MyD88-Deficient Mice Exhibit Decreased Parasite-Induced Immune Responses but Reduced Disease Severity in a Murine Model of Neurocysticercosis

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The symptomatic phase of neurocysticercosis (NCC), a parasitic disease of the central nervous system (CNS) in humans, is characterized by inflammatory responses leading to neuropathology and, in some cases, death. In an animal model of NCC in which mice were intracranially inoculated with the parasite Mesocestoides corti, the infection in mice lacking the myeloid differentiation primary response gene 88 (MyD88−/−) resulted in decreased disease severity and improved survival compared with that in wild-type (WT) mice. The CNS of MyD88−/− mice was more quiescent, with decreased microgliosis and tissue damage. These mice exhibited substantially reduced primary and secondary microglial nodule formations and lacked severe astrogliotic reactions, which were seen in WT mice. Significantly reduced numbers of CD11b+ myeloid cells, αβ T cells, γδ T cells, and B cells were present in the brains of MyD88−/− mice in comparison with those of WT mice. This decrease in cellular infiltration correlated with a decrease in blood-brain barrier permeability, as measured by reduced fibrinogen extravasation. Comparisons of cytokine expression indicated a significant decrease in the CNS levels of several inflammatory mediators, such as tumor necrosis factor alpha, gamma interferon, CCL2, and interleukin-6, during the course of infection in MyD88−/− mice. Collectively, these findings suggest that MyD88 plays a prominent role in the development of the hyperinflammatory response, which in turn contributes to neuropathology and disease severity in NCC.

Neurocysticercosis (NCC) is the most common parasitic disease of the central nervous system (CNS), occurring as a result of infection of the brain with the larval stage of the tape worm parasite Taenia solium (56). In humans, the disease has a long asymptomatic phase, typically 3 to 5 years, followed by the symptomatic phase, consisting of clinical signs such as epilepsy (43), increased intracranial (i.e.,) pressure, obstructive hydroencephalus, stroke, and encephalitis (55, 56). More than 25% of all epileptic cases diagnosed in adults worldwide are due to NCC (19). The sequential progression from asymptomatic to symptomatic NCC depends upon the degeneration of larvae, caused by either therapeutic treatment or normal attrition. This leads to the induction of a strong inflammatory response, causing a chronic granulomatous reaction and the manifestation of symptoms of the disease (41, 57). The immune response in the CNS of symptomatic patients consists of an overt TH1 phenotype (39) or a mixed TH1, TH2, and TH3 phenotype, depending upon the absence or presence of granuloma formation (38). Specifically, the TH1 hyperinflammatory response prevailing in the CNS during the symptomatic phase is thought to be responsible for the severe neuropathology and mortality associated with NCC (55). Direct evidence that the inflammatory/TH1 response contributes to the neuropathology and severity of NCC, however, is limited. Nevertheless, along with antiparasitic drugs, the treatment of NCC patients with immunosuppressive/anti-inflammatory factors such as corticosteroids helps to control the host inflammatory response and associated neuropathology (32). Long-term treatments with steroids, however, lead to problematic side effects that may become life-threatening. Therefore, despite recent advances made in detection and therapy, effective treatment of NCC remains a major challenge, as cysticidal treatment itself results in the symptoms that one is trying to control and/or the manifestation of other complications. Therefore, it is important to understand the pathophysiological basis of the CNS inflammatory response in NCC and to identify critical molecules responsible for such responses.

The myeloid differentiation primary response gene 88 (MyD88) is an important regulator of the host inflammatory response (50, 51). The protein produced by the MyD88 gene is an adaptor molecule necessary for signal transductions originating from the interleukin-1 receptor (IL-1R)/IL-18R family of receptors and the Toll-like receptor (TLR) family of proteins (35). Once engaged, TLRs signal through a common pathway involving MyD88 (42), leading to the subsequent downstream activation of the NF-κB and mitogen-activated protein (MAP) kinase pathways and inducing a TH1 proinflammatory response (28). Previous studies have demonstrated that MyD88 knockout mice exhibit defective proinflammatory responses and display dramatic defects in...
antimicrobial immunity in a variety of infectious disease models, highlighting the importance of this molecule in influencing a wide array of host responses and disease control (2, 8, 18, 45, 52). A contrasting situation occurs in onchocerciasis, an infection of the eye caused by another helminth parasite, Onchocerca volvulus. In this case, MyD88 plays a pivotal role in the development of a persistent hyperinflammatory response eventuating in corneal haze and hence contributing to the development of river blindness (22, 26). An important unanswered question, therefore, is whether MyD88-dependent mechanisms are involved in inflammatory responses that contribute to the observed pathology and severity of NCC or play an essential role in the containment of this disease.

