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Induction of Distinct Neurologic Disease Manifestations during Relapsing Fever Requires T Lymphocytes

Hongqi Liu,* Denise Fitzgerald, † Bruno Gran, ‡,§ John M. Leong, * and Kishore R. Alugupalli*

Relapsing fever borreliosis is a multisystemic infection characterized primarily by bacteremia but can extend to the CNS. The incidence of CNS disease manifestations in humans depends on the infecting relapsing fever Borrelia species. In the murine model of Borrelia hermsii infection we found high incidence of distinct signs of CNS disease that ranged from a flaccid tail to complete paralysis of hind limbs. Infiltration of large number of T cells into the spinal cord of B. hermsii-infected mice and the upregulation of MHC class II and CD80 on infiltrating macrophages and on microglial cells suggested a role for T cell and Ag-presenting cell interactions in this pathogenesis. Indeed, B. hermsii infection did not induce CNS disease manifestations in T cell-deficient mice (TCR-β×δ−/−), although it resulted in bacteremia comparable to wild-type (Wt) level. Moreover, the infiltration of immune cells into the spinal cord of TCR-β×δ−/− mice was reduced and the resident microglial cells were not activated. Histopathological analysis of lumbar sections of the spinal cord confirmed severe inflammation in Wt but not in TCR-β×δ−/− mice. Induction of CNS disease was dependent on the B. hermsii strain as well as on the ability of the host to control bacteremia. Mice that are impaired in controlling B. hermsii, such as CD14+/− mice, exhibited more severe CNS disease than Wt mice. This study demonstrates that distinct neurologic disease manifestations develop during relapsing fever and that T cells play a critical role in the induction of neuropathogenesis.


Materials and Methods

Mice and infections

These studies have been reviewed and approved by Institutional Animal Care and Use Committee. Mice were maintained in a specific pathogen-free facility of Thomas Jefferson University and housed in microisolation cages with free access to food and water. C57BL/6J (wild-type [Wt]), B6.129S-Cd14tm1Frm/J (CD14−/−), and C57BL/6J-TCR-β−/−Mum1-TCR-δ+/+Mum1 (TCR-β×δ−/−) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Eight- to 12-wk-old male or female mice were infected i.p. with 5 × 10^4 B. hermsii strain DAH, RAL, or FRE and bacteremia was monitored by dark-field microscopy (18).
Clinical scoring

*B. hermsii*-infected mice were scored daily for CNS disease manifestations, using a well-established EAE clinical scoring protocol (19): 0, no detectable signs; 1, completely limp tail and/or weakness of one hind limb; 2, weakness of both hind limbs; 3, paralysis of one hind limb; 4, paralysis of both hind limbs; and 5, death related with this disease.

Flow cytometry

To determine the immune cell infiltration of spinal cord, mononuclear cells were isolated as detailed below. Following extensive transcardial perfusion with Dulbecco’s phosphate buffered saline (DPBS), spinal cords were removed from mice by flushing and pooled for mononuclear cell isolation (five mice/group). Tissues of spinal cord in 40 ml staining media (RPMI 1640-deficient w/HEPES, 0.5 M EDTA and 3% newborn calf serum) were mechanically dissociated through a 100-μm strainer and washed with DPBS. The resultant pellet was resuspended in 6 mL 60% Percoll (for five mice) and fractionated on a 60/30% Percoll gradient (GE Healthcare, Uppsala, Sweden) by centrifugation at 300 × g for 20 min at room temperature with brake off. Microglia, infiltrating mononuclear, and other cells were harvested from the 30/60% interface, washed, and labeled with mixture of fluorescent-labeled Abs for flow cytometric analysis. To identify and to analyze the activation status of microglia and macrophages, the previously described harvested cells were stained with anti-CD45–PerCP (clone: 30-F11, BD Pharmingen, San Diego, CA), anti-CD11b–APC (Clone:M1/70, eBioscience, San Diego, CA), anti-MHC class II (MHCII)–FITC (clone: M5/114.15.2, eBioscience) and anti-CD80–PE (Clone:16-10A1, eBioscience). Anti-CD4–PerCP (clone: RM4-5, Pharmingen), anti-CD8–PE (clone:53-6.7, eBioscience), anti-B220–FITC (clone: RA3-6B2, eBioscience), and anti-NK1.1–APC (clone: PK136, eBioscience) were used to determine specific lymphocyte infiltration into the spinal cord. After blocking the Fc receptors with 2.4G2 Ab, cells were stained with the previously described conjugated Abs and were washed twice with staining medium, and the preparations were run on a FACSCalibur (Becton Dickinson, Mountain View, CA) using CELLQuest software for acquisition of the data (Becton Dickinson). Data were analyzed using FlowJo software (Treestar, San Carlos, CA).

