Chemical Attenuation of *Plasmodium* in the Liver Modulates Severe Malaria Disease Progression

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Cerebral malaria is one of the most severe complications of malaria disease, attributed to a complicated series of immune reactions in the host. The syndrome is marked by inflammatory immune responses, margination of leukocytes, and parasitized erythrocytes in cerebral vessels leading to breakdown of the blood–brain barrier. We show that chemical attenuation of the parasite at the very early, clinically silent liver stage suppresses parasite development, delays the time until parasites establish blood-stage infection, and provokes an altered host immune response, modifying immunopathogenesis and protecting from cerebral disease. The early response is proinflammatory and cell mediated, with increased T cell activation in the liver and spleen, and greater numbers of effector T cells, cytokine-secreting T cells, and proliferating, proinflammatory cytokine-producing T cells. Dendritic cell numbers, T cell activation, and infiltration of CD8+ T cells to the brain are decreased later in infection, possibly mediated by the anti-inflammatory cytokine IL-10. Strikingly, protection can be transferred to naive animals by adoptive transfer of lymphocytes from infected mice.

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Abbreviations used in this article: BBB, blood–brain barrier; CAT, chemical attenuation treatment; Ctx, cycle threshold; DC, dendritic cell; ECM, experimental cerebral malaria; EEF, exoerythrocytic form; HCM, human cerebral malaria; MHC, MHC class I; NCX, neglected cerebral malaria; pRBC, parasitized erythrocyte; qRT-PCR, quantitative RT-PCR.

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T cells (11–13). However, parasite attenuation has not yet been extensively examined in the context of cerebral malaria, despite evidence that it may afford some protection. For example, in humans, a malaria vaccine candidate, RTS,S, has been shown to cause partial protection against severe and cerebral malaria, possibly because of impairment of infectious merozoite release by infected liver cells (14–16). Furthermore, in the mouse model, it has previously been demonstrated that antimalarial treatment of infected mice just before the onset of ECM symptoms, the usual end point of infection, attenuates infection by decreasing parasitemia and delaying the onset of cerebral pathology (4).

Few studies have examined the pre-erythrocytic stage or the interaction of immune responses between liver and blood that occurs in early infection on the immunopathogenesis of ECM. Some initial work recently indicated a potential role for the liver stage in modulation of disease severity, with impaired liver-stage development and merozoite formation correlating with a reduced incidence of cerebral pathology in a transgenic parasite lacking a Plasmodium-specific apicoplast protein (17). However, unlike vaccine-like sterilizing immunity induced by attenuated strains, few studies have examined very early cellular immune responses associated with parasite attenuation and their roles in protection from cerebral pathology. One study demonstrated a correlation between protection from cerebral pathology and increased β2-microglobulin–specific IFN-γ production at 24 h postinfection (18). The authors hypothesized that CD8+ T cells were the source of the IFN-γ. A subsequent study demonstrated that when irradiated blood-stage parasites were administered as a vaccine, ECM did not develop upon further challenge with normal parasites (19). Similarly, protected animals produced early IFN-γ, which the authors hypothesized is produced by splenic lymphocytes.

We investigated the role of early immune responses and protection from cerebral malaria in the context of attenuating parasite infection. In doing so, we developed a reproducible model for attenuating the malarial liver stage as a means of manipulating the host immune response to alter the clinical outcome of ECM. We propose that this model causes modulation of cerebral disease by the induction of CD8+ T cell responses earlier than it would occur in natural infection, causing deregulation of downstream, systemic Th1 responses and reduced CD8+ T cell priming.

Materials and Methods

Ethics statement

All animal experiments were performed according to European regulations concerning FELASA category B and GV-SOLAS standard guidelines. Animal experiments were approved by German authorities (Regierungspräsidium Karlsruhe, Germany), § 8 Abs. 1 Tierschutzgesetz (TierSchG) under the license G-6/09 (“MALBI: Malaria Biologie und Immunologie” (“The Biology and Immunology of Malaria”)). For all experiments, C57BL/6 mice were purchased from Janvier, France, and kept under specified-pathogen-free conditions within the animal facility at Heidelberg University (Interfaculty Biomedical Facility). IL-10−/−, IL-2−/−, and IL-2−/− mice were purchased from Janvier, France, and kept under specified-pathogen-free conditions within the animal facility at Heidelberg University (Interfaculty Biomedical Facility).

Plasmodium life cycle and rodent methods

Anopheles stephensi mosquitoes infected with P. berghei ANKA c1f5ey1 (MBF) or P. berghei ANKA c1f5ey1 (MBF) QF1 were provided by the laboratory of Dr. Thomas Schelling (University of Bonn, Germany). Animals were matched for sex and age.