The goal of this study was to identify the overall effect of the absence of the MyD88-dependent signaling pathway, using a well-characterized murine model of NCC developed in our laboratory (14). In the present study, we compared the susceptibilities and immunopathology of MyD88−/− and wild-type (WT) mice infected i.c. with Mesocestoides corti. The contribution of MyD88 signaling to CNS inflammation was assessed by measuring infiltration of various immune cells into the brain, blood-brain barrier (BBB) permeability, and proinflammatory cytokine responses in the CNS of MyD88−/− and WT mice.

MATERIALS AND METHODS

Mice. Female mice aged 3 to 5 weeks were used in this study. MyD88−/− mice in the C57BL/6 background (originally from S. Akira, Osaka University, Osaka, Japan) were obtained from Michael Bertin (Department of Microbiology and Immunology, University of Texas Health Science Center, San Antonio, TX). C57BL/6 mice were obtained from the National Cancer Institute animal program (Bethesda, MD) and used as WT controls. WT controls and MyD88−/− mice were bred in the University of Texas at San Antonio (UTSA) animal facility. Experiments were conducted under the guidelines of the IACUC, UTSA, University of Texas System, the U.S. Department of Agriculture, and the National Institutes of Health.

Antibodies. Phycoerythrin-conjugated antibodies purchased from BD Pharmingen (San Diego, CA) included GL3 (pan-anti-γ), H57-597 (pan-anti-α), M1/70 (anti- McCoy), ID3 (anti-CD19), R35-95 (anti-rat immunoglobulin G2a [IgG2a], κ chain), B6-13 (anti-hamster IgG2, κ chain), and HA/48 (anti-hamster IgG2, A chain). Purified 2.4G2 (anti-CD16/32), mouse fibrinogen, anti-mouse IL-6, anti-mouse gial fibrillary acidic protein (anti-mouse GFAP), and biotinylated anti-mouse CD11b were also purchased from BD Pharmingen. Biotinylated anti-mouse neuronal nuclear protein was purchased from U.S. Biological (Swampscott, MA). Anti-mouse GFAP was conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. For indirect immunofluorescence (IF), appropriately fluorescently conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used to detect specific primary antibodies against immune mediators and cell surface markers. Biotinylated primary antibodies were detected using Alexa Fluor 488-labeled streptavidin (Molecular Probes).

Murine model of NCC. In this study, we used a mouse model of NCC developed in our laboratory (14, 15). M. corti metacestodes were maintained by serial intraperitoneal inoculation of 8- to 12-week-old female BALB/c mice. Metacestodes were aseptically harvested, and murine NCC was induced by i.c. injection of 50 μl of Hanks balanced salt solution (HBSS) containing about 40 parasites into 3- to 5-week-old mice under short-term anesthesia, as described previously. Mock-infected control mice were injected by the i.c. route with 50 μl sterile HBSS, using the same protocol. Before i.c. inoculation, mice were anesthetized with a 50:50 mixture of ketamine HCl and xylazine (30 mg/ml ketamine and 4 mg/ml xylazine in phosphate-buffered saline [PBS]) given intramuscularly. Animals were sacrificed at the indicated times after inoculation and analyzed for parasite burden and various immune parameters. Before sacrifice, animals were anesthetized with 100 μl of the above mixture and perfused through the left ventricle with 10 ml cold PBS.

Tissue processing. The brain was immediately dissected from perfused animals, embedded in optimal-cutting-temperature resin, and snap-frozen. Serial horizontal cryosections of 10 μm in thickness were placed on silane prepped slides (Sigma-Aldrich, St. Louis, MO). One in every four slides was fixed in formalin for 10 min at room temperature and stained with hematoxylin and eosin (H&E). The remainder of the slides were air dried overnight and fixed in fresh acetone for 20 s at room temperature. Acetone-fixed sections were wrapped in aluminum foil and stored at −80°C or processed immediately for immunohistochemistry or IF.

IF and H&E staining. After fixation in 10% formalin for 10 min at room temperature, slides were washed twice in deionized water, dehydrated for 30 s in 100% ethanol, stained for 30 s in hematoxylin, and washed in distilled water for 2 min. Tissue sections were stained with eosin for 15 s, followed by 2 min of treatment (each) with 95% and 100% ethanol. Slides were allowed to air dry and then submerged in xylen for 3 min and mounted using Cytoseal mounting medium (Stephens Scientific, Riverdale, NJ). The number and location of parasites were determined by microscopic examination of the stained tissues. Tissues were also analyzed for the presence or absence of mononuclear infiltration.

Luxol blue staining. Luxol blue staining of brain sections from mock- and parasite-infected WT and MyD88−/− mice was performed as previously described (27). Briefly, dissected brains were stored in 10% buffered formalin overnight for histological processing. Brains were dehydrated through graded alcohol, cleared with xylene, and embedded in paraffin. Transverse sections were cut at a 5-μm thickness and were placed on silane prepped slides. Sections were deparaffinized in xylene for 5 min, rehydrated in 95% ethanol for 5 min at room temperature, and stained in Luxol fast blue (a solution of Luxol fast blue [La- rington, PA] at 60°C overnight. Slides were washed for 1 min in 95% ethanol in water and differentiated in 0.05% lithium carbonate (Sigma-Aldrich, St. Louis, MO) followed by 70% ethanol for 3 to 4 min. These sections were rinsed in deionized water for 30 s, counterstained with cresyl etch violet (Sigma-Aldrich, St. Louis, MO), dehydrated through a 95% and 100% alcohol series for 5 min each, cleared in xylene, and cover slipped with synthetic resin.

