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Maternal immune activation in late gestation increases neuroinflammation and aggravates experimental autoimmune encephalomyelitis in the offspring

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ABSTRACT

Multiple sclerosis (MS) is characterized by an autoimmune response against myelin antigens driven by autoreactive T cells. Several lines of evidence indicate that environmental factors, such as previous infection, can influence and trigger autoimmune responses. However, the importance of the gestational period, particularly under inflammatory conditions, on the modulation of MS and related neuroinflammation by the offspring is unknown. This study aimed to evaluate the impact of prenatal exposure to lipopolysaccharide (LPS) during late gestation on the neuroinflammatory response in primary mixed glial cultures and on the progression of experimental autoimmune encephalomyelitis (EAE, an animal model of MS) in the offspring. LPS (*Escherichia coli* 0127:B8, 120 μg/kg) was administered intraperitoneally to pregnant C57BL/6J mice on gestational day 17, and the offspring were assigned to two experiments: (1) mixed glial cultures generated using the brain of neonates, stimulated *in vitro* with LPS, and (2) adult offspring immunized with MOG_{35-55}. The EAE clinical symptoms were followed for 30 days. Different sets of animals were sacrificed either during the onset (7 days post-immunization [p.i.]), when spleen and lymph nodes were collected, or the peak of disease (20 days p.i.), when CNS were collected for flow cytometry, cytokine production, and protein/mRNA-expression analysis. The primary CNS cultures from the LPS-treated group produced exaggerated amounts of IL-6, IL-1β and nitrites after *in vitro* stimulus, while IL-10 production was lowered compared to the data of the control group. Prenatal exposure to LPS worsened EAE disease severity in adult offspring, and this worsening was linked to increased CNS-infiltrating macrophages, Th1 cells and Th17 cells at the peak of EAE severity; additionally, exacerbated gliosis was evidenced in microglia (MHC II) and astrocytes (GFAP protein level and immunoreactivity). The IL-2, IL-6 and IL-17 levels in the spleen and lymph nodes were increased in the offspring of the LPS-exposed dams. Our results indicate that maternal immune activation during late gestation could predispose the offspring to increased neuroinflammation and potentiate the autoimmune response and clinical manifestation of EAE.

1. Introduction

Multiple sclerosis (MS), the most prevalent inflammatory demyelinating disease of the central nervous system (CNS), affects approximately 0.1% of the global population, mostly young adults in Europe and North America (Hauser and Oksenberg, 2006). Both MS and its murine model, experimental autoimmune encephalomyelitis (EAE), are characterized by an autoimmune response against CNS proteins driven by autoreactive T cells, which infiltrate the CNS, causing gliosis, demyelination, axon degradation and neuronal death (Brown and Sawchenko, 2007; Murphy et al., 2010; Neumann, 2003; Rodriguez, 2007).

Many studies have employed the EAE model to analyze the cell types involved in the pathogenesis of the disease, such as T $CD4^+$ (Kroenke and Segal, 2007) and $CD8^+$ (Goverman et al., 2005) cells as well as astrocytes and microglia (Glass et al., 2010; Murphy

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et al., 2010). Prior to the onset of the disease, antigen-presenting cells (APC) polarize T cells toward Th1 and Th17 subtypes in response to myelin antigens, initiating the peripheral immune response that culminates in massive cell infiltration into the CNS. In the target organ, those infiltrating cells interact with glial cells to orchestrate the immune response to myelin proteins during the progression of the disease.

Despite all the knowledge that has been gathered on MS pathogenesis, the underlying cause and risk factors for the autoimmunity remain poorly understood. The genetic background seems to influence, but not determine, susceptibility to MS (Sospedra and Martin, 2005). Environmental factors, such as sunlight exposure (Handel et al., 2010) and previous infections (Sospedra and Martin, 2005), could play a role in predisposing an organism to MS. However, the involvement of the prenatal period in this phenomenon and particularly the impact of acute maternal immune activation in the offspring's autoimmunity are currently unknown.

A recent body of evidence from our laboratory has shown that prenatal immune stimulation by lipopolysaccharides (LPS) impacts behavior and neurochemistry in later life (Zager et al., 2014, 2012). Recent findings demonstrated that adult offspring of LPS-stimulated mice presented enhanced peripheral Th1 cytokines and increased delayed-type hypersensitivity responses (Zager et al., 2013). Additionally, it has been shown that prenatal immune activation by the viral mimetic polyriboinosinic–polyribocytidylic acid (PolyI:C) induces preferential development of Th17 cells by the offspring (Mandal et al., 2010a,b), supporting the hypothesis of the presence of altered adaptive immunity in the offspring of immune-stimulated dams.