Rapid and sensitive detection of blood-stage parasites from tail blood by PCR

For the rapid and sensitive detection of the presence of P. berghei blood-stage parasites in the peripheral blood after the completion of the liver-stage development, we developed a simple and fast PCR-based method. At the indicated time points, 10 μl tail blood was collected in 500 μl Alsever’s solution or PBS and cells were collected by centrifugation for 5 min at 1800 rpm. Erythrocytes were lysed in 500 μl 0.1% saponin in PBS and free parasites collected by centrifugation for 5 min at 8000 rpm. Cells were washed once with 500 μl PBS and collected by centrifugation for 5 min at maximum speed. The pellet was taken up in ~20 μl ddH2O and snap-frozen for at least 5 min at −80˚C and kept frozen until used for PCR detection. Parasites were detected with primers specific for 18S rRNA (Forward 5′-AACCATTTAAATAAAGCCAAATACATCCTAAC-3′ and Reverse 5′-GGAGATTGGTTTTGACGTTTATGTG-3′) using a 10 min 94˚C hot start PCR protocol.

Rapid Murine Coma and Behavior Scale

As described previously (21), animals were assessed for 10 parameters of cerebral symptoms, such as coordination and motor performance, and scored 0–2, where a 0 score correlates with lowest function and 2 the highest. Animals were assessed daily and scored from 0 to 20 according to this scale.

Cytokine bead array analysis

Cytokine bead array analysis was performed as previously described using a Th1/Th2/Th17 CBA kit (BD Biosciences). For analysis of cytokine concentration, organ homogenates were isolated at the designated time point postinfection. In brief, liver was gently pressed through a sieve, then washed with 1× PBS containing 1% FCS and 2 mM EDTA and centrifuged (10 min, 1500 rpm). Supernatants were taken for cytokine analysis and stored at −20˚C in protease inhibitor mixture (1× protease inhibitor [Roche], 0.05% Tween 20 in PBS) until needed. Organ homogenates and serial dilutions of cytokine standards were incubated with 50 μl beads diluted in dilution buffer. PE-detection reagent mix was added and samples were incubated for 2 h in the dark. Samples were subsequently washed in wash buffer; 300 events/cytokine were acquired on a FACSCalibur, and gating on the total bead population identified by a forward scatter-side scatter profile. Cytokine concentrations were analyzed by FCAP Array software (BD Biosciences).

Flow cytometry

For flow-cytometry analysis, mice were sacrificed and perfused intracardially with 1× PBS. Organs were prepared as single-cell suspensions via homogenization through a 70-μm cell strainer in PBS. Liver lymphocytes were isolated by resuspension in 33% Percoll and collection in the cell pellet after centrifugation at 2000 rpm. Cells were then subjected to erythrocyte lysis in erythrocyte lysis buffer (0.037 g EDTA, 1 g KHCO3, 8.26 g NaCl in 1 L ddH2O, pH 7.4). Cells were then washed and counted in a hemocytometer. For T cell cytokine analysis, cells were stimulated ex vivo with 1 μM ionomycin (Sigma-Aldrich) and 50 ng/ml PMA (Sigma-Aldrich) with 10 μg/ml brefeldin A to inhibit Golgi function for 5 h at 37˚C. Afterward, and where cells were not stimulated, cells were stained and analyzed for expression of CDA8a (clone 53-6-7/PerCP; BD Biosciences), CD4 (clone GK1.5/FITC; eBioscience), CD44 (clone IM7/allophycocyanin; BD Biosciences), CD62L (clone MEL-14/PE; BD Biosciences), CD25 (clone PC61.5/allophycocyanin; eBioscience), and CD69 (clone H1.2F3/PE-Cy7; eBioscience) markers. For intracellular staining, after surface staining, cells were fixed in 2% PFA, washed, permeabilized with 0.2% sonication lysis buffer, and stained for intracellular cytokines TNF-α (clone MP6-XT22/allophycocyanin; eBioscience), IFN-γ (clone XMG1.2/FITC; eBioscience), and IL-2 (clone JES6-5H4/PE; eBioscience) or included matched isotype controls as appropriate. Data were collected on a FACSJuno and analyzed by CellQuest Pro software (BD Biosciences) or FCS Express (De Novo Software). Brain leukocyte isolation was conducted by cutting brains into pieces and digesting in 0.5 mg/ml collagenase A (Sigma-Aldrich) at 37˚C for 30 min. Organs were subsequently passed through a sieve to obtain a single-cell suspension. Cells were pelleted and resuspended in 3 ml 30% Percoll underlaid by 3 ml 37% and 3 ml 70% Percoll. Cells were centrifuged at 2000 rpm for 20 min at room temperature, and cells were collected from the interphase.

Evans blue quantification

Quantification of vascular leakage was performed as described previously (22). In brief, mice were injected i.v. with 150 μl of 1.5% Evans blue dye in PBS and imaged at the indicated time points.
(Sigma-Aldrich) and sacrificed 2 h later to assess vascular leakage: brains were isolated in formaldehyde and incubated for 48 h at 37 C. Absorbance was measured at 620 nm in an ELISA reader with Evans blue concentration assessed via standard curve starting at 400 mg/ml and expressed as milligram dye per gram of brain tissue.