Brain mononuclear cell isolation. Leukocytes from mouse brains were isolated as described by irani and Griffin (24), with some modifications (13, 15). Each perfused brain was gently minced through a fine 70-μm Nitex screen (Sefar America, Depew, NY) by use of a syringe plunger and collected in 10 ml HBSS (Invitrogen, Carlsbad, CA) containing 0.05% collagenase D (Roche Diagnostics, Indianapolis, IN), 0.1 μg/ml l-glutamine, 3.4-aminomido-7-amino-2-heptanone-HCl (Sigma-Aldrich), 10 mg/ml DNase I (Sigma), and 10 mM HEPES buffer, pH 7.4 (Invitrogen). The mixture was gently rocked at room temperature for 1 h and allowed to settle by gravity for 30 min to deplete undigested debris. The supernatant was collected, pelleted at 200 × 100 g for 5 min, and resuspended in 3 ml Ca2+/Mg2+-free HBSS (Invitrogen) per brain. The suspension was layered onto 10 ml of a density gradient containing 3 parts Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ) and 1 part RPMI medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma), 10 mM HEPES (Invitrogen) and 50-μg gentamicin (Invitrogen)/ml. Each gradient was centrifuged at 300 × g for 30 min. The white brain pellet was collected by aspiration with a Pasteur pipette, washed twice with 10 ml of RPMI medium, and centrifuged, and the interface of tissue debris were removed. The entire 10 ml of gradient medium was then diluted fivefold with HBSS and centrifuged at 300 × g for 10 min. Cells were washed three times in 1 ml 0.1% bovine serum albumin (Sigma-Aldrich) in HBSS and counted. Cells were then diluted to 2 × 107 cells/ml and processed for IF staining of cell surface antigens for flow cytometric analysis (fluorescence-activated cell sorter [FACS] analysis).

FACS analysis. Our previous studies have demonstrated that infiltrating leukocytes consisting of macrophages, γ/δ T cells, and NK cells are detected in the CNS during murine NCC by day 3 postinfection (p.i.), followed by αβ T cells by 1 week p.i. and B cells by 3 weeks p.i. (14). Additionally, the early infiltrating γ/δ T cells appear to play a key role in amplifying further infiltration of leukocytes by 1 week p.i. and afterward (13). Therefore, in the present study, leukocyte infiltration in WT and MyD88−/− mice was analyzed by FACS analysis at 1 week p.i. and 3 weeks p.i. Single-cell suspensions of harvested mononuclear cells from brain perfusion of WT and MyD88−/− mice were counted, stained with 10 μl 10 cells/ml in staining buffer (10% fetal calf serum in PBS) and preincubated with 1 μg of the 2.4G2 antibody for 5 to 10 min on ice prior to staining. Fifty microliters of cell suspension (equal to 106 cells) was dispensed into each tube or well along with a previously determined optimal concentration of cell surface-specific antibody in 50 μl of staining buffer. Each test tube was mixed gently and incubated for 30 min in the dark in an ice bath. Cells were stained with fluoro- chrome-labeled antibodies of the appropriate isotype control for nonspecific binding, while cells stained in the absence of primary antibodies served as negative controls. After the incubation period, cells were washed by adding 2 ml of staining buffer followed by centrifugation for 7 min (300 to 400 × g) at 4°C. The washing was repeated two more times, for a total of three washes. Cell pellets were suspended in 500 μl of staining buffer and analyzed on a BD LSR
II flow cytometer (BD Biosciences). FlowJo (Three Star) software was used to analyze the FACS data.

IF. IF microscopy was used to determine the presence of immune mediators and/or specific cell types in brains of parasite-infected mice at various times postinfection (3 days, 1 week, and 3 weeks). Brains from mock-infected animals were used as controls, and IF staining was done as described before (6, 13, 30). All steps were carried out at room temperature. Briefly, sections were incubated with specific primary antibodies in PBS with 3% host serum to prevent nonspecific binding. After 1 h, sections were washed seven times for 3 min each time and incubated with appropriate secondary antibodies for 30 min. Sections were then washed seven times for 3 min each time in 50 mM Tris-HCl, pH 7.6, with 0.1% Tween 20. For double-IF staining, the abovementioned procedures were sequentially repeated for each additional staining step. The sections were mounted using FluorSave reagent (Calbiochem, La Jolla, CA) containing 0.3 μM 4′,6-diamidino-2-phenylindole (DAPI)–diacetate (Molecular Probes). Additional control staining was performed to rule out any nonspecific staining. In each case, sections were blocked with saturating concentrations of appropriate host serum antibodies to eliminate false-positive staining due to FcR-mediated nonspecific binding. Staining in the absence of primary antibodies provided additional negative controls.