Histopathology

After an initial transcardial perfusion with DPBS, animals were perfused with 4% paraformaldehyde and spinal cords were removed. Tissues were processed and blocked in paraffin wax. The 5-μm sections were cut and stained with H&E for assessment of inflammation. Sections were assessed for inflammation as follows: 0, no inflammation; 1, a few inflammatory cells; 2, organization of perivascular infiltrates; and 3, increasing severity of perivascular cuffing with extension into the adjacent tissue (20).

Statistics

Statistical analysis was carried out using GraphPad Prism Version 4 (GraphPad Software, San Diego, CA). Differences between control and infected groups were evaluated by Student *t* test (two tailed, unpaired) and a *p* value of <0.05 was deemed significant.

Results

*B. hermsii* infection causes distinct neurologic manifestations in immunocompetent mice

Although neurologic complications are well documented during RF in humans, the critical parameters required for the progression of neurologic disease are not known. To understand the CNS pathogenesis during RF, we infected C57BL/6 mice with DAH, a clinical isolate of *B. hermsii*, and visually monitored them daily for signs of neurologic disease. Surprisingly, a high percentage of *B. hermsii*-infected mice exhibited symptoms that are similar to those of neurologic disease manifestations described for classical EAE, such as tail/hind limb weakness or paralysis (19). In addition, a minor fraction of (~5%) of infected mice showed vestibular dysfunction characterized by uncontrolled axial rotation when lifted by tail (not shown).

To quantify the neurologic manifestations associated with *B. hermsii* infection, we evaluated infected mice using a grading system described for studying EAE (19). During the primary bacteremic episode (day 2–4 postinfection [data not shown]) no discernable signs of neurologic manifestations were detected. However, by 1 wk postinfection, the tails of a majority of mice became flaccid with or without weakness in their hind limbs (Fig. 1). By 2–3 wk postinfection, symptoms ranged from a flaccid tail to hind limb paralysis. The incidence of these manifestations remained high during second and third week postinfection, but gradually diminished by 6 wk postinfection (Fig. 1). No relapse and remittance of the CNS disease was noted. Unlike in EAE, where gender plays a role in the severity of the disease in certain mouse strains (21), *B. hermsii* infection resulted in indistinguishable manifestations in male and female mice. These data revealed that *B. hermsii* infection of immunocompetent mice results in high incidence of a neurologic disease manifestations similar to but distinct from EAE.

The DAH strain is the most well-studied clinical isolate of *B. hermsii* and grows to high density in Wt mice (58.9 ± 34.9 × 10⁷/ml blood). To examine whether the induction of CNS disease is also triggered by other clinical isolates of *B. hermsii*, we infected Wt mice with two additional strains. In Wt mice, the peak bacteremia of strain RAL is 4.3 ± 2.9 × 10⁷/ml blood, whereas FRE reaches a density of 17.4 ± 12.4 × 10⁷/ml blood. We found that Wt mice infected with RAL exhibited CNS disease (score: 1.4 ± 1.14), whereas none of the FRE-infected mice suffered any detectable CNS disease symptoms. These results indicate that the induction of CNS disease during *B. hermsii* infection is strain-dependent.

