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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

package for comparison analysis among groups. A probability value (p value) less than 0.05 was considered as statistically significant.

3. Results

3.1. Neonatal endotoxin exposure reduces the EAE morbidity and severity induced by GPSCH in adult rats

Nine days postimmunization, some animals began to show decreased activity and feeding behavior, as well as loss of body weight. On 12 dpi, two out of 10 nSaline-SCH rats displayed neurological impairment, including tail paralysis and hindlimb paresis, while the onset of those signs was delayed for 2 days in the nLPS-SCH group (Fig. 1). The EAE signs gradually led to complete paralysis of hindlimbs, tetraplegia and a moribund state. The signs peaked at the 17 dpi in the nSaline-SCH group, and at 18 dpi in the nLPS-SCH group. The mean EAE scores were also significantly reduced in the nLPS-SCH group ($p < 0.05$; Fig. 1, Table 1). Significant differences in decreased morbidity were noted for nLPS-SCH group compared with the nSaline-SCH group ($p < 0.01$).

3.2. Neonatal endotoxin exposure inhibits the CNS inflammatory cell infiltration and demyelination of EAE induced by GPSCH in adult rats

The pathological incidence of the nSaline-SCH group was consistent with morbidity due to EAE. In the nLPS-SCH group, pathological changes were detected in all rats displaying symptoms of EAE; however, there was only one additional rat that presented pathological changes without signs of EAE (Table 1). A significant statistical difference was noted in the pathological incidence between the two groups ($p < 0.01$). Severe infiltration and perivascular cuffing with mononuclear cells were noted in the cerebrum, cerebellum and lumbar spinal cord in the nSaline-SCH group rather than the nLPS-SCH rats (Fig. 2). The histological inflammatory scores from all the segments were significantly lower in nLPS-SCH group compared with those in the nSaline-SCH group ($p < 0.05$; Table 2). Consistent with the histopathologic scores, demyelination of the CNS in the nLPS-SCH group was significantly less compared with that in the other group (Fig. 2).

3.3. Neonatal endotoxin exposure attenuates the activation of microglia and astrocytes induced by GPSCH in adult rats

In the cerebrum, cerebellum, brain stem, cervical, thoracic and lumbar spinal cord of the nSaline-SCH rats, more markedly activated Iba-1-positive cells that were thicker, of larger size and had increased intensity were observed compared with those in the nLPS-SCH rats ($p < 0.05$; Fig. 3, Table 3).

The activation of astrocytes was also significantly suppressed in the nLPS-SCH group compared with that of the nSaline-SCH group. In the nSaline-SCH rats, compared with the nLPS-SCH animals, more GFAP-stained cells were noted ($p < 0.01$; Table 4), which were notably activated and of increased intensity (Fig. 3).

Table 1
Effects of neonatal endotoxin exposure on the EAE development of adult rats induced by GPSCH.

Groups	EAE morbidity	Histological incidence	Latency (days, mean ± SE)	Rats of EAE scores (n)					
				0	1	2	3	4	5
nSaline-SCH	10/10	10/10	13.80 ± 1.69	0	1	3	3	3	0
nLPS-SCH	4/12*	5/12*	14.25 ± 1.89	8	2	0	1	1	0*

* $p < 0.01$, compared with nSaline-SCH group.

3.4. Neonatal endotoxin exposure down-regulates the levels of IL-17 and IFN-γ, but up-regulates IL-10 levels induced by GPSCH in the CNS of adult rats

The mean levels of IL-17 and IFN-γ in the cerebrum, cerebellum, brain stem, cervical and lumbar spinal cord segments were significantly lower in the nLPS-SCH rats than that in their nSaline-SCH counterparts ($p < 0.05$). In contrast, higher levels of IL-10 were detected in the cerebrum, cerebellum, brain stem, cervical and lumbar spinal cords of the nLPS-SCH rats compared with their nSaline-SCH counterparts ($p < 0.05$; Fig. 4A–C).

3.5. Neonatal endotoxin exposure down-regulates the levels of RORγt and T-bet, but up-regulates STAT-6 and Foxp3 in the cerebrum of adult rats induced by GPSCH

The mean levels of transcription factors RORγt and T-bet in the cerebrum were significantly lower in the nLPS-SCH rats than that in the nSaline-SCH ones ($p < 0.05$). In contrast, higher levels of transcription factors STAT-6 and Foxp3 were detected in the cerebrum of the nLPS-SCH rats ($p < 0.05$; Fig. 4D–G).