Analysis of fibrinogen extravasation. The integrity of the BBB was assessed 1 week and 3 weeks after i.c. inoculation of M. corti metacestodes (4, 6). Adjacent brain cryosections from infected and noninfected WT and MyD88+/− mice were stained using antibodies against fibrinogen or CD11b. CD11b was used to provide a frame of reference for the location of blood vessels, around which infiltration of leukocytes takes place. At least five images of compromised pial blood vessels (and control pial vessels from mock-infected mice) were captured using identical camera settings so that differences in the pixel intensities of the images were solely due to differences in fibrinogen extravasation. Disruption of the BBB was determined by measuring the area (number of pixels) and fluorescence intensity (average intensity of pixels) of fibrinogen surrounding vessels exhibiting leakage. This was done using the imaging software IP lab 4.0 (BD Biosciences Bioimaging, Rockville, MD). The relative extent of fibrinogen extravasation was calculated by multiplying the number of pixels (area) by the average intensity of pixels. Statistical analysis was performed with Student's t test, using Sigma Plot 8.0 (Systat Software, San Jose, CA).

Statistical analysis. We used Student's t test for comparison of means of different groups in Sigma Plot 8.0 (Systat Software, San Jose, CA). A P value of <0.05 was considered to be statistically significant. Statistical differences in the mortality of parasite-infected mice with NCC were analyzed using Kaplan-Meier survival analysis.

RESULTS
MyD88+/− mice exhibit reduced morbidity and mortality. WT and MyD88+/− mice were infected i.c. with 40 M. corti metacestodes to evaluate the role of MyD88 in murine NCC. The development of disease severity in these animals was then compared. CNS infection with M. corti metacestodes produces severe neurological signs, such as tilted head, walking in circles, spinning when held from the tail, and weight loss between 1 and 3 weeks p.i., that worsen at later time points (15). In the present study, the WT mice displayed these signs of infection. In contrast, the majority of MyD88+/− mice did not exhibit severe neurological signs during the first 5 weeks p.i. By this time point, 64.3% of M. corti-infected WT mice (9 of 14 mice) succumbed to the infection (Fig. 1). In contrast, only 21.4% of MyD88+/− mice (3 of 14 mice) succumbed to the infection. Thus, the lack of MyD88 in mice resulted in reduced disease severity and improved survival during murine NCC.

MyD88+/− mice exhibit reduced parasite loads in brain parenchyma. To examine whether the reduction in neurological symptoms and mortality of MyD88-deficient mice was associated with effective clearance of parasites and/or their segregation to specific brain areas, serial horizontal sections of infected WT and MyD88+/− mouse brains were stained with

FIG. 2. CNS infection with M. corti in MyD88+/− mice is mostly localized in extraparenchymal regions. After 1 week and 3 weeks of infection, WT and MyD88+/− mice were sacrificed. The location and number of parasites were obtained by microscopic examination of serial H&E-stained brain sections. (A) Total numbers of parasites present in the brains of infected WT and MyD88+/− mice (n = 4). (B) Total numbers of parasites present in parenchymal and extraparenchymal regions in individual brains were calculated (n = 4). Parasites in MyD88+/− mice were localized mostly in meninges, ventricles, and subarachnoid spaces. Significant differences in parasite distribution were observed in MyD88+/− versus WT animals and are denoted by asterisks (***, P < 0.001).
H&E, and the number of *M. corti* metacestodes was determined by microscopic analysis. Brains of WT and MyD88<sup>/−</sup>/MyD88<sup>/−</sup> mice harvested at 1 week and 3 weeks p.i. exhibited similar parasite counts (Fig. 2A). However, the distribution of organisms was altered. In WT mice, the majority of organisms had invaded the parenchyma at 1 and 3 weeks p.i., whereas in MyD88<sup>/−</sup>/MyD88<sup>/−</sup> mice the majority of organisms were located in extraparenchymal areas (Fig. 2B).

**MyD88<sup>/−</sup>/MyD88<sup>/−</sup> mice display reduced CNS pathology after *M. corti* infection.** We evaluated H&E- and Luxol fast blue-stained sections of infected brains to determine changes in nervous tissue integrity (Fig. 3 and 4). Figure 3A-1 and A-2 depict normal brain tissue morphology in control animals inoculated i.c. with HBSS. In comparison, infected WT brain tissue displayed a strong inflammatory response characterized by the presence of a large number of infiltrating immune cells (Fig. 3B-1 and C-1), including infiltrates surrounding the parasites in the parenchyma (Fig. 3D-1). Some areas contained sites of necrosis. In contrast, infected MyD88<sup>/−</sup>/MyD88<sup>/−</sup> brain tissue exhibited reduced CNS inflammation, with smaller numbers of infiltrating immune cells, compared to WT mice (Fig. 3B-2 and C-2). Smaller foci of immune cells surrounded parenchymal parasites in the MyD88<sup>/−</sup>/MyD88<sup>/−</sup> mice (Fig. 3D-2).

