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Intracerebral *Mycobacterium bovis* bacilli Calmette–Guerin infection-induced immune responses in the CNS ☆

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ABSTRACT

To study whether cerebral mycobacterial infection induces granuloma and protective immunity similar to systemic infection, we intracerebrally infected mice with *Mycobacterium bovis* bacilli Calmette–Guerin. Granuloma and IFN-γ$^+$CD4$^+$ T cell responses are induced in the central nervous system (CNS) similar to periphery, but the presence of IFN-γIL-17 double-positive CD4$^+$ T cells is unique to the CNS. The major CNS source of TNF-α is microglia, with modest production by CD4$^+$ T cells and macrophage. Protective immunity is accompanied by accumulation of Foxp3$^+$CD4$^+$ T cells and PD-L2$^+$ dendritic cells, suggesting that both inflammatory and anti-inflammatory responses develop in the CNS following mycobacterial infection.

1. Introduction

Mycobacterium tuberculosis (*Mtb*) has become the most common intracellular bacterium to infect the central nervous system (CNS) (Drevets et al., 2004). Despite the serious consequences following mycobacterial infection of the CNS, our understanding of the neuro- and immuno-pathogenesis of cerebral mycobacterial infection is limited.

Current knowledge of the immune response elicited by mycobacterial infection has come mainly from studies with respiratory or non-respiratory mycobacterial infection models (Flynn, 2006; Gupta and Katoch, 2005). Since these infection models do not express evident pathology in the CNS, an intracerebral infection model is needed to study the immune response against mycobacterial infection in the CNS. Previously, a rabbit model of intracisternal acute mycobacterial infection demonstrated prominent mononuclear cell infiltration, extended perivascular inflammation, and high TNF-α levels in the cerebrospinal fluid (CSF) (Tsenova et al., 1998, 1999). However, the cell-mediated immune responses that accompany this have not been fully characterized due to the rapid course of disease. In mice, infiltration of mononuclear cells and activation of microglia were observed in parallel with mycobacterial growth in the CSF following intracerebral (i.c.) inoculation with *Mycobacterium bovis* bacilli Calmette–Guerin (BCG) (Mazzolla et al., 2002). I.c. inoculation of FVBN mice with *Mtb* H37Rv resulted in granuloma formation and robust lymphocytic infiltration in the CNS (Rock et al., 2008). While all these models contribute to the understanding of mycobacterial pathogenesis in the CNS, further studies are needed to gain a better understanding of cell-mediated immune responses and bacterial containment in the CNS.

In the present study, we investigated cell-mediated immune responses during i.c. BCG infection with emphasis on the difference between CNS and peripheral immunity. To do so, we

Abbreviations: BCG, *Mycobacterium* bovis bacille Calmette–Guerin; CLN, cervical lymph node; CNS, central nervous system; CFU, colony forming unit; CSF, cerebrospinal fluid; DC, dendritic cell; i.c., intracerebral; IHC, immunohistochemistry; MFI, mean fluorescence intensity; *Mtb*, *Mycobacterium tuberculosis*.

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infected C57BL/6 mice i.c. with live BCG and studied the cellular composition and distribution of infiltrating cells into the CNS. The protective immune responses in the brain during the course of i.c. BCG infection were characterized by assessing mycobacterial growth, evaluating the phenotype of infiltrating lymphocytes, and quantifying the production of inflammatory cytokines, including TNF-α, IFN-γ, and IL-17. We demonstrate that CNS infiltrating $CD4^+$ T cells produced IFN-γ or IL-17. Additionally, approximately half of the IL-17-producing cells also produced IFN-γ. TNF-α was produced by $CD4^+$ T cells, macrophages, and microglia. Development of protective immune responses was accompanied by increased accumulation of regulatory $Foxp3^+CD4^+$ T cells and PD-$L2^+$ dendritic cells (DCs). Taken together, these data demonstrate that cerebral BCG infection induces protective immunity, potentially contributing to the development of therapies against cerebral mycobacterial infections.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at the Animal Care Facility at the University of Wisconsin, Madison. All experiments were carried out in accordance with guidelines of the National Institutes of Health and the University of Wisconsin Medical School Animal Care and Use Committee.

2.2. Bacteria

Frozen stocks were grown and prepared for infection as previously described (Hogan et al., 2001). The Pasteur strain of BCG (Staten Serum Institute, Denmark), kanamycin-resistant GFP-expressing BCG, or kanamycin-resistant dsRed-expressing BCG were grown in Middlebrook 7H9 supplemented with 0.05% Tween 80 and 10% oleic acid–dextrose-catalase supplement (Difco, Detroit, MI) in the presence or absence of kanamycin (50 μg/mL) and stored in frozen aliquots at −80 °C. For infections, ampoules were thawed, the inoculum was diluted in PBS, and briefly exposed to sonic oscillations to obtain a single-cell suspension. BCG Pasteur was transformed with an Hsp60 promoter-driven GFP construct containing the kanamycin resistance gene (a gift from Dr. Glen Fennelly, Albert Einstein University, New York). Kanamycin-resistant dsRed-expressing BCG was a gift from Dr. Lalita Ramakrishnan (University of Washington, WA).