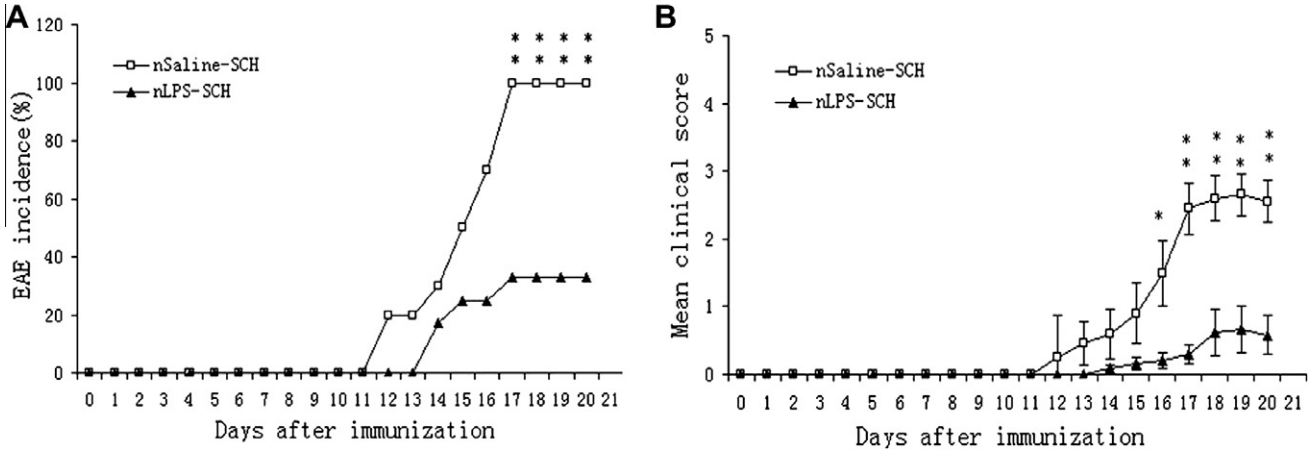

Fig. 1. The long-lasting effects of neonatal endotoxin exposure on EAE morbidity and severity induced by GPSCH in adult rats. (A, B) The onset of EAE began on 12 dpi and the mean EAE score reached the peak around 17 dpi, lasted for 3–5 days and was followed by a gradual recovery. (A) Significant differences were noted for EAE morbidity ($p < 0.01$) from 17 to 20 dpi. (B) The mean EAE scores were significantly reduced in the nLPS-SCH group compared with the nSaline-SCH group ($p < 0.05$) from 16 to 20 dpi. Results are expressed as the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, compared with the nSaline-SCH group.