Quantitative real-time PCR

Quantification of parasite load by quantitative real-time PCR (qRT-PCR) was conducted as previously described (23). In brief, mice were killed 42 h after sporozoite infection and species were removed, homogenized, and subjected to RNA isolation via the RNeasy kit (QIAGEN) with 


cDNA synthesized by the RETROscript (Ambion) kit as per manufacturer’s instructions. qRT-PCR was conducted with the ABI 7500 (Applied Biosystems) and Power SYBR Green PCR MasterMix (Applied Biosystems). Relative liver parasite levels were quantified by determining the mean cycle threshold (Ct) value of the parasite 18S ribosomal subunit using gene-specific primers (GenInfo Identifier, 160641) (forward: 5'-AACGATTAAATAAGCGAAATACCTTAC-3'; reverse: 5'-GGA-GATGGGTTTGCAGTTATG-3'), normalized to the mean Ct mouse GAPDH values (GenInfo Identifier, 28199966) (forward: 5'-CTCCTCGCTA-GACAAAAATG-3'; reverse: 5'-TTATGGCCAAACTTC-3'). Relative copy numbers were determined via the ΔCt method.

Whole-body bioluminescence imaging

The transgenic P. berghei line 676m1c11 (Pb GFP-Luc, mpn) (20) was used for the whole-body imaging procedure. Luciferase activity was visualized through whole-body imaging using an in vivo Imaging System (IVIS 100; Caliper Life Sciences) as previously described (24). In brief, animals were anesthetized using isoflurane and their abdomen shaved. D-Luciferin (Synchem Laborgemeinschaft OHG, Germany) was dissolved in PBS and 2.5 mg/mouse injected i.p. After administration of Δ-luciferin, bioluminescence imaging was directly acquired with an exposure time of 180 s and analyzed using Living Image 2.50.1 (Xenogen, Hopkinton, MA). Units are expressed as photons per second per centimeter squared per steradian (p/s/cm²/sr).

Depletion experiments

IL-10R Ab, clone 1B1.3A was purchased from Biolegend and administered i.p. at 250 µg at each day -1 or 5 as indicated and 200 µg daily thereafter until the end point of the experiment. CD8-depleting Ab clone 53-6.7 was purchased from Biolegend and administered 150 µg 3 d before the experiment commencement and every 3 d thereafter until the end point of the experiment. Anti-CD25–depleting Ab clone PC61 was purchased from Biolegend and 250 µg was administered i.p. 8 d before sporozoite inoculation. Anti-CD4–depleting Ab clone GK1.5 purified from hybridoma culture was a kind gift from the University of Bonn, and 300 µg was administered i.p. 1 d before the first isopentaquine shot and every 3 d thereafter.

Histology

Brains were carefully removed immediately after death and snap-frozen in liquid nitrogen. Ten-micrometer-thick coronal sections were cut on a cryotome (Leica Microsystems, Vienna, Austria) and directly mounted onto SuperFrost/Plus slides (Microm International, Walldorf, Germany). Sections were stained routinely with H&E (Thermo Fisher Scientific, Waltham, MA) and Giemsa (Thermo Fisher Scientific). Finally, slides were blinded and three randomly selected sections per coronal plane were subjected to histological analysis. Examinations were done on an Olympus BX45 research microscope (Olympus, Tokyo, Japan). For semiquantitative analysis of parasite infiltration, histological sections of organs were scored in a blind manner using the following pathological scores (scores are given in parentheses after each category): 1) perivascular infiltration: no infiltrates (0), mild (<25%; 1), moderate (25–50%; 2), or severe (>50%; 3); 2) necrosis/hemorrhage: no (0), mild (1), or severe (2); 3) vessels: free (0), luminal myeloid cells (1), or massive blockage (2); 4) parasitism: 50 vessels were counted in each brain and the percentage of vessels with infected erythrocytes was scored as follows: no parasitism (0), mild parasitism of 25% (1), moderate parasitism of 26–50% (2), and severe parasitism >50% (3); and 5) hemolysin deposition: no (0), mild (1), moderate (2), severe (3).

Immunohistochemical staining of liver slices

Liver samples were harvested from ECM and negated cerebral malaria (NCM) mice 42 h postinfection with 10,000 P. berghei ANKA cl15cy1 sporozoites and fixed with 4% paraformaldehyde in PBS overnight at 4 C. They were washed and subsequently sliced into 50–µm slices using a Vibratome (VT1000S, Leica). Slices were permeabilized and blocked overnight at 4°C in 0.5% v/v Triton X-100 plus 1% w/v BSA and then incubated in the same solution containing goat anti-P. berghei UIS4 (1:1750, kindly provided by M. Seabra) and mouse anti-P. berghei HSP70 (1:250) for ~2 h at room temperature. After washing with 1× PBS, slices were incubated with a secondary donkey anti-goat Ab conjugated to Alexa Fluor 488 (1:500; Jackson ImmunoResearch Laboratories) and donkey anti-mouse conjugated to Alexa Fluor 555 (1:500). Cell nuclei were stained with Hoechst 33258 (Molecular Probes/Invitrogen) and F-actin with Alexa Fluor 660 Phalloidin (Molecular Probes/Invitrogen). Forty nonconsecutive stained liver slices were placed on microscope slides and mounted with 24 × 60-mm coverslips using Fluoromount-G (Southern Biotech). Images were acquired with a Zeiss Axiovert 200M to count and measure EEFs. Numbers of EEFs were normalized to the total liver slice area observed. Illustrative pictures were taken using a Zeiss LSM 510 META Point Scanning confocal microscope. All images were analyzed with ImageJ 1.42b software (National Institutes of Health) (25).

