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Neonatal endotoxin exposure suppresses experimental autoimmune encephalomyelitis through regulating the immune cells responsivity in the central nervous system of adult rats

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ABSTRACT

Early-life exposure to bacterial endotoxin (lipopolysaccharide, LPS) affects the susceptibility to a variety of systemic organic inflammation in adulthood. To determine the long-term effects of neonatal LPS exposure on inflammatory responses in the central nervous system (CNS) in adulthood, we examined the effects on the development of experimental autoimmune encephalomyelitis (EAE) in adult rats as well as the potential regulatory immune mechanisms involved. The results showed that neonatal LPS exposure significantly reduced the morbidity ($p < 0.01$) and severity ($p < 0.05$) of EAE in adult rats, and decreased inflammatory cell infiltration and demyelination in the CNS compared with neonatal saline controls ($p < 0.05$). Neonatal LPS-treated animals showed reduced activation of microglia and astrocytes, as detected by immunocytochemistry, accompanied by down-regulation of the pro-inflammatory cytokines interleukin-17 and interferon-γ but up-regulation of anti-inflammatory cytokine interleukin-10 in the CNS ($p < 0.05$). At the same time, cerebrum mRNA levels of the transcription factors T-bet and RORγt were lower in neonatal LPS-compared with saline- treated animals ($p < 0.05$) accompanied with increased STAT-6 and Foxp3 levels in the neonatal LPS-treated group ($p < 0.05$). These findings suggest that early-life exposure to LPS could provide an important neuroprotective effect on the development of EAE in adult rats due to modulation of inflammatory responses in the CNS.

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is a $CD4^+$ T cell-mediated inflammatory demyelinating disease that affects the central nervous system (CNS) [1]. EAE is widely used as an animal model of multiple sclerosis (MS) [2]. The pathogenesis of MS/EAE involves breakdown of the blood–brain barrier, infiltration of autoreactive $CD4^+$ T cells and monocytes into the CNS, activation of glial cells, demyelination, axonal degeneration and neuronal loss, as well as changes in expression of pro- and anti-inflammatory cytokines [1,3,4]. Although the pathogenesis of EAE/MS is not well-understood, interactions between activated glial cells and inflammatory mediators released by infiltrating cells are believed to contribute to inflammatory disease progression and tissue damage [5].

Endotoxin (Lipopolysaccharide, LPS), derived from the cell wall of Gram-negative bacteria, is well-recognized as an activator of the neuroendocrine–immune system [6]. Treatment with LPS in the rat during the first week of life is increasingly used as a model of neonatal bacterial infection [7–10]. Immune stimuli such as LPS exposure during early neonatal life can alter the developmental trajectory and permanently affect the adult immune system and neuroendocrine activities, including responses to further immune challenge [8–10]. All these physiological changes appear to influence the susceptibility of the adult organism to subsequent pathological challenges [10].

In this study, we examined the long-term effects of neonatal LPS exposure on the development of EAE in Sprague–Dawley (SD) rats. We determined the characteristics and components of the immune response in the CNS following neonatal LPS treatment. After immunization, we measured the activation of glia cells, the expression of pro- and anti-inflammatory cytokines, interleukin-17 (IL-17) and interferon-γ (IFN-γ), as well as the anti-inflammatory cytokine interleukin-10 (IL-10), all of which are known to significantly contribute to EAE pathogenesis. We also measured the levels of

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transcription factors mRNA, which the differentiation of $CD4^+$ T cells subsets require (T-bet, STAT-6, RORγt and Foxp3 for Th1, Th2, Th17 and Treg cells, respectively).

2. Materials and methods

2.1. Animals and neonatal endotoxin exposure

Four litters ($n = 44$) of SD rat pups (both male and female) provided by the Laboratory Animal Center of Wenzhou Medical College, were used in this experiment. All litters were culled to 10–12 pups on the day of birth (postnatal day 0). They were maintained under standard animal-housing conditions (14-h light, 10-h dark cycle, with lights on at 07:00 h; temperature 22 ± 2 °C) and provided with food and water ad libitum. All rat pups, on days 3 and 5 postpartum, were randomly divided into two groups, including neonatal LPS-treated, adult spinal cord homogenate (SCH)-induced (nLPS-SCH) and neonatal saline-treated, adult SCH-induced (nSaline-SCH) groups. The rats were injected intraperitoneally with either 0.05 mg/kg of LPS in 0.05 ml of sterile saline (Lipopolysaccharide, serotype *Escherichia coli* 055:B5; Sigma–Aldrich, USA) [7,10]—or an equivalent volume of sterile saline vehicle. All litters were weaned at 21 days of age and housed in same-sex groups consisting of four to six animals per cage until 8 weeks of age when EAE was induced. The experimental procedures were done in accordance with the Care and Use of Laboratory Animals guidelines stipulated by the US National Institutes of Health Guide (1996). All efforts were made to minimize animal suffering and reduce the number of animals requested for the study.

2.2. Immune induction of EAE in adult rats

Rat EAE was induced by guinea pig spinal cord homogenates (GPSCH) as we described previously [11]. Briefly, rats aged 8 weeks were subjected to neonatal LPS- or saline-treatment, and then they were immunized by subcutaneous injection of 400 μl emulsion of GPSCH (50% w/v in saline) and complete Freund's adjuvant into limb footpads. Then, the inguinal grooves in these animals were injected subcutaneously with 0.1 ml pertussis vaccine suspension (Shanghai Institute of Biological Products, PR China) at 0 and 48 h postimmunization.