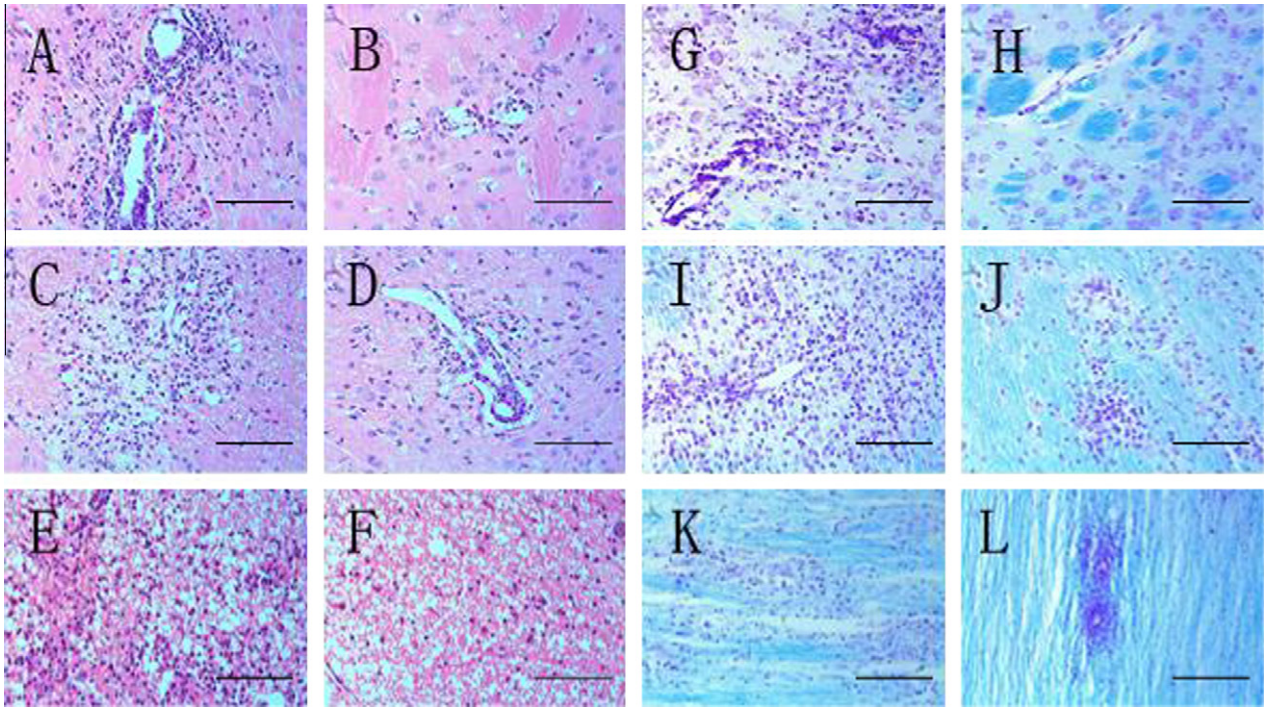

Fig. 2. Neonatal endotoxin exposure alleviates the CNS inflammatory cell infiltration and demyelination of EAE induced by GPSCH in adult rats. (A)–(F) show sections with inflammatory cell infiltration and perivascular cuffing by mononuclear cells in the CNS of each group. (A), (C) and (E) show the cerebrum, cerebellum and lumbar cord of nSaline-SCH rats. (B), (D) and (F) are rats from the nLPS-SCH group. (G)–(L) show sections with demyelination in the CNS of each group. (G), (I) and (K) show cerebrum, cerebellum and lumbar spinal cord sections of the nSaline-SCH group. (H), (J) and (L) show sections from the nLPS-SCH group. Bars: A–L 25 μm.

Table 2
Effect of neonatal endotoxin exposure on histopathological scores of EAE rats (mean ± SE).

Groups	Cerebrum	Cerebellum	Brain stem	Cervical cord	Thoracic cord	Lumbar cord
nSaline-SCH	3.00 ± 0.39	3.40 ± 0.34	3.30 ± 0.30	3.60 ± 0.31	2.70 ± 0.54	3.40 ± 0.31
nLPS-SCH	0.50 ± 0.26*	1.00 ± 0.43*	1.08 ± 0.47*	1.00 ± 0.44*	0.58 ± 0.31*	0.92 ± 0.45*

* $p < 0.01$, compared with nSaline-SCH.

4. Discussion

In this study, we first found that neonatal LPS exposure led to lower morbidity and less severity, as well as fewer pathological lesions in adult EAE rats. Consistent with a recent report, we suggested that LPS exposure during the first week of life reduced the susceptibility to EAE both in adult rats and mice [14]. In EAE, inflammation is associated with perivascular infiltration into the CNS by autoreactive T cells and monocytes. These activated autoreactive T cells stimulate resident glial or other cells in the CNS to increase their activities, to release more pro-inflammatory cytokines and chemokines and consequently to induce demyelination and axonal injury [15]. It has been known that activation of astrocytes and microglia play a critical role in the initiation and progression of EAE/MS [3,4,11,16]. These activated glial cells produce pro-inflammatory cytokines that may contribute to disease progression and related oligodendrocyte cell death [16,17]. Our results showed a significant reduction in the number of perivascular infiltrates and the activation of microglia and astrocytes in the CNS of the neonatal LPS-treated EAE rats. These findings suggested that the effect of neonatal LPS exposure on reducing the susceptibility and severity to EAE could be partially attributed to the inhibition of microglia and astrocyte activation, which may subsequently reduce the release of key inflammatory mediators, infiltration of inflammatory cells, and oligodendrocyte cell death [16,17]. However, the exact physiological mechanism involved in this lower response by glial cells to an immune challenge mediated by neonatal LPS treatment proposed previously by Ellestad et al. [14] remains to be investigation.