**FIG. 3. Brain pathology associated with *M. corti* infection in WT and MyD88<sup>/−</sup>/MyD88<sup>/−</sup> mice.** (A-1) H&E staining of brain cryosection showing normal parenchymal tissue in an HBSS-inoculated WT mouse. (A-2) Normal parenchymal tissue in an HBSS-inoculated MyD88<sup>/−</sup>/MyD88<sup>/−</sup> mouse. (B-1) Parenchymal blood vessel in a WT mouse at 3 weeks p.i., associated with areas of infiltrating immune cells (*). (B-2) Parenchymal blood vessel in a MyD88<sup>/−</sup>/MyD88<sup>/−</sup> mouse at 3 weeks p.i., exhibiting a reduced number of infiltrating immune cells (*). The inset C-1' represents a 2× magnification of the selected area depicting immune cells with morphology indicative of activated cells. (C-2) Indusium griseum of WT mouse at 1 week p.i., associated with massive infiltration of immune cells compared to WT mouse (*). The inset C-2' represents a 2× magnification of the selected area depicting immune cells of quiescent morphology compared to the WT mouse. (D-1) Parenchymal parasite in a WT mouse at 3 weeks p.i., associated with infiltrating immune cells. P, parasite. (D-2) Parenchymal parasite in a MyD88<sup>/−</sup>/MyD88<sup>/−</sup> mouse at 3 weeks p.i., associated with reduced infiltrating immune cells. P, parasite. Results are from one representative experiment of three independent experiments (n = 4 mice per time point), and the images were captured at a magnification of ×200.

**FIG. 4. Brain pathology associated with *M. corti* infection in WT and MyD88<sup>/−</sup>/MyD88<sup>/−</sup> mice.** (A-1) Luxol blue staining of brain cryosection showing normal parenchymal tissue in an HBSS-inoculated WT mouse. (A-2) Normal parenchymal tissue in an HBSS-inoculated MyD88<sup>/−</sup>/MyD88<sup>/−</sup> mouse. (B-1) Parenchyma of WT mouse at 3 weeks p.i., exhibiting what appears to be microglial nodule formation associated with neuronal death. (B-1') The boxed area in panel B-1 was magnified (×4) to show details of microglial/macrophage nodule formation in WT mice. (B-2') Parenchyma of MyD88<sup>/−</sup>/MyD88<sup>/−</sup> mouse at 3 weeks p.i., depicting reduced nodule formation compared to that in WT mouse. (B-2') The boxed area in panel B-2 was magnified (×4) to show the absence of similar nodule formation in MyD88<sup>/−</sup>/MyD88<sup>/−</sup> mice. Results are from one representative experiment of three independent experiments (n = 4 mice per time point), and the images were captured at a magnification of ×200.
MyD88−/− mice exhibit reduced numbers of infiltrating immune cells in the CNS. Our previous studies demonstrated that the predominant immune cells initially recruited to the brain after parasite inoculation are macrophages and γδ T cells (3 to 5 days p.i.), followed by αβ T cells (1 week p.i.) and B cells (3 weeks p.i.). The maximum infiltration of immune cells is usually detected between 2 and 5 weeks p.i. in the M. corti-infected mouse brain. To determine whether MyD88−/− mice displayed any differences in infiltrating immune cells during NCC, the total leukocytes from whole brains were isolated at the peak of inflammation (3 weeks p.i.) and quantified by trypan blue staining. Brains of MyD88-deficient mice at that time exhibited significantly smaller numbers of leukocytes than did those of WT mice (P < 0.005) (Fig. 5). In a manner consistent with our previous observations (5), the infiltrating leukocytes in brains of mock control mice were barely detectable (Fig. 5).

To further elucidate the difference in recruitment of various immune cell types in MyD88-deficient mice in NCC, we used flow cytometric analysis to determine the numbers of CD11b+ myeloid cells, γδ T cells, αβ T cells, and B cells at both an early stage of infection (1 week p.i.) and the peak of inflammation (3 weeks p.i.) (Fig. 6). CD11b+ myeloid cells represented the predominant leukocyte population at both times p.i. in M. corti-infected brains of WT as well as MyD88−/− mice (Fig. 6A to C). In comparison with the WT infected mice, the number of CD11b+ cells was significantly lower in MyD88−/− mice both at 1 week (Fig. 6A and C) and at 3 weeks p.i. (Fig. 6B and C). As seen for CD11b+ cells, there were significantly smaller numbers of γδ T cells and αβ T cells detected in MyD88−/− mice at 1 week (Fig. 6A and C) as well as at 3 weeks p.i. (Fig. 6B and C) compared to the WT mice. As reported previously, in murine NCC brains B cells were detected only after 3 weeks p.i. (4). Likewise, CD19+ B cells were detected at 3 weeks p.i. in WT or MyD88−/− infected mice (Fig. 6B), but not at 1 week p.i. (data not shown). However, the number of B cells detected in MyD88−/− mouse brains was threefold lower than in the WT mice (Fig. 6B and C). Taken together, these data demonstrate that the absence of MyD88 leads to a significant reduction in leukocyte recruitment into the brain during NCC.