Infiltration of immune cells in the spinal cords of *B. hermsii*-infected mice

The CNS is an immune privileged site, and in classical EAE the spinal cord is the critical part of the CNS affected by immune cell infiltration. Because the signs of CNS disease during RF (Fig. 1) resemble those of classical EAE (22) as opposed to atypical EAE, which is associated mainly with the pathology of the brain, we analyzed the cells in the spinal cord by flow cytometry. As expected, in uninfected Wt mice, we identified mainly resident microglial cells (CD45^loCD11b^) (Fig. 2). In infected mice, we found massive infiltration of macrophages (CD45^loCD11b^) and
In the absence of T cells, inflammatory lesions are not generated (Fig. 4A). Lack of CNS disease cannot be attributed to a reduced bacterial burden, because the severity and the duration of bacteremic episodes in Wt and TCR-β−/- mice were not significantly different (e.g., first episode $34 \pm 5$ versus $27 \pm 2$; second episode $11 \pm 2$ versus $10 \pm 3$ million bacteria/ml blood [Fig. 4B]). These results demonstrate that T cells are critical for the induction of CNS disease manifestations. Immune cell infiltration and APC activation do not occur in the absence of T cells

The previously described results led us to hypothesize that in the absence of T cells there might not be a significant infiltration of macrophages and other cells into the spinal cord of mice. Examination of spinal cords of infected TCR-β−/- mice indeed revealed a greatly reduced infiltration of macrophages, B220+ and NK cells compared with Wt controls (Fig. 5A). Furthermore, neither the infiltrating macrophages nor the resident microglial cells in the spinal cords of infected TCR-β−/- mice showed upregulated MHCII or CD80 expression (Fig. 5B). These results demonstrated that T cells are required for the activation and infiltration of immune cells in the CNS.

In the absence of T cells, inflammatory lesions are not generated in the spinal cord of B. hermsii-infected mice

Histopathological evaluation of cervical, thoracic and lumbar sections of the spinal cords revealed an extensive perivascular and parenchymal inflammation in lumbar spinal cord sections of the infected Wt mice (Fig. 6). The inflammation was predominant in the anterior and lateral cord. As expected there was no inflammatory infiltration in the infected TCR-β−/- mice (Fig. 6). Luxol fast blue staining of spinal cord sections did not reveal demyelination (data not shown), a hallmark of EAE. These results independently confirmed the previously described data that T cells are required for the induction of inflammatory cell infiltration into the spinal cord and the development of distinct neurologic disease manifestations during B. hermsii infection.
CD14 deficiency exacerbates the CNS disease manifestations during B. hermsii infection

CD14, a glycosylphosphatidylinositol-anchored receptor involved in facilitating the sensing of microbes and microbial components, is expressed on a variety of cells, including macrophages (27). We have previously shown that mice deficient in CD14 suffer 5- to 10-fold more severe B. hermsii bacteremia than Wt mice (28). To examine whether CD14-deficiency also affects neurologic disease manifestations, we infected CD14−/− mice with B. hermsii DAH and found that they suffer exacerbated neurologic complications when compared with Wt mice (Fig. 7A). A significantly higher number of mice suffered paralysis of one or both hind limbs. The average clinical score during the peak of disease in CD14−/− mice (~3.5) is significantly higher than that in Wt mice (~2.0). Furthermore, the incidence of disease seen in CD14−/− mice was higher than that in the Wt mice. By 6 wk postinfection, the clinical score in CD14−/− mice dropped to 2.0, and persisted at this level until the end of experiment (Fig. 7A). Consistent with an exaggerated disease in CD14−/− mice, we found significantly more severe pathology in these mutant mice compared with Wt controls (p < 0.05) (Fig. 7B).

Because an increase in bacterial burden and CD14 deficiency appear to increase the CNS disease severity at least in the case of strain DAH infection, we examined whether deficiency of CD14 also increases the CNS disease severity due to RAL and possibly FRE infections. Although strain FRE failed to induce CNS disease in Wt mice, it caused severe CNS disease in CD14−/− mice (score: 3.2 ± 1.64). CNS disease severity also increased in the case of RAL infection (score: 3.0 ± 1.4). These results suggest that although the induction of CNS disease is dependent on the B. hermsii strain, the ability of host to control this bacterium also contributes to this disease.
FIGURE 6. In the absence of T cells, inflammatory lesions are not generated in the spinal cord of B. hermsii-infected mice. Lumbar sections of spinal cord from B. hermsii-infected Wt (n = 15) and TCR-β × δ−/− (n = 4) mice were stained with H&E and inflammation status in peripheral regions was evaluated (original magnification ×125).