Recent data have established that the imbalance between pro- and anti-inflammatory cytokines contributes to the pathogenesis of autoimmune diseases [10]. Meanwhile, the IFN-γ-producing Th1 cells and IL-17-producing Th17 cells were pathogenic T cells in MS/EAE [18], while IL-10-producing effector Th2 and Treg could ameliorate MS/EAE progress. Therefore, inhibition of Th1 and Th17 cytokines or induction of Th1/Th17 to Th2/Treg cytokines shift is critical in the design of strategies to induce tolerance to disease [19]. Interestingly, in this study we observed that neonatal LPS-treated EAE rats displayed profoundly down-regulated expression of IL-17 and IFN-γ and up-regulated expression of IL-10 in the CNS, while the lower levels of transcription factors RORγt and T-bet and higher levels of STAT-6 and Foxp3 were detected in the cerebrum as compared with those of the control.

IL-17 is a pro-inflammatory cytokine produced mainly by Th17 cells and is well characterized for its role in infection and autoimmune disease [20,21]. It can activate T cells and other immune cells to produce a variety of cytokines, chemokines and cell-adhesion molecules [22]. Given that IL-17 is a critical effector cytokine in EAE, the fact that neonatal LPS-treated EAE rats showed decreased IL-17 production compared with that of control is of particular