MyD88−/− mice display reduced BBB permeability after M. corti infection. The deficiency in the number of immune cells in MyD88−/− mice could reflect a deficiency in leukocyte trafficking into the brain and/or a defect at subsequent stages of cell expansion. Leukocyte trafficking during the course of NCC has previously been correlated with the breakdown of the BBB (3–7). Therefore, we investigated the impact of MyD88 deficiency on BBB permeability in M. corti-infected brains by testing for the presence of the serum protein fibrinogen in brain tissue by IF. Multiple brain cryosections of WT and MyD88−/− animals were analyzed. Mock-infected animals (both WT and MyD88−/−) exhibited small amounts of fibrinogen staining (red) that was largely confined to the vessels (Fig. 7A-1 and A-2). Upon infection, pial vessels of infected WT animals displayed substantial extravasation of fibrinogen at 1 week p.i. (Fig. 7B-1), accompanied by cellular infiltration (*). The extravasation remained high, as indicated by extensive staining for fibrinogen in perivascular areas adjacent to the pial vessels, at 3 weeks p.i. (Fig. 7C-1). In contrast, infected MyD88-deficient mice exhibited substantially less immunoreactivity for fibrinogen at both 1 and 3 weeks p.i. than did infected WT mice (Fig. 7B-2 and C-2). Measurement of the mean pixel intensity of fibrinogen staining confirmed that extravasation of fibrinogen was significantly lower in parasite-infected MyD88−/− mice than in the WT controls (Fig. 7D). These findings suggest that the reduced number of immune cells detected in infected MyD88-deficient mice correlates with decreased BBB permeability of CNS vasculatures.

MyD88−/− mice exhibit reduced IL-6 protein expression in proximity to blood vessels. Our previous studies revealed that IL-6 is detected in astrocytes proximal to the pial vessels that are actively involved in the infiltration of leukocytes into the CNS during murine NCC (4). Thus, the production of IL-6 was assayed in parasite-infected brain cryosections of WT and MyD88−/− mice by in situ IF microscopy (Fig. 8). In mock-infected/normal mouse brains, IL-6 was detected at low basal levels (Fig. 8A-1 and A-2). Infected WT mice displayed a progressive increase in the level of IL-6 protein, as measured by both the intensity of staining and number of positive cells. At 1 week p.i., an increased expression of IL-6 was evident in periventricular and leptomeningeal areas of the brain (Fig. 8B-1). These induced effects were even more pronounced at 3 weeks p.i. (Fig. 8C-1). The upregulated expression was detected primarily in the astrocytes present in periventricular and leptomeningeal areas of the brain, including the glia limitans, in proximity to pial vessels (Fig. 8D-1). Although the infection in MyD88−/− mice induced increases in IL-6 protein expression in similar brain anatomical regions and cell types, the protein was detected at substantially lower levels than those in WT mice at each time point analyzed (Fig. 8B-2, C-2, and D-2). Thus, the results from this study suggest that MyD88-deficient NCC mice express reduced levels of IL-6 in astrocytes present in proximity to the pial vessels.
MyD88$^{-/-}$ mice express lower levels of inflammatory mediators after *M. corti* infection. As reported previously, infection of the CNS by *M. corti* leads to an upregulation of various proinflammatory cytokines/chemokines (4, 13–15), which appear to contribute to the immunopathogenesis of this disease. Among them, the Th1 cytokines tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ), produced by γδ T cells, correlated with exacerbating neuropathology and disease severity (15), while early induction of CCL2 (<1 week) correlated with cellular infiltration into the CNS during murine NCC (13). Thus, to determine the involvement of MyD88 in the CNS levels of these proin-
Inflammation cytokines after *M. corti* infection, enzyme-linked immunosorbent assay was performed on brain homogenates from WT and MyD88−/− mice. In normal/mock-infected mouse brains, TNF-α, IFN-γ, and CCL2 were at low basal levels in WT and MyD88−/− mice (Fig. 9). Upon parasite infection, WT mice exhibited increased levels of TNF-α, IFN-γ, and CCL2 at 1 week p.i., which were further upregulated at 3 weeks p.i. (Fig. 9). *M. corti* infection in MyD88−/− mice exhibit reduced disease in murine NCC.
FIG. 8. MyD88−/− mice present reduced brain expression of IL-6. In situ IF staining was performed on frozen sections of mock-infected and parasite-infected brains of WT and MyD88−/− mice at 1 and 3 weeks p.i. IL-6 expression was visualized in red (rhodamine RXX), astrocytes (GFAP+; green; Alexa Fluor 488), and nuclei acids (blue) were stained with DAPI. (A-1) IL-6 expression in brain of a mock-infected WT mouse. (A-2) IL-6 expression in HBSS-inoculated MyD88−/− mouse brain. (B-1) IL-6 expression in brain of a parasite-infected WT mouse at 1 week p.i. (B-2) IL-6 expression in brain of a parasite-infected MyD88−/− mouse at 1 week p.i. (C-1) IL-6 expression in brain of a parasite-infected WT mouse at 3 weeks p.i. (C-2) IL-6 expression in brain of parasite-infected MyD88−/− mouse at 3 weeks p.i. (D-1) IL-6 is predominantly expressed in brain astrocytes of a parasite-infected WT mouse at 3 weeks p.i. IL-6 is visualized in red (rhodamine RXX), and astrocytes are in green (Alexa Fluor 488). Arrowheads show colocalization of IL-6 and GFAP (yellow-orange). Magnification, ×400. (D-2) IL-6 expression is reduced in brain astrocytes of a MyD88−/− mouse at 3 weeks p.i. Arrowheads show colocalization (yellow-orange). Magnification, ×400. Results are from one representative experiment of three independent experiments (n = 4 mice per time point).
mice resulted in marginal increases in the levels of these cytokines over the basal control levels of the mock-infected mice. However, compared to those in the WT infected mice, the levels of TNF-\(\alpha\), IFN-\(\gamma\), and CCL2 in MyD88\(^{-/-}\) mice were significantly lower \((P < 0.001)\), suggesting that induction of these inflammatory mediators in NCC is largely dependent on MyD88 signaling.

