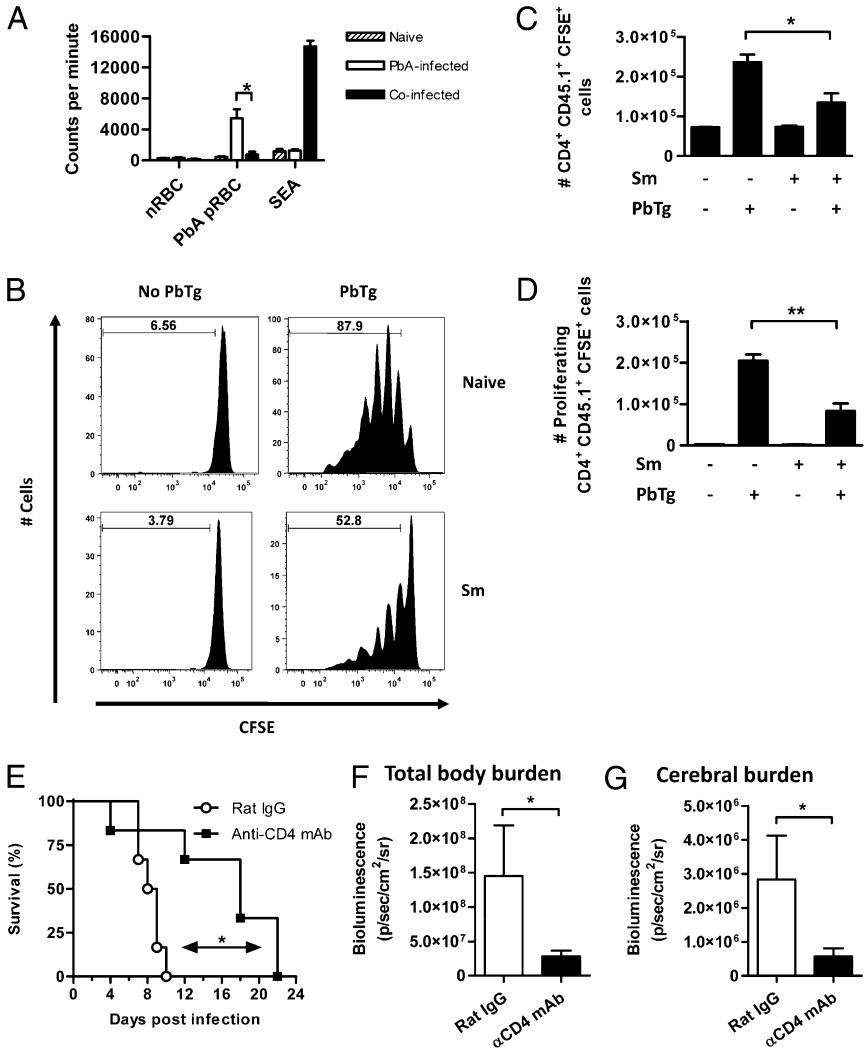


**FIGURE 6.** *S. mansoni* coinfection impairs PbA-specific CD4$^+$ T cell responses. PbA-specific CD4$^+$ T cell proliferation in PbA-infected and coinfected mice after stimulation in vitro with PbA pRBC Ag (*A*). Naive or *S. mansoni*-infected C57BL/6 mice ($n$ = 4) were challenged with PbA*luc* pRBCs. Four days later, CD4$^+$ T cells were purified from spleen cells and cultured in vitro with nRBCs, PbA pRBC Ag, or SEA in the presence of irradiated syngeneic APCs. The values represent mean ± SEM [$^3$H]thymidine uptake from triplicate cultures. In vivo proliferation (*B*), absolute numbers (*C*), and expansion (*D*) of CFSE-labeled CD4$^+$ transgenic OT-II cells in the spleens of PbA-infected and coinfected mice ($n$ = 4) following infection with PbTg. Splenic CD4$^+$ T cells purified from CD45.1$^+$ OT-II mice were CFSE-labeled and transferred into CD45.2$^+$ recipient PbA-infected or coinfected mice. In vivo proliferation of CFSE-labeled cells was measured 4 d p.i. Survival (*E*), total body (*F*), and brain (*G*) bioluminescence of *S. mansoni*-coinfected mice following in vivo depletion of CD4$^+$ T cells. Mice with acute *S. mansoni* infection were infected with PbA*luc* and administered anti-CD4 mAb or rat IgG day −1 and day 4 p.i., and bioluminescence imaging was performed when rat IgG control mice developed ECM (day 7 p.i.). Each bar represents the mean ± SEM of four to six mice. Data are from one representative experiment of at least two performed. $*p < 0.05$; $**p < 0.01$.