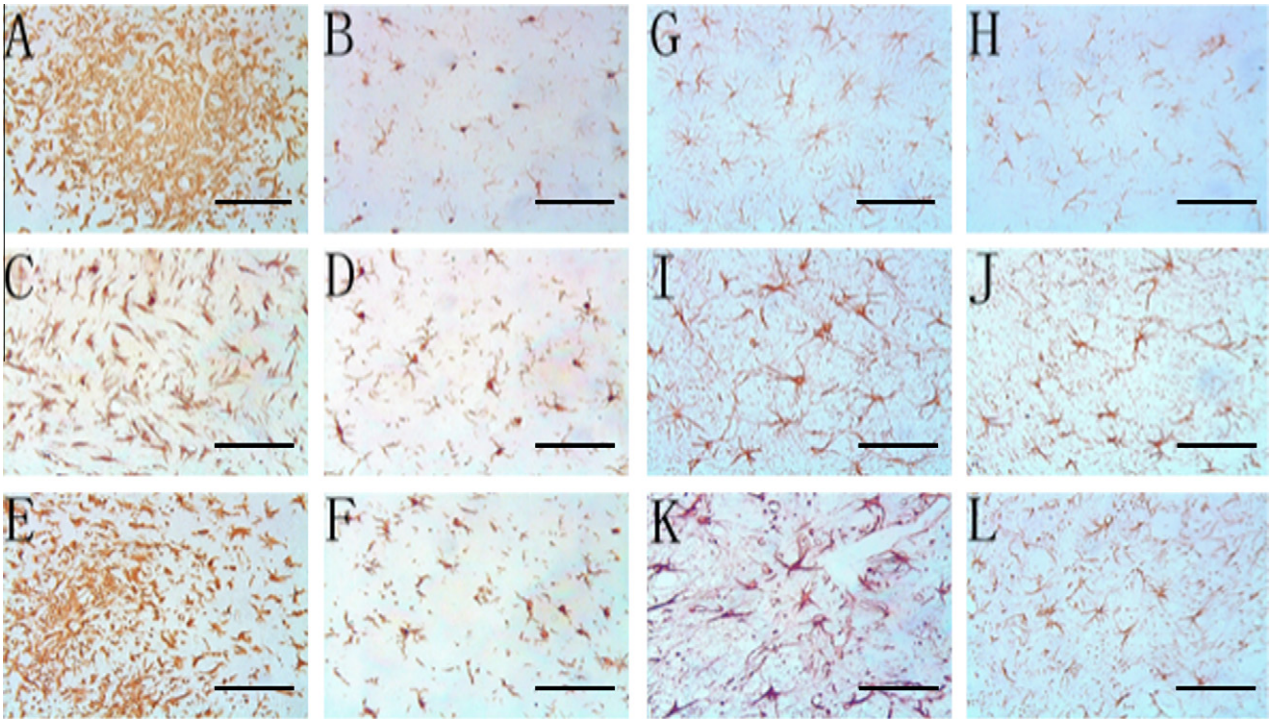

Fig. 3. Neonatal endotoxin exposure attenuates the activation of Iba-1-positive microglia and GFAP-positive astrocytes in adult rats induced by GPSCH. (A)–(F) showed Iba-1-positive cells of each group: (A)–(B) cerebrum, (C)–(D) cerebellum, (E)–(F) lumbar spinal cord sections of the nSaline-SCH and nLPS-SCH groups, respectively. (G)–(L) represent GFAP-positive cells of each group: (G), (I) and (K) cerebrum, cerebellum and lumbar cord sections in the nSaline-SCH group, and (H), (J) and (L) are from the nLPS-EAE group. Bars (A)–(L): 25 μm.

Table 3
Effect of neonatal LPS exposure on the number of Iba-1 positive cells in CNS (mean ± SE).

Groups	Cerebrum	Cerebellum	Brain stem	Cervical cord	Thoracic cord	Lumbar cord
nSaline-SCH	45.30 ± 2.13	48.00 ± 2.02	47.90 ± 1.65	48.80 ± 1.04	43.90 ± 2.98	47.30 ± 1.94
nLPS-SCH	21.67 ± 3.67*	24.92 ± 3.55*	25.08 ± 3.67*	24.33 ± 3.55*	22.08 ± 3.84*	23.58 ± 4.04*

* $p < 0.01$, compared with nSaline-SCH.

Table 4
Effect of neonatal LPS exposure on the number of GFAP-positive cells in CNS (mean ± SE).

Groups	Cerebrum	Cerebellum	Brain stem	Cervical cord	Thoracic cord	Lumbar cord
nSaline-SCH	25.30 ± 0.75	28.20 ± 0.71	21.40 ± 0.65	17.80 ± 0.47	16.20 ± 0.55	18.00 ± 0.54
nLPS-SCH	21.67 ± 1.30*	23.50 ± 1.12**	17.83 ± 0.90**	13.67 ± 1.12**	11.75 ± 0.99**	13.75 ± 1.04**

* $p < 0.05$, compared with nSaline-SCH group.
** $p < 0.01$, compared with nSaline-SCH group.

interest. Moreover, the Th17-associated transcription factor RORγt exhibited reduced expression in the cerebrum of neonatal LPS-treated EAE animals. Neuroprotection against EAE due to neonatal LPS exposure may be mediated partially through re-modulation of Th17 cell-immune responses including down-regulation of the production of IL-17 in the CNS.

IFN-γ is produced by T cells and NK cells. It can activate microglia to act as effector cells that damage CNS cells via phagocytosis, and it can activate the secretion of some active molecules including NO, superoxide and pro-inflammatory cytokines [23,24]. It has been demonstrated that IFN-γ plays an integral role in EAE pathogenesis [25]. In this study, neonatal LPS-treated EAE rats showed lower IFN-γ levels and lower IFN-γ-producing Th1 cells associated transcription factor T-bet expression in the CNS as compared to that of the control animals, indicating that down-regulation of IFN-γ and the suppressed Th1 cells contributed partially to the neuroprotection afforded by LPS treatment.

It is known that IL-10, which is mainly produced by Th2 and Treg cells, has potent anti-inflammatory and immunosuppressive activities, plays a key role in inflammation and is associated with remissions of EAE [26]. Our observations are compatible with previous finding that neonatal LPS exposure may affect the expression of IL-10 in adult, which are inversely correlates with disease severity [14]. Importantly, transcription factors Foxp3 and STAT-6, the important marker respectively of Treg and Th2 cells, exhibited reduced expression in the cerebrum of neonatal LPS-treated EAE animals, indicates that Treg and Th2 cells and their cytokine IL-10 likely play a key role in reducing neuroinflammation during EAE.