2.3. Injection

Intracerebral injection was performed as previously described (Lee et al., 2008; Ling et al., 2003). 1×10^5 CFU of BCG in 30 μL of PBS or PBS alone was injected into the ventral-posterior region of the right frontal lobe of mice at a depth of 1.5 mm from the surface of the skull using an insulin syringe (28 and 1/2G) via a penetrating depth controller.

In some experiments, 1×10^7 CFU of BCG in 100 μL of PBS was intraperitoneally (i.p.) injected.

2.4. Organ load

At 3 and 5 weeks post kanamycin-resistant recombinant dsRed-expressing BCG infection, bacterial organ load was determined by plating serial dilutions of organ homogenates on Middlebrook 7H10 agar plates (Difco, Franklin Lakes, NJ) supplemented with 10% OADC (Difco, Franklin Lakes, NJ) and 50 μg/mL kanamycin. Colonies were counted after 3 weeks of incubation at 37 °C. Data are presented as total CFU per gram tissue for individual mice after $\log_{10}$ transformation.

2.5. Mononuclear cell isolation and flow cytometry

Mononuclear cells from the CNS were isolated and stained for flow cytometry as previously described (Karman et al., 2004; Lee et al., 2008; Ling et al., 2003). Mice were anesthetized with a ketamine and xylazine mixture and perfused transcardially with cold PBS. Organs were removed, weighed, and put on ice in Hank's buffered salt solution (HBSS) (Mediatech Inc., Herndon, VA). Spleen and cervical lymph nodes (CLN) were homogenized between frosted glass slides, and the resultant cell suspension was pelleted by centrifugation. Spleen red blood cells were lysed in lysis buffer, and the remaining cells were washed 3 times with HBSS and counted. To isolate mononuclear cells from the CNS, brains and spinal cords were removed from perfused animals, weighed, minced, transferred to Medicon inserts, and ground in a MediMachine (Becton Dickinson, Mountain View, CA) for 20–30 s. The cell suspension was washed with HBSS, and cells were resuspended in 70% Percoll (Pharmacia, Piscataway, NY), and overlaid with 30% Percoll. The gradient was centrifuged at 2250 *g* for 30 min at 4 °C. The interface was removed and washed once for further analysis. Absolute numbers were calculated based on the percentage of specific T cells from the total cell population acquired as determined using flow cytometric analysis and the weight of tissues.

For intracellular cytokine staining, single-cell suspensions from various tissues were cultured at 37 °C in complete RPMI 1640 supplemented with GolgiStop (BD Biosciences, San Jose, CA) in the presence of either 20 μg/mL MOG_{35-55} peptide or 5 μg/mL anti-CD3 (145-2C11) antibody for 5 h. After surface staining with anti-CD4 (RM4-5) and anti-LFA-1 (2D7) antibodies cell suspensions were fixed and permeabilized by Cytofix/Cytoperm solution (BD Biosciences, San Jose, CA) followed by staining with anti-IFN-γ (XMG1.2), anti-TNF-α (MP6-XT22), and anti-IL-17 (TC11-18H10) antibodies. Fluorochrome-labeled antibodies against CD4, CD8, LFA-1, CD45, CD11c, CD40, MHCII, IFN-γ, TNF-α, IL-17, and appropriate isotype controls were purchased from BD Biosciences (San Jose, CA). Fluorochrome-labeled antibody against PD-L2 was purchased from eBioscience (San Diego, CA). Anti-CD3 and anti-CD11b antibodies were produced from hybridomas. Stained cells were collected using a dual-laser FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) and analyzed with FlowJo software (TreeStar, Ashland, OR). For $Foxp3^+$ staining, anti-Foxp3 (FJK-16s) antibody (eBioscience, San Diego, CA) was used according to the manufacturer's protocol.

2.6. Tissue section preparation

Brains were removed from infected mice, fixed in 10% formalin, and embedded in paraffin. 10 μm sections were cut and processed for hematoxylin and eosin (H&E) or Ziehl–Neelsen (ZN) staining to detect infiltrating cells or acid fast bacilli, respectively.

2.7. Fluorescent microscopy

Frozen sections were prepared for fluorescent microscopy as previously described (Lee et al., 2008). Mice were perfused with 50 mL of PBS, followed by perfusion with 50 mL of 3% paraformaldehyde in PBS. In the case of frozen sections, spinal cord was removed and postfixed in 3% paraformadehyde/25% sucrose solution in PBS before freezing in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) on dry ice. Sections (5 μm) were stored at −80 °C until further use. Frozen sections were thawed for 10 min at room temperature and blocked with 1% BSA solution in PBS for 15 min. Sections were stained with antibodies for CD4-, CD11b-, or CD11c-conjugated with allophycocyanin, PE, and allophycocyanin, respectively, for 1 h at room temperature, followed by extensive washes with PBS. Images were acquired with a camera (Optronics Inc., Goleta, CA) mounted on a fluorescence microscope (Olympus BX41, Olympus) at 100× and 400× magnification. PictureFrame software (Optronics Inc.,