Statistical analysis

Data were tested for normal distributions using the D’Agostino and Pearson omnibus normality test. In two group comparisons, statistical significance was determined using t test or the two-tailed Mann–Whitney U test, depending on distribution of the data. For three or more group comparisons, statistical significance was determined using a one-way ANOVA, with Dunn post hoc analysis for normally distributed data, or a Kruskal–Wallis test, with Dunn post hoc analysis for nonparametric data. Significant disparity between slopes was determined by linear regression analysis using GraphPad Prism 5 software using calculations equivalent to analysis of covariance as outlined by Zar (26). Asterisks signify degrees of significance: ***p < 0.001, **p < 0.01, *p < 0.05, and ns indicates p > 0.05.

Results

Experimental animals subjected to chemical attenuation treatment sustain high parasitemias, die of hyperparasitemia-associated anemia, and do not experience development of ECM

We hypothesized that chemical attenuation of the parasite during its liver-stage development could lead to a change in disease outcome. Therefore, we first administered subtherapeutic doses of isopentaquine, an 8-aminoquinoline with a liver-stage action comparable with that of primaquine (27–29). Primaquine and primaquine derivatives are thought to interfere with the cellular respiration, generation of free radicals, and deregulation of electron transport in liver-resident parasites (30).

By subtherapeutic prophylactic administration of isopentaquine before i.v. infection of 10,000 P. berghei ANKA salivary gland sporozoites (Fig. 1A), mice became blood-stage–positive but did not show any typical cerebral symptoms, as measured by the Rapid Murine Coma and Behavior Scale described previously (21), and the onset of paralyzation was delayed by 3–4 d (Fig. 1B and 1C). This NCM outcome was characterized by death from hyperparasitemia-associated anemia at days 25–30 postinfection (Fig. 1B and 1C).

To investigate this delay, we subjected mice to chemical attenuation treatment (CAT) and subsequently infected them with luciferase-expressing P. berghei ANKA parasites (20, 31). In both ECM wild-type controls and mice subjected to CAT we observed a bioluminescent signal localized to the liver up to 48 h postinfection, correlating with parasite biomass (Supplemental Fig. 1). At 72 h postinfection the signal was disseminated throughout the whole body of the mouse, indicative of some moribund that are able to establish blood-stage infection having been released from the liver at this point. However, this parasitemia is submicroscopic. From 72 to 96 h postinfection there was a significant reduction in parasite biomass throughout the whole body in animals subjected to CAT (Fig. 1 and Supplemental Fig. 1D). Furthermore, from 48 h to 72 h postinfection at the point of schizogony and merozoite release, and from 72 to 96 h postinfection during blood-stage replication, there was a significant decrease in the rate of biomass growth of 68 and 67% (p = 0.002, p = 0.0005), respectively, in animals subjected...
to CAT (Fig. 1D). The liver stage following CAT was investigated by qRT-PCR analysis of *P. berghei* 18S rRNA transcript levels. Liver-stage development in NCM, characterized by multiple nuclear divisions (32), was decreased as shown by quantitative transcriptional analysis at 48 h postinfection (Fig. 1E). PCR analysis of small volumes of blood (≈10 μl) taken every 12 h from mice subjected to CAT and infected with wild-type *P. berghei* ANKA sporozoites and sacrificed at 42 h postinfection. Livers were extracted and RNA isolated and cDNA synthesized. Relative liver load was calculated via ΔΔCt analysis based on parasite-specific 18S rRNA transcription. Animals (groups of 4), assigned to chemically attenuated (NCM) and positive control (ECM) mice, were infected with 10,000 *P. berghei* ANKA sporozoites and sacrificed at 42 h postinfection. (F) Liver slices were performed and immunofluorescence analysis conducted by staining for *P. berghei* UIS4, *P. berghei* HSP70, Hoechst, and Phalloidin. Number of liver stages was determined in animals subjected to CAT (NCM) and untreated controls (ECM) at 42 h postinfection. (G) Representative immunofluorescence images from both samples are presented. (H) Size distribution of liver-stage parasites was determined by immunofluorescence analysis in animals subjected to CAT (NCM) and untreated controls (ECM). ***p < 0.001. ns, p > 0.05.
succumbed to cerebral symptoms, to assess the integrity of the vascular endothelium in experimental animals. Upon injection of Evans blue, BBB disruption leads to extravasation of dye into the brain tissue, which can be visualized and assessed quantitatively and qualitatively. Control animals that experienced development of ECM displayed evidence of cerebral pathology—a dark, evenly distributed staining—compared with very faint staining in brains from chemically attenuated NCM animals (Fig. 2A) that was not statistically significantly different from naive brains from noninfected animals by colorimetric analysis (Fig. 2B), indicating that the BBB and vascular endothelium remained intact in animals subjected to CAT. Cryosections of brain tissue were isolated from NCM and ECM mice at the same time point postinfection, and histological analysis revealed no indicators of cerebral damage in the NCM mice, in contrast with the ECM controls, which displayed leukocyte infiltration, parenchymal hemorrhages, subarachnoidal bleeding, and adherence of leukocytes to venule walls (Fig. 2C). NCM mice displayed an intact epithelium, no indicators of subarachnoidal bleeding, and intact blood vessels with no evidence of sequestration (Fig. 2C). ECM is associated with leukocyte sequestration in the brain (33–37), and we accordingly quantified CD8⁺ and CD4⁺ T cells infiltrating the brains of ECM and NCM mice. Interestingly, NCM mice showed dramatically fewer CD8⁺ infiltrates but a comparable number of CD4⁺ infiltrates (Fig. 2D). This correlates with the observation that CD8⁺ T cells in the brain-sequestered leukocytes are essential for the cerebral symptoms and mortality in ECM (38). In addition to leukocyte infiltration, protection from cerebral malaria is associated with reduced parasite biomass and sequestration in the brain (39), and we accordingly quantified CD8⁺ and CD4⁺ T cells infiltrating the brains of ECM and NCM mice. Interestingly, NCM mice showed dramatically fewer CD8⁺ infiltrates but a comparable number of CD4⁺ infiltrates (Fig. 2D). This correlates with the observation that CD8⁺ T cells in the brain-sequestered leukocytes are essential for the cerebral symptoms and mortality in ECM (38). In addition to leukocyte infiltration, protection from cerebral malaria is associated with reduced parasite biomass and sequestration in the brain (39). Thus, we determined the degree of parasite infiltration within the brains of protected and nonprotected mice. Semiquantitative histological analysis of brain sections revealed an approximate 50% reduction in parasite infiltration in NCM mice relative to ECM controls (Fig. 2E).