but instead promoted parasite sequestration in a CD4$^+$ T cell-dependent manner.

### *The generation of nonpathogenic, antiparasitic CD4$^+$ T cell responses by vaccination*

Our findings above indicate that pathogenic CD4$^+$ T cell responses generated following PbA infection promote parasite tissue sequestration, even in the presence of helminth coinfection. Vaccination represents an important approach for shaping immune responses for pathogen challenge and protection against disease. Hence, we investigated whether nonpathogenic, antiparasitic CD4$^+$ T cell responses could be generated by vaccination with an experimental, whole blood stage malaria vaccine known to confer CD4$^+$ T cell-mediated protection in non-ECM malaria models (52). Vaccination with PbA lysate adsorbed to alum resulted in significantly increased PbA-specific CD4$^+$ T cell responses (Fig. 7*A*). However, although vaccinated mice had significantly reduced parasite burdens compared with those of unvaccinated mice at the time of ECM (Fig. 7*B*), only half of the vaccinated mice were protected against ECM (Fig. 7*C*, 7*D*). Interestingly, vaccinated mice that showed no clinical signs had lower parasite burdens than those that displayed clinical signs of ECM. Importantly, vaccinated mice with higher parasite burdens were those that succumbed to ECM, whereas those with reduced burdens were protected from ECM, emphasizing the strong correlation between parasite burden and disease severity.

We have shown previously that modulation of Treg function with an anti-CD25 mAb (PC61) prior to PbA infection resulted in enhanced PbA-specific CD4$^+$ T cell activation, reduced PbA tissue biomass, and protection from ECM (38). We therefore hypothesized that anti-CD25 mAb treatment at the time of vaccine priming would increase PbA-specific CD4$^+$ T cell responses and protect against ECM. To test this, we administered anti-CD25 mAb 1 d prior to vaccination. After PbA challenge, these mice had increased PbA-specific CD4$^+$ T cell responses relative to those of unvaccinated controls and control vaccinated mice (Fig. 7*A*). This enhanced response was associated with further reductions in total body parasite biomass and complete protection against ECM (Fig. 7*B–D*). However, anti-CD25–treated, vaccinated mice that had survived ECM developed acute anemia (Fig. 7*E*). This suggests that although the vaccine protected against ECM, no such protection was afforded against other severe malaria complications. Finally, to confirm that vaccine-induced malaria-specific CD4$^+$ T cells primed under conditions of anti-CD25 mAb treatment were responsible for the observed reduction in PbA biomass, we depleted CD4$^+$ T cells 5 d p.i. (at the onset of ECM symptoms in unvaccinated controls) and then measured PbA biomass in these mice 5 d later. The PbA biomass in CD4$^+$ T cell-depleted, vaccinated mice was significantly greater compared with that of control-treated, vaccinated mice (Fig. 7*F*), confirming an important role for these cells in controlling PbA biomass following vaccination. Together, these results demonstrate that PbA-specific CD4$^+$ T cell