2.3. Evaluation for EAE symptoms

After immunization with GPSCH, rats were weighed and examined daily for symptoms and signs of EAE. To minimize the subjective error, three independent investigators with no knowledge of the procedures evaluated the symptoms and signs of EAE in each animal. The neurobehavioral changes of EAE were scored according to the criteria as follows: grade 0, no obvious signs; grade 1, tail paralysis; grade 2, paresis of hind legs; grade 3, complete paralysis of hind legs; grade 4, tetraplegia; and grade 5, moribund state or death [12].

2.4. Assessment for EAE histopathological changes

To assess the degree of CNS inflammation and demyelination, half of the animals in the nLPS-SCH ($n = 12$) and nSaline-SCH ($n = 10$) groups were deeply anesthetized by intraperitoneal injection of 3.6% chloral hydrate (Sigma Chemical Co., St. Louis, MO) on 21 days postimmunization (dpi; close to the peak stage of EAE signs) [11] and perfused with of cold 4% paraformaldehyde. The cerebrum, cerebellum, brain stem and spinal cords were dissected out and immersed in paraformaldehyde and embedded in paraffin wax for sectioning. Sections were stained with hematoxylin–eosin to detect inflammatory cell infiltration and luxol fast blue for demyelination. Histopathological severity of inflammatory cells infiltration was evaluated by two blinded observers according to the following criteria [13]: grade 0, no inflammation; grade 1, cellular infiltrates only adjacent to blood vessel and meninges; grade 2, mild cellular infiltrates in parenchyma (1–10/section); grade 3, moderate cellular infiltrates in parenchyma (11–100/section); and grade 4, serious cellular infiltrates in parenchyma (>100/section).

2.5. Immunohistochemical analyses of glial activation

Five-micrometer sections (cerebrum, cerebellum, brain stem, cervical, thoracic and lumbar cord segments) were dehydrated in a series of increasing alcohol concentration and then treated with 0.03% H_2O_2 to inactivate the endogenous peroxidase and with high pressure for antigen retrieval. They were blocked with goat serum and incubated with mouse anti-rat ionized calcium binding adaptor molecule 1 (Iba-1) antibody, a marker for microglia/macrophages (Abcam, US, 1:1000 dilution) or rabbit anti-rat glial fibrillary acidic protein (GFAP) antibody, a marker for astrocytes (Santa Cruz Biotechnology, Inc., US, 1:100 dilution). After washing, sections were incubated with the appropriate peroxidase-linked secondary antibody (biotinylated anti-mouse or anti-rabbit IgG). The number of positive cells per section in five randomly selected high-power fields (400×) was counted and averaged by two independent, blinded investigators.

2.6. ELISA analyses IL-17, IFN-γ and IL-10 protein levels

The animals used for ELISA analyses in the nLPS-SCH ($n = 12$) and nSaline-SCH ($n = 10$) groups were sacrificed and perfused with physiological saline at 21 dpi. The CNS was dissected out and tissue samples were prepared by homogenization in cell-lysis buffer supplemented with phenyl-methyl-sulphonyl fluoride. After being centrifuged, the supernatants were used for determination of IL-17, rat IFN-γ and rat IL-10 levels (R&D Systems, Minneapolis, MN). Optical densities were measured using a Model 680 microplate reader (Bio-RAD, Hercules, CA) at 450 nm. Total protein was determined using the BCA assay (Bio-RAD, Hercules, CA).

2.7. Real-time RT-PCR analyses for mRNA levels of T-bet, STAT-6, Foxp3 and RORγt

Total RNA was isolated from the cerebrum (50–100 mg) with Trizol (Invitrogen, USA), and then reverse-transcribed into cDNA using MMLV reverse transcriptase (Epicentre, USA). Primer sequences used for real-time PCR were as followers: T-bet, forward 5′ATGCCAGGGAACCGCTTAT3′ and reverse 5′TGGCTCACCGTCATT CACC3′; STAT-6, forward 5′CCAAGAAACCCAAGGATGAG3′ and reverse 5′TGGAATGAGACTGTGGAGGATA3′; Foxp3, forward 5′GGA CAACCCAGCGATGA3′ and reverse 5′CTTGGCAGTGCTTGAGAAAC3′; RORγt, forward 5′CGCACCAACCTCTTCTCACG3′ and reverse 5′GAC TTCCATTGCTCCTGCTTTC3′; GAPDH, forward 5′GGAAAGCTGTGGC GTGAT3′ and reverse 5′AAGGTGGAAGAATGGGAGTT3′. Real-time PCR was done using Rotor-Gene 3000 Real-time PCR instrument (Corbett Research, Australia). Semiquantitative analysis was performed by monitoring in real-time the increase of fluorescence of the SYBR-green dye (Invitrogen, USA) on a PCR Thermal Cycler (Takara Biotechnology, Dalian).

2.8. Statistical analyses

The independent-samples *t*-test and the χ^2-test were used in conjunction with the SPSS 12.0 (Windows) covariance software