**FIGURE 2.** Animals subjected to CAT feature absence of pathology and decreased BBB damage. Animals (groups of 5) were assigned to ECM, NCM, and control groups as described and infected with 10,000 *P. berghei* ANKA sporozoites and sacrificed at day 8 postinfection upon the onset of cerebral symptoms. (A) Evans blue extravasation in formamide at day 8 postinfection was compared between chemically attenuated and ECM control mice by visual appearance. (B) Evans blue extravasation in formamide at day 8 postinfection was compared between chemically attenuated and ECM control mice by measurement of absorbance at 620 nm in comparison with control mice. (C) Sections were isolated from brain tissue, stained with H&E, and analyzed for indicators of cerebral pathology. Representative images (left panels) display parenchyma and parenchymal hemorrhage, in NCM and ECM groups, respectively, and (right panels) subarachnoidal space and subarachnoidal bleeding in NCM and ECM groups, respectively. (D) Whole number cerebral CD8⁺ and CD4⁺ T cell infiltration at day 8 postinfection was analyzed by flow cytometry. (E) Brain parasite infiltration and parasitemia were assessed by histology and scored from 1 to 3, and measured by percentage, respectively. **p < 0.001, *p < 0.05. ns, p > 0.05.

The host immune response to the liver stage induces protection from ECM

We next examined whether the early host immune response, during which delayed, attenuated liver-stage development occurs, is responsible for the protective phenotype in NCM by parasite transfer experiments. To investigate whether the absence of cerebral pathology in NCM is due to a modification of the parasite at the blood stage, we isolated 10⁶ pRBCs from NCM mice and transferred them by i.v. injection into naive animals. All recipient mice displayed clinical symptoms and died of ECM (Fig. 3A and Supplemental Fig. 2), thus indicating that the intraerythrocytic development after CAT remains unaffected and viable in its potential to cause ECM. Furthermore, to determine whether residual a malarial compound affects the parasite at the blood stage during patency, animals were subjected to CAT and then challenged i.v. with 10⁶ or 10³ pRBCs at either day 5 or 51 h postinfection to mimic the onset of both microscopic and submicroscopic patency in NCM. However, animals were not protected and succumbed to cerebral pathology (Fig. 3B). We therefore assume that an immune response to the very early stages is responsible for the protective phenotype in NCM, and that this prepatent period, during which delayed, attenuated liver-stage development occurs, is crucial for the outcome of ECM.

Overall increase in proinflammatory immune responses in experimental animals subjected to CAT at day 4 postinfection

We dissected the immune response during this period of prepatency 4 d postinfection. We observed a marked increase in total IFN-γ protein in the liver (4286 ± 1100 pg cytokine/g organ) of NCM mice compared with both ECM mice (35.96 ± 6.528 pg cytokine/g organ; p < 0.005) and control mice (4.622 ± 0.6982 pg cytokine/g organ; p < 0.005, n = 4, Mann–Whitney). Because IFN-γ is mainly produced by T lymphocytes and, to a minor extent, by NK and
NKT cells, we performed flow-cytometry analysis of lymphocytes from liver and spleen tissue followed by intracellular cytokine staining. We observed only a minor difference in the NK cell populations in these organs and no difference in their intracellular cytokine staining (data not shown). However, we observed a pronounced increase in the expression of activation marker CD44 on both CD8⁺ and CD4⁺ T cells in the liver and CD8⁺ T cells in the spleen at day 4 postinfection (Fig. 4). In turn, a higher expression of CD4⁺CD44⁺CD62L⁻ effector memory T cells was evident within the liver and decreased numbers of CD4⁺CD44⁺CD62L⁻ central memory T cells (Fig. 4A and 4B) were apparent in mice subjected to CAT. Within the spleen, there was a markedly higher number of CD8⁺ T effector cells in animals subjected to CAT compared with ECM control mice (Fig. 4C and 4D). Intracellular cytokine staining of liver-derived CD8⁺ and CD4⁺ T lymphocytes revealed an increased tendency toward IFN-γ⁺ cells, in addition to IL-2 and TNF-α at day 4 postinfection (Fig. 5). To assess early T cell activation in NCM, we examined CD69 and CD25 at this time point. We observed a decrease in CD25 and CD69 expression on CD8⁺ and CD4⁺ splenic T cells in NCM and none in the liver (Supplemental Fig. 3A–D). CD69 is an early lymphocyte activation marker associated with active, tissue-invading cells (19), whereas CD25 forms part of the IL-2R and has been associated with several cell phenotypes, including early T cell activation (40) and regulatory T cells. These data suggest that early NCM immunopathogenesis...
after CAT is marked by the early activation of the host immune system.