DISCUSSION

The chronic stage of both human and murine NCC is associated with manifestation of the TH1 inflammatory response. The persistence of an inflammatory response in the CNS during NCC appears to play a prominent role in the severe neuropathology and mortality associated with this disease (57). The present study demonstrates a critical role played by MyD88-associated signaling in promoting the severity of NCC. MyD88\(^{-/-}\) mice were less susceptible to infection and exhibited reduced pathological signs compared to infected WT mice. The presence of both primary and secondary microglial nodules that reflect engulfment of degenerating neurons and necrotic brain tissue by glial cells appeared to be lacking in the infected brains of MyD88\(^{-/-}\) mice. These results strongly support the role of MyD88-associated signaling in amplifying the disease severity of murine NCC.

MyD88 is a primary adaptor molecule that interacts with many host pattern recognition receptors (35). Recruitment of MyD88 leads to the activation of MAP kinases and the transcription factor NF-\(\kappa\)B (28). This in turn allows the regulated expression of a wide range of proinflammatory factors by macrophages and other cell types. Many of these mediators have important roles in the amplification of acute inflammatory responses, innate immune function, and the development of acquired immunity. The cytokines produced in the CNS during M. corti infection are indicative of a TH1 type of response (14, 15). Furthermore, a decreased expression of the TH1 cytokines TNF-\(\alpha\) and IFN-\(\gamma\) in M. corti-infected mice that lack \(\gamma\delta\) T cells is associated with reduced pathology and longer survival times (15). Thus, lower brain levels of TH1 inflammatory responses that are associated with reduced CNS symptoms in M. corti-infected MyD88\(^{-/-}\) mice strongly support the idea that TH1 cytokines such as TNF-\(\alpha\) and IFN-\(\gamma\) contribute to the CNS pathology during NCC. Within that context, \(\gamma\delta\) T cells are a major TH1 cytokine-producing cell type in the CNS after M. corti infection (15). The significant reduction in \(\gamma\delta\) T-cell numbers in the CNS of MyD88-deficient mice after M. corti infection correlates with the reduction in TH1 cytokine responses. Moreover, \(\gamma\delta\) T cells from M. corti-infected mice also express multiple TLRs (30). Indeed, \(\gamma\delta\) T cells produce large amounts of proinflammatory cytokines and chemokines upon stimulation with TLR ligands (unpublished results). Thus, it is possible that MyD88-dependent responses in \(\gamma\delta\) T cells directly contribute to TH1 responses and inflammation-mediated pathology in NCC.