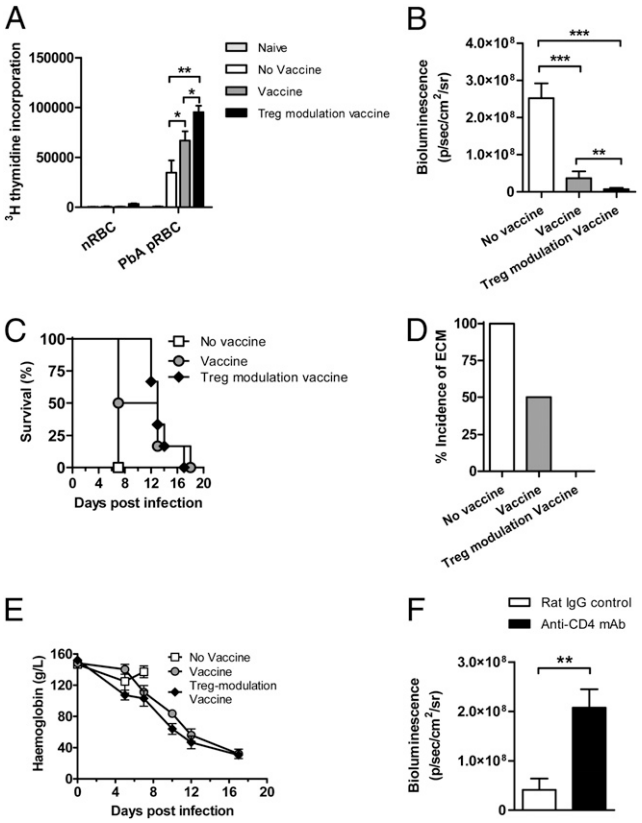


**FIGURE 7.** Vaccination generates antiparasitic, nonpathogenic CD4$^+$ T cell responses. PbA-specific CD4$^+$ T cell proliferation in unvaccinated, vaccinated, or vaccinated mice in which Tregs were modulated at priming ($n$ = 4) following challenge with $10^5$ PbA*luc* pRBCs (*A*). Four days after PbA*luc* infection, CD4$^+$ T cells were purified from spleen cells and cultured in vitro with nRBCs or PbA pRBC Ag in the presence of irradiated syngeneic APCs. The values represent mean ± SEM [$^3$H]thymidine uptake from triplicate cultures. Total body bioluminescence (*B*), survival (*C*), ECM incidence (*D*), and hemoglobin levels (*E*) were measured following vaccination and PbA*luc* infection. Total body bioluminescence of PbA-infected vaccinated mice, primed under conditions of Treg modulation, depleted of CD4$^+$ T cells at day 5 p.i., and imaged on day 10 p.i. (*F*). Each bar represents the mean ± SEM of four to six mice. Data are from one representative experiment of two performed. $*p < 0.05$; $**p < 0.01$; $*** p < 0.001$.

responses can be modulated by vaccination to reduce parasite sequestration and avoid ECM.

## Discussion

The sequestration of *P. falciparum* to various tissues has been acknowledged as a major risk factor for severe malaria syndromes, including CM (6, 10). In this study, we have employed an ECM model to study parasite tissue sequestration and have identified host cells and cytokines that contribute to or inhibit this process. Furthermore, we have shown that helminth coinfection can have a major influence on parasite tissue sequestration, again via mechanisms dependent on the host immune response. Critically, we were able to demonstrate that the host immune response could be modified by vaccination to protect against ECM rather than contribute to disease.

The spleen is a major lymphoid organ and blood filtration tissue during malaria (4). However, we found that splenectomized mice survived ECM and had significantly reduced PbA biomass, indicating that removal of pRBCs by the spleen during PbA infection is not a critical function for prevention of ECM and furthermore that immune responses generated in this tissue site contribute to ECM pathogenesis. We identified both CD4$^+$ and CD8$^+$ T cells as mediators of PbA tissue sequestration. The importance of these T cell subsets for ECM development has long been known (12–14, 57), but their impact on parasite growth and/or survival has not been appreciated. In the absence of either T cell subset, there was a markedly reduced parasite biomass, indicating that CD4$^+$ and CD8$^+$ T cells are critical for either promoting PbA tissue sequestration and/or suppressing the removal of PbA pRBCs from the circulation by phagocytic cells. We believe that the latter possibility is unlikely because blood parasitemia continues to increase in mice depleted of either CD4$^+$ or CD8$^+$ T cells and these animals die with hyperparasitemia (12–15). Therefore, a more likely explanation is that T cells and/or their products condition tissues to allow parasite sequestration.