Animals subjected to CAT exhibited a decrease in T effector cells, T cell activation marker expression, and CD8⁺CD11c⁺ dendritic cell numbers at day 8 postinfection

Host immunopathogenesis diverged significantly from ECM mice at the fulminant stage of disease. At days 8–9 postinfection, NCM mice showed a decrease in splenic CD4⁺CD44⁺CD62L⁺ T effector subsets, and a decrease in expression of both CD69 and CD25 activation markers in both splenic CD8⁺ and CD4⁺ T cells (Supplemental Fig. 3E and 3F). T cell activation in ECM occurred in response to a systemic Th1 environment and Ag presentation by CD11c⁺ dendritic cells (DCs) (41, 42). We observed a lower number of CD11c⁺highCD8⁺ DCs in our NCM model in liver and a pronounced decrease in spleen at day 8 postinfection (Fig. 6).

Adoptive transfer of splenocytes from animals subjected to CAT leads to 70% protection against ECM and is ablated upon depletion of CD8⁺ T cells, but not CD25⁺ or CD4⁺ T cells from late-phase infection

To investigate the transferability of the early immune environment in NCM, we performed an adoptive transfer of $2 \times 10^7$ liver-derived lymphocytes at 48 h after sporozoite inoculation into naive

FIGURE 5. Animals subjected to CAT feature increased cytokine expression by CD8⁺ T cells and increased TNF-α expression by CD4⁺ T at day 4 postinfection. Animals (groups of 5) were assigned to ECM and NCM groups as described, infected with 10,000 *P. berghei* ANKA sporozoites, and sacrificed at day 4 postinfection. Livers were harvested and lymphocytes isolated and cultured ex vivo with PMA/Ionomycin and brefeldin A. (A) Cells were stained for CD8 and IFN-γ, TNF-α, and IL-2. (B) Cells were stained for CD4 and IFN-γ, TNF-α, and IL-2. *p < 0.05, **p < 0.01.
animals, which were challenged with 10,000 P. berghei ANKA sporozoites 24 h later. This did not confer any difference in protection between NCM- and ECM-recipient animals. In contrast, adoptive transfer of $2 \times 10^7$ whole splenocytes from NCM donors to naive mice led to around 70% protection of mice against cerebral symptoms compared with 0% protection in control animals that received splenocytes from ECM donors (Fig. 7A). Strikingly, this protection could not be conferred if donor NCM mice were depleted of CD8$^+$ T cells (Fig. 7A). We therefore assume that NCM protection is essentially mediated by splenic CD8$^+$ T cells, as part of a systemic modulation of the early host immune response. Quantification of parasite liver load by qRT-PCR at 42 h postinfection in adoptive transfer recipients revealed no significant difference between adoptive transfer recipients from NCM and ECM donors, respectively (Supplemental Fig. 4A), indicating that adoptively transferred splenocytes do not have a cytolytic effect on infected hepatocytes. Because it has been shown that CD4$^+$ T cells are required for the induction of cerebral pathology in C57BL/6 mice and their early depletion leads to protection from cerebral pathology (43, 44), we administered CD4-depleting Ab to animals subjected to CAT from day 6 postinfection to determine the role of CD4$^+$ T cells at late infection. All animals were protected from cerebral pathology. Similarly, depletion of CD25 from early infection did not alter the NCM phenotype (Supplemental Fig. 4B and 4C).

Protection arising from CAT cannot be elicited in mice deficient of IL-10, indicating that NCM protection may depend upon IL-10

Given the presumed downregulation of the Th1 response during fulminant infection in CAT mice, because of the absence of cerebral pathology, we investigated the role of the anti-inflammatory cytokine IL-10. IL-10 is a master regulator of immunity to malaria and is important in ECM immunopathology, controlling Th1 responses (45, 46). Because protection by CAT is associated with stronger proinflammatory responses at earlier time points in contrast with ECM controls, which decrease at later infection, we investigated the role of IL-10 in the NCM model. We subjected IL-10$^{tm1Cgrn}$ knockout mice and WT mice treated with anti–IL-10R Ab to CAT and subsequently challenged with 10,000 P. berghei ANKA sporozoites. Animals had ECM pathology and died at days 10–12, indicating the requirement of IL-10 in NCM (Fig. 7B). We next injected chemically attenuated mice with IL-10R Ab at day 5 postinfection, and protection was still abrogated (Fig. 7C). This suggests that the protective role of IL-10 in NCM is stage specific and mediated from the later stages, not the pre-erythrocytic stage (Fig. 8). To determine whether protection could be conferred to IL-10 knockout mice by adoptive transfer of splenocytes from chemically attenuated wild-type mice, we subjected IL-10 knockout mice to adoptive transfer as described earlier. All animals succumbed to cerebral pathology (Supplemental Fig. 4D).