FIG. 9. MyD88\(^{-/-}\) mice exhibit defects in the induction of several proinflammatory mediators. The brains from mock-infected control and M. corti-infected WT and MyD88\(^{-/-}\) mice were harvested at 1 and 3 weeks p.i. and homogenized in PBS with protease inhibitors (100 mg/ml), whereupon TNF-\(\alpha\), IFN-\(\gamma\), and CCL2 protein levels were quantitated by sandwich enzyme-linked immunosorbent assay (data are means ± standard errors). Results shown are averages for three infected and three mock-infected control mice for each time point. Significant differences are denoted by asterisks (***, \(P < 0.001)\).
The reduced neuropathology of MyD88-deficient (and γδ T cell−/−) mice coincides with a smaller number of parasites in brain parenchyma. Although the exact mechanisms contributing to tissue invasion by M. corti are unknown, we speculate that the extent of inflammatory responses elicited by infiltrating immune cells may influence the outcome. The initial immune response in WT mice is inflammatory and takes place in extraparenchymal spaces (meninges and subarachnoid spaces). Such responses likely cause tissue pathology, allowing easier access of the parasite to the parenchyma. A reduced level of such responses in MyD88−/− mice may help to minimize invasion of the parasite. It is also likely that parenchymal parasites would be more injurious, particularly in critical areas, as has been reported for human patients (32, 55, 56).

Inflammation is a complex process consisting of various host determinants. One of the important steps of inflammation is leukocyte migration to the site of infection or injury. In murine NCC, parasite infection results in infiltration of large numbers of macrophages, γδ T cells, and αβ T cells within the first week of infection, followed by B cells by 3 weeks p.i. (4). The results presented here show a reduction in infiltration of immune cells, including macrophages, αβ T cells, and γδ T cells, after M. corti infection in MyD88-deficient mice compared with WT mice. This reduction in cellular infiltration correlates with decreased fibrinogen leakage compared to that in WT mice, reflecting reduced BBB breakdown in the MyD88-deficient mice. The BBB is a selective barrier formed by the endothelial cells that line cerebral microvessels (1, 40). It regulates the transit of blood-borne molecules and immune cells into the brain. During a pathological insult, the BBB becomes more permeable. Failure of the BBB is a critical event in the development and progression of several neurological inflammatory diseases (12, 21, 36, 58). Although the mechanisms of BBB disruption during CNS diseases are not fully deciphered, specific growth factors such as IL-6 and vascular endothelial growth factor have been shown to play crucial roles in affecting the permeability of the BBB in various clinical settings (9, 11). Our previous studies have shown that IL-6 expression by astrocytes in the glial limitans in the proximity of pial vessels correlates with leukocyte migration through pial vessels during murine NCC (4). It is likely that significantly lower levels of IL-6 expression in astrocytes in MyD88-deficient mice contribute to decreased BBB permeability, eventuating in reduced leukocyte trafficking. In vitro studies are currently under way to define such interactions.

The data presented here indicate a role for MyD88 in the production of proinflammatory responses that contribute to the disease severity in murine NCC. This is in contrast to the well-established role for MyD88 signaling in mediating production of proinflammatory cytokines leading to host resistance in various infections (31, 37, 44, 46, 49, 53, 54). Nevertheless, emerging findings in parasite diseases such as river blindness and cerebral malaria indicate that MyD88-mediated responses contribute to disease severity (17, 25). Similar functions of MyD88 in NCC and river blindness are of interest since the associated immunopathogenesis is similar in nature. In both cases, the infection does not produce any detectable inflammatory response or disease syndrome while the parasites are alive, suggesting an active suppression of immune responses by the live parasite. However, the death/degeneration of the parasites results in the induction of inflammatory responses that are the cause of the associated pathology and disease severity in river blindness (20) and NCC (32). Thus, in these extracellular helminth infections, the activation of the MyD88 signaling pathway results in the induction of inflammatory responses and appears to be the cause of the associated pathology and disease symptoms. However, the involvement of any particular TLR in murine NCC has yet to be determined. Nonetheless, TLRs 1 to 13, except for TLR5 and -10, are differentially expressed in the CNS during murine NCC (29, 30). However, MyD88 also interacts with several other TIR (Toll-like-1R) domain-containing proteins within the IL-1R/IL-18R family, such as the IL-1R1 and IL-1R accessory protein (10). Besides, MyD88 also has been reported to interact with several non-TIR domain-containing receptors, such as IFN-γ receptor 1 (47), phosphatidylinositol 3-kinase (34), IFN regulatory factor 1 (IRF1) (33), IRF5 (48), and IRF7 (23). Determining pattern recognition receptors that are responsible for the induction of MyD88-dependent inflammatory responses in M. corti-infected mouse brains is of current interest in our laboratory.

An adequate amount of inflammation is required to mount a successful antimicrobial response to any kind of infection. However, sustained inflammation is the cause of tissue damage and associated pathology, particularly in CNS diseases (16). The common treatment for NCC consists of corticosteroids to suppress inflammatory responses that accompany degeneration of the parasite after antihelminth drug treatment. Treatment of corticosteroids leads to unwanted and undesirable side effects. A better understanding of the exact MyD88 signaling-dependent pathways leading to unwanted inflammation could potentially lead to other therapeutic inhibitors, particularly if they could be targeted specifically to the CNS.

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