Cytokines are important immune mediators and regulators with both protective and pathogenic functions in malaria (7, 11). We identified IFN-γ and LTα as critical mediators of PbA tissue sequestration. Both cytokines can activate microvascular endothelial cells (6, 60), and hence, one possibility is that changes to the adherent properties of these cells by IFN-γ and LTα may result in greater PbA adherence. However, this remains speculative because the nature of interactions between PbA and endothelial cells are unclear and to date few parasite molecules or host receptors that mediate these interactions in vivo have been identified. Furthermore, our studies on perfused tissues do not exclude the possibility that at least some of the PbA tissue sequestration observed could arise from blockages in microvasculature caused by pRBCs or the accumulation of pRBCs in tissue hemorrhages. Nevertheless, future studies on the roles of IFN-γ and LTα in promoting adherence of *P. falciparum* and endothelial cells, where parasite molecules and host receptors are better characterized, may be warranted, because this may lead to new opportunities to suppress this pathogenic process in humans.

Our efforts to block soluble LTα using soluble human TNFR2-Fc failed to prevent ECM in PbA-infected mice, despite mice deficient in this cytokine being resistant to ECM. Blockade with human TNFR2-Fc had no effect on PbA biomass, but this is likely explained by TNF activity also being blocked, because our results show that this cytokine plays an important antiparasitic role in the ECM model. Thus, the positive effects of blocking pathogenic LTα were countered by the negative effects of neutralizing antiparasitic TNF. The further delineation of LTα and TNF activities in ECM and CM must await the development of reagents with greater selective actions on TNF and TNFR family member interactions. These studies are clearly important because of the opposing effects that this family of cytokines and receptors has on parasite growth and/or malaria pathogenesis.

We also identified an important role for IL-10 in the regulation of PbA tissue sequestration. Previous studies have shown that IL-10 is important for preventing pathology associated with different experimental malaria models (61) but also can cause parasite persistence and thus contribute to the establishment of some chronic infectious diseases (62). We recently showed that modulation of Treg function with an anti-CD25 mAb resulted in reduced parasite biomass and protection from ECM, but this protection was lost in IL-10–deficient mice (38). These data support an important regulatory role for IL-10 in malaria and also highlight the dangers of blocking IL-10 activity to improve antiparasitic immune responses. Clearly, there is a fine balance between the tissue-protective role of IL-10 during infectious disease and the unwanted action of this cytokine in suppressing antiparasitic immunity. A greater understanding of how this balance is established and maintained is crucial for the design

and implementation of strategies aiming to safely harness the therapeutic potential of IL-10.