Discussion

In most studies focusing on murine cerebral immunopathogenesis, the pre-erythrocytic and early erythrocytic stages have been largely overlooked. In contrast, our study uncovers a previously uneluciated role for the pre-erythrocytic stage in ECM and emerging T cell responses. Classically, upon infection of erythrocytes and clearance in the spleen, the host undergoes a Th1 response that involves the production of proinflammatory cytokines and the recruitment of innate immune cells such as monocytes and neutrophils and their chemotaxis to the brain microvasculature (47–49). A diverse range of cytokines, especially the proinflammatory cytokine IFN-γ, also increases expression of adhesion molecules such as ICAM-1 and LFA-1 (50, 51). DCs capture and present Ags during the rupture of pRBCs and their clearance in the spleen. This, together with the overriding Th1 environment and costimulatory signals provided by adhesion molecules (52), causes T lymphocytes to become activated and migrate via chemotaxis to the brain microvasculature. It is this adaptive response, and T cell activation, that occurs in late blood-stage infection that leads to BBB damage and pathology.

Some experimental ECM models with “unnaturally early” IFN-γ responses have been associated with protection from ECM (18, 19), where inflammatory responses may diverge from their normal role of mediating splenic T cell activation and the development of pathology during the fulminant stage of disease (3). We believe that chemical attenuation in the NCM model causes protection via a similar dynamics (Fig. 8). The pre-erythrocytic parasite development following CAT is marked by a significant

![FIGURE 6. Animals subjected to CAT display fewer CD8$^+$CD11c$^+$ DCs at day 8. Animals (groups of 5) were assigned to ECM and NCM groups as described, and infected with 10,000 P. berghei ANKA sporozoites, and sacrificed at days 4 and 8 postinfection. Liver and spleen were harvested, and lymphocytes isolated and stained for CD8 and CD11c to determine whole number of CD8$^+$CD11c$^+$ DC population. **p < 0.01.](image)

![FIGURE 7. The NCM phenotype can be adoptively transferred and relies on CD8$^+$ T cells and IL-10. (A) NCM and ECM mice were either depleted or not of their CD8$^+$ T cells, infected with 10,000 P. berghei ANKA sporozoites, and sacrificed at day 2 postinfection. Splenic lymphocytes from the depleted and undepleted donor groups were isolated, pooled, and adoptively transferred into recipient mice (groups of 5). Recipient mice were infected with 10,000 P. berghei ANKA sporozoites 24 h posttransfer and survival tracked (experiment performed in triplicates). (B) IL-10$^{tm1Cgrn}$ knockout mice were subjected to CAT (NCM), infected with 10,000 P. berghei ANKA sporozoites, and survival tracked (experiment performed in triplicates). (C) NCM mice were administered anti–IL-10R Ab i.p. at either day 0 or 5 postinfection and compared with control mice, infected with 10,000 P. berghei ANKA sporozoites, and survival was tracked (experiment performed in duplicates).](image)
with submicroscopic parasitemia. Interestingly, liver-stage development appears to be essential for protection from cerebral pathology, because both low- and high-dose challenge of pRBCs following CAT failed to protect from ECM.

As revealed by immunofluorescence microscopy, the number of liver-stage parasites does not differ in protected animals. However, most liver stages in protected animals are considerably less developed than in nonprotected controls, whereas only a small number of EEFs appears to show normal development, suggesting that they may have escaped attenuation marked by an unchanged parasitophorous vacuolar membrane. This attenuated growth correlates with an equally idiosyncratic early immune response. Indeed, following CAT and sporozoite challenge, attenuated parasite development induces T cell activation within both liver and spleen, and increased IFN-γ, TNF-α, and IL-2 production by CD8+ lymphocytes at an earlier time point than in a normal ECM infection. This early peak appears to cause modulation of later inflammatory immunopathogenesis in the host, during the phase where splenic T cells would normally become activated and migrate to the brain at the crucial time point between days 8 and 10 postinfection (days 4–6 postblood infection) (3). This may be similar to other experimental ECM models in which early IFN-γ production was associated with protection (18, 19).