Previous study has demonstrated that early-life exposure to endotoxin may alter the maturation and differentiation of naive T helper cells [27] and permanently modify the function of T helper cells activity, consequently influence the Th1/Th2 balance in adulthood [28]. In our study, attenuation of inflammatory Th1 and Th17 immune responses and induction of biased Th2 and Treg

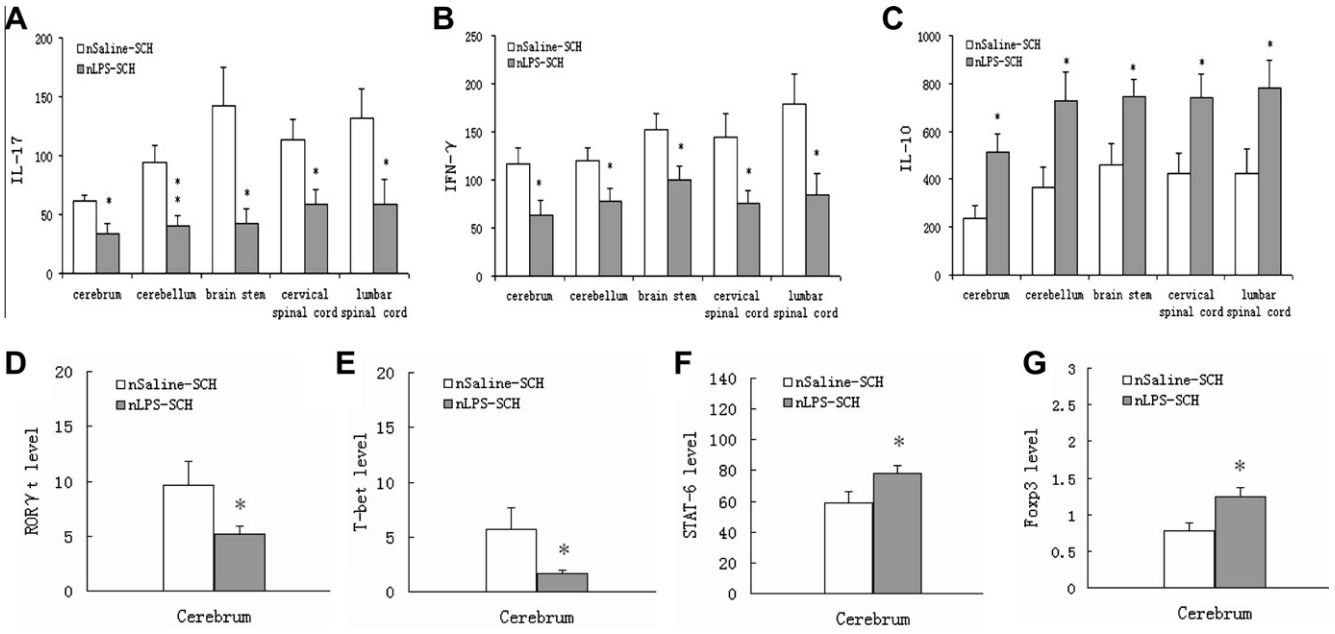

Fig. 4. (A–C) The expression of IL-17, IFN-γ and IL-10 in the cerebrum, cerebellum, brain stem, cervical and lumbar spinal cord segments in both nLPS-SCH and nSaline-SCH groups on 21 dpi by ELISA analysis. (D)–(G) The expression of T-bet, RORγt, STAT-6 and Foxp3 in the cerebrum of both nLPS-SCH and nSaline-SCH groups on 21 dpi. Results are expressed as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with that of the controls.

immunities were apparent in the CNS of neonatal LPS-treated EAE rats. Therefore, the current study provides evidence to suggest that neonatal LPS exposure can exert long-term and robust modulatory effects on immune systems interactions act through programming T cell development toward a Th2 and Treg phenotype, leading to altered disease courses through an alteration in the balance of cytokines production, favouring the creation of an anti-inflammatory cytokine milieu in the CNS.

In conclusion, our data support the view that endotoxin exposure in the first week of life protects against EAE in adult rats through its action on the immune system by decreasing recruitment of inflammatory cells into the CNS, reducing effector mechanisms of glia cells within the CNS, modulation of balance between pro- and anti-inflammatory cytokines production in the CNS, and consequently directly impacting development of T cell-mediated autoimmune response.

Earlier findings that were similar to those presented here have led to the proposal of the "hygiene hypothesis", which suggests that exposure to exogenous infectious or environmental antigens diminishes the subsequent risk of inflammatory and autoimmune diseases. Hence, it is conceivable that early injection of LPS during neonatal life might exert robust and sustained effects on immune function and consequently modulate the immune response in adulthood. These findings also provide targets for future therapies.

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