Helminth infections can greatly influence the host immune system (58), and importantly, these infections often occur in areas of endemic and seasonal malaria transmission (63). Therefore, it is important that we understand their impact on the development of immunity to malaria and any effects that they may have on malaria pathogenesis. We found that an established *S. mansoni* infection resulted in greater PbA tissue sequestration, but this was associated with impaired development of PbA-specific $CD4^+$ T cell responses. These effects were only observed in mice with high-level helminth infections and not when lower numbers of *S. mansoni* cercariae were used to establish infections (Supplemental Fig. 3 and data not shown). Therefore, helminth burden and/or the inoculums used to establish infection is likely to be an important factor in determining whether there is any impact on the generation of malaria-specific immunity and/or the likelihood of developing severe malaria syndromes. Nevertheless, it was intriguing to note that despite suppressed PbA-specific $CD4^+$ T cell responses in mice with *S. mansoni* infections the enhanced PbA tissue sequestration in these animals was still dependent on $CD4^+$ T cells. Although we cannot rule out the possibility that the suppressed malaria-specific $CD4^+$ T cell response was sufficient to mediate the enhanced PbA tissue sequestration and susceptibility to ECM, our data leave open the possibility that helminth-specific $CD4^+$ T cell responses or even bystander $CD4^+$ T cell activation could contribute to the enhanced PbA biomass and susceptibility to ECM in coinfected mice. Regardless, our data support previous suggestions (64) that the integration of helminth control programs into malaria control strategies will be beneficial, particularly for individuals harboring high worm burdens, because this will help to improve the development of malaria-specific immunity and possibly reduce the risk of developing severe malaria syndromes, such as CM, due to the accumulation of high malaria parasite burdens.

Our finding that concomitant *S. mansoni* and PbA infection failed to protect against ECM contradicts a recent study in which a protective effect was observed (65). This difference in susceptibility is likely to be due, in part at least, to differences in experimental design. For example, the previous study examined the impact of pre-existing *S. mansoni* infection on the development of ECM in an outbred mouse strain, as opposed to inbred C57BL/6 mice employed in the current study. Furthermore, mice were challenged with a 2-fold lower PbA inoculum than that used here. Both mouse genetic background and parasite inoculums will impact on the effect that helminths have on susceptibility to malaria infection.

Vaccination represents one of the most promising strategies to control malaria (66–68). Despite our incomplete understanding about the types of immune responses that are necessary for safe and effective control of infection, vaccine development and testing are proceeding rapidly due to an urgent need for this important public health tool (68). Nevertheless, it is important to recognize that immune responses generated by vaccination have the potential to promote disease rather than control infection (69). We found that anti-CD25 mAb treatment at the time of vaccine priming resulted in the generation of a highly effective malaria-specific $CD4^+$ T cell response that suppressed PbA tissue sequestration and prevented ECM development. Thus, the specific immune conditions established during vaccine priming had a major impact on the quality of the subsequent malaria-specific $CD4^+$ T cells response. It is important to note that despite preventing ECM the anti-CD25 mAb combined vaccine strategy did not result in ultimate control of parasite growth and mice eventually succumbed to hyperparasitemia and anemia. We believe that this failure to control parasite growth after protection from ECM could be attributed to either inappropriate downregulation of malaria-specific $CD4^+$ T cell responses, as previously described in this model (70, 71), or a failure to generate an effective antiparasitic Ab response, as reported in several different experimental malaria models (11, 72). These two possibilities are not mutually exclusive, and we are currently developing ways to improve our malaria vaccine formulation to enhance both aspects of our vaccine-induced immunity.

In conclusion, we have identified specific components of the host immune system that contribute to and inhibit parasite tissue sequestration in an experimental model of CM. Our findings suggest that sequestration of parasites in vital organs, including the brain, marks the onset of ECM and that ECM susceptibility is strongly associated with the total parasite biomass. This observation is in keeping with previous studies in acute *P. falciparum* malaria showing an association among total body parasite biomass, disease severity, and clinical outcome (35). Importantly, the data show that for ECM to be prevented, parasite sequestration needs to be limited and parasite biomass significantly reduced early in infection. This process appears to be at least partly dependent on TNF and IL-10. We also have been able to evaluate the impact of helminth coinfection on the generation of malaria-specific immunity and ECM pathogenesis. Importantly, we were able to show that vaccination could be used to generate potent antiparasitic $CD4^+$ T cell responses without induction of pathology. Together, these advances in our understanding about the pathogenesis of malaria have important implications for developing therapeutic approaches to treat severe malaria syndromes, the development of safe and effective malaria vaccines, and the implementation of these important public health tools.

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## Disclosures

The authors have no financial conflicts of interest.

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