Early NCM immunopathogenesis following CAT is marked by the early activation of the host immune system, possibly by isopentaquine-killed parasites, in turn leading to increased accumulation of splenic T effector cells and a pronounced intrahepatic and splenic Th1-environment. NCM hosts display decreased CD11c+CD8+ DC numbers, downregulation of T cell activation, no cerebral infiltration, and a complete absence of cerebral pathology. The connection between CD11c+CD8+ DCs and ECM is well established. Parasites associate with these DCs, which capture dying cells and exogenously process Ag for cross-presentation via MHCI (53). They have been shown to prime naïve CD8+ T cell proliferation and CTL effector functions (54) with the induction of specific CD8+ T cell responses abrogated in their absence (55). Recent work suggests CD11chighCD8+ DCs are responsible for ECM immunopathogenesis (42). Although we did not test the capacity for Ag presentation in DCs, we observed a decreased number and concurrent reduction in T cell activation at the fulminant stage of infection, a component of the overall downregulation in Th1 responses. We speculated that this downregulation may be mediated by the anti-inflammatory cytokine IL-10 during the fulminant stage of disease in a response that we hypothesize represents the host bringing its early proinflammatory response under control, at the stage where T cell priming and/or chemotaxis to the brain would occur. IL-10 plays an essential role in ECM immunopathology that can both impede pathogen clearance and ameliorate immunopathology (45, 46). Furthermore, it has been demonstrated that neutralization of IL-10 leads to ECM symptoms in otherwise resistant mice (56). Although we did not observe an increased level of IL-10 protein in mice subjected to CAT, we were unable to induce NCM protection in those lacking the cytokine, perhaps due in part to increased ECM pathology in its absence. This strongly indicates the requirement of IL-10 for the NCM phenotype. Because regulatory T cells are associated with the production of IL-10 and the limitation of inflammatory pathology (57) and have been associated with protection from ECM (58), we speculated that they might be responsible for the production of IL-10 in the NCM model or otherwise mediate T cell responses. Although depletion of CD25 had no effect on the protective phenotype (Supplemental Fig. 4B), some reports have indicated that CD25+ cells are required for development of cerebral pathology in susceptible mice (59, 60), so we cannot exclude their role in NCM, and the source of IL-10 remains unknown. Recent literature reports an IL-10–producing CD4+CD25+ Foxp3+ regulatory Th3 phenotype.
T cell subset associated with the modulation of pathology in *Plasmodium yoelii* (61), although depletion of CD8+ cells from day 6 postinfection had no effect on the protective phenotype observed in NCM animals.

The early inflammatory peak in the NCM phenotype caused by CAT appears to be largely caused by CD8+ T cells, including the effector memory subset, because adoptive transfer of splenocytes from mice subjected to CAT cannot confer protection to naive animals in their absence. Adoptive transfer into IL-10mutCgn knockout mice was not able to confer protection, perhaps unsurprisingly, because NCM protection is dependent upon IL-10. Although we cannot comment as to epitope specificity of these CD8+ T cells, we assume that they are specific to pre-erythrocytic parasite Ag. The Ag specificity of T cells that migrate to the brain in ECM is still mostly undescribed, although a recent publication identified one conserved CD8 epitope cross-presented by the brain microvessels (5). Although it is attractive to speculate that a shared antigenic repertoire may exist between the parasitized hepatocyte and the infected erythrocyte, and is displayed via MHC I on the cytokine-activated brain endothelial cell (62), it is conceivable that CD8+ T cell–mediated protection leading to NCM is not restricted to any one particular Ag. Indeed, it is possible that early T cell priming, regardless of Ag, causes a more generalized, systemic downregulation of the Th1 response, Ag presentation, and T cell activation where and when it matters, at the onset of fulminant disease. Furthermore, it is possible that a reduced parasite load in the liver due to CAT induces the priming of differential CD8+ specificities to those that mediate ECM. Follow-up experiments will isolate protective CD8+ T cells and determine their precise epitope specificity, longevity, and effect after adoptive transfer.

Quantification of liver burden after adoptive transfer and sporozoite infection revealed no significant difference between recipients of adoptively transferred splenocytes from protected animals and ECM control animals, indicating that adoptively transferred CD8+ T cells are not engaged in cytolytic parasite killing. However, a recent study in P. yoelii reported that adoptive transfer of splenic and hepatic lymphocytes renders CD8+ T cells immotile and unable to make contact with *Plasmodium*-infected hepatocytes (63). Thus, we cannot exclude that parasite killing by CD8+ T cells does occur in animals subjected to CAT. After adoptive transfer, the inability of immobilized CD8+ T cells to make contact with infected hepatocytes may render them reliant on soluble mediators to modulate cerebral immunopathogenesis. This may explain why adoptive transfer does not lead to 100% protection. Our work has far-reaching implications in dissecting the complex sequence of interrelated events that form the immunological basis of HCM pathology, thought to begin with pRBC sequestration or rupture (3). We suggest a role for the early intrahepatic host immune responses that can alter the course of downstream-systemic immunopathogenesis. The liver is an immunoprivileged organ typified by tolerogenic, limited T cell responses (64). We suggest that liver tolerance may explain why such potentially protective pre-erythrocytic responses do not naturally occur in HCM. The decrease in severe pathology, including cerebral symptoms, and yet onset of blood infection in the RTS,S vaccine trials are so far unexplained. RTS,S consists of sequences from the C-terminal region of the CS protein (65) and is designed to produce a pre-erythrocytic T cell response directed against the infected liver. It is conceivable that this provokes T cell responses akin to those in the NCM model, and we hypothesize that these data may provide a mechanistic basis for the unexplained decrease in severe and cerebral pathology observed in the RTS,S malaria vaccination trials and other pre-erythrocytic malaria interventions (10, 17).

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**Disclosures**

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**References**