Discussion
CNS disease manifestations in RF patients and in animal models of RF have been well documented, but the critical factors involved in the induction of these manifestations are not clear. In the current study, we have found distinct neurologic disease manifestations during an experimental B. hermsii infection. Ascending paralysis of the hind limbs is the major manifestation in this model and the incidence of these symptoms was very high in even immunocompetent mice. Examination of the spinal cord revealed a severe infiltration of immune cells in the lumbar sections. We have identified T cells as the essential contributors for the induction of this disease. We have recently shown that TLR-mediated signaling is critical for IgM-mediated control of this disease. We have also shown that the infiltration of spinal cord predominantly involves CD4+ T cells, distinguishing the current findings from any of those previously reported (3, 13, 14).

A high incidence of CNS disease manifestations in RF patients has not been reported (2); however, in the current study we found that the majority of the B. hermsii strain DAH-infected mice suffer CNS disease manifestations. A likely reason for the low incidence in humans could be due to the fact that RF patients are typically treated with antibiotics during the very early stages of the infection (i.e., primary bacteremic episode). In fact, such treatment has been shown to eliminate even residual brain infection in mice (30). The absence of the infectious agent and a suboptimal T cell response might preclude high incidence of the neurologic manifestations in humans. In addition, it is possible that not all strains of B. hermsii could induce CNS disease in immunocompetent individuals. For example, although strain FRE failed to induce disease in Wt mice, it induced severe disease manifestations in CD14-deficient mice, suggesting that the susceptibility of the host also plays a role in the progression of CNS disease.

FIGURE 7. B. hermsii infection causes more severe neurologic symptoms in CD14-deficient mice. A, C57BL/6j (Wt; n = 10) and CD14−/− (n = 10) mice were infected and CNS disease manifestations were scored as in Fig. 1. B, Lumbar sections of spinal cord from B. hermsii-infected Wt (n = 15) and CD14−/− (n = 10) mice were stained with H&E and inflammation status in peripheral regions was scored (see Materials and Methods). Each dot symbol represents pathological score of an individual mouse. Mean values are indicated with a bar. Statistically significant differences in the severity of disease between Wt and CD14−/− mice were indicated with p values <0.05 (*).
bacteremia in CD14−/− mice is likely responsible for the increased severity of the CNS disease (28). In addition to an increased bacterial burden, *Borrelia* lipoproteins induce an altered pro- and anti-inflammatory cytokine profile in the absence of CD14-mediated signaling (32, 33) that might also contribute to a more severe neuropathology. For example, in classic EAE, in which a defined amount of autoantigen, myelin oligodendrocyte gp, and CFA are used to induce CNS inflammation, a more severe phenotype is observed in CD14−/− mice than in WT mice (34), suggesting that the CD14 deficiency itself can also alter the inflammatory response during *B. hermsii* infection.

Many microbial organisms have been associated with the induction of autoimmune diseases of the CNS (35–37). *B. burgdorferi*, a related spirochete and the agent of Lyme disease, has a number of outer membrane and cytosolic proteins similar to those of *B. hermsii*, for example, variable major proteins and flagellin (38). Analysis of T cell clones isolated from the cerebrospinal fluid of a patient with chronic Lyme neuroborreliosis revealed reactivity to *B. hermsii* of outer membrane and cytosolic proteins similar to those of *B. burgdorferi*, for example, variable major proteins and flagellin (38). Analysis of T cell clones isolated from the cerebrospinal fluid of a patient with chronic Lyme neuroborreliosis revealed reactivity to *B. hermsii* of outer membrane and cytosolic proteins similar to those of *B. hermsii*, for example, variable major proteins and flagellin (38). Analysis of T cell clones isolated from the cerebrospinal fluid of a patient with chronic Lyme neuroborreliosis revealed reactivity to *B. hermsii*.

The authors have no financial conflicts of interest.