Based on previous findings, disruption of the maternal cytokine balance during critical gestational periods influences the development of the offspring's immune response (Mandal et al., 2010a,b; Zager et al., 2013). Thus, the aim of the present study was to investigate the impact of prenatal immune activation by LPS during late gestation on neuroinflammatory responses in primary mixed glial cultures from offspring as well as on EAE progression in adult offspring.

2. Methods

2.1. Animals

Male and female C57BL/6J mice from our own colony, weighing 20–30 g each, were used. The animals were housed in standard polypropylene cages (4 animals/cage, except where indicated) at a controlled room temperature (22 ± 2 °C) and humidity level (65–70%), with artificial lighting (12 h light/12 h dark cycle) and with free access to Nuvilab® rodent chow (Nuvital Company, São Paulo, Brazil) and filtered water. Sterilized and residue-free wood shavings were used as animal bedding. The animals used in this study were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources of the School of Veterinary Medicine, University of São Paulo, Brazil (protocol #1683/2009, FMVZ–USP).

2.2. Prenatal treatment and experimental design

Fig. 1 show the experimental design used in this study. Four independent experiments were performed according to Good Laboratory Practice (GLP) standards and quality assurance methods. To obtain the mice offspring used in this study, 2 virgin females were housed with an experienced male, and the presence of a vaginal plug was considered as gestational day (GD) 0. After mating, pregnant dams were housed in pairs until prenatal treatment. LPS derived from *Escherichia coli* serotype O127:B8 (Sigma, St. Louis, MO, USA) was freshly dissolved in 0.9% sterile saline and was administered intraperitoneally (i.p.) to pregnant mice at a dose of 120 μg/kg on GD 17 (~4 μg/animal). The dose and gestational period were chosen based on previous studies in which maternal inflammation was achieved without inducing preterm delivery or influencing offspring growth or sensory-motor reflex development (Golan et al., 2005, 2006; Hava et al., 2006; Zager et al., 2012, 2013).

These dams were allowed to give birth and nurture their offspring normally, and an average of 12 litters per treatment was used to obtain offspring for the study. No cross-fostering was performed because cross-fostering *per se* alters the behavioral and corticosterone responses to LPS (Gomez-Serrano et al., 2001). Nevertheless, our LPS treatment did not influence the number of pups per litter, the physical or reflexological development of the offspring, or the maternal behavior (data not shown). When necessary, the litters were standardized to a maximum of 8 pups per dam. The animals were weighed once a week from birth to weaning and again before the experiments, with no differences observed between the prenatal treatments at any time points.

To optimize the use of the obtained offspring and to attend the requirements of our Ethical Committee on this study protocol, the male offspring was utilized in the primary culture, whereas the female offspring was assigned to EAE experiments, since females are more susceptible to EAE than males (Voskuhl and Palaszynski, 2001). For the EAE experiments, female offspring were subjected to regular nurture and weaning on postnatal day 21, when the pups were distributed into different groups based on their prenatal treatment. No more than one pup from the same litter was placed into the same cage or same group.

2.3. Experiment 1: primary mixed glial culture

The primary glial cells culture was performed as described elsewhere (McQuillan et al., 2010). Briefly, brains of male neonates were collected from 1 to 3 day old neonates, and the cortices were dissected with the aid of a magnifying glass for the removal of meninges, cerebellum and midbrain. These components were passed through a cell strainer (70 μm) in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA). After these procedures, the brains of three neonates were pooled, using a maximum of one pup from each dam per flask and distinguishing the prenatal treatment. This mixed neuron × glia preparation was chosen rather than purified microglia because it has been shown that purified microglia has an activated phenotype with marked upregulation of CD40 and CD11b (McQuillan et al., 2010).

After mechanical dissociation of the tissues, the cells were subjected to pre-plating for 45 min to remove fibroblasts. After this stage, the cells were maintained in DMEM supplemented with 10% of fetal calf serum (FCS, Invitrogen, USA) in culture flasks (25 cm^2) pre-coated with poly-L-lysine (Sigma–Aldrich, St. Louis, USA) in a humidified 5% CO_2 environment. The medium was changed every 3 days, and the cells were grown for 12 days, when they reached confluence. The cells were then trypsinized (Trypsin 0.05%; Gibco, Life Technologies, Grand Island, USA), resuspended in DMEM and counted in a Neubauer chamber. The cells (5×10^5 per well) were incubated in 6-well plates pre-coated with poly-L-lysine, and after reaching confluence, the cells were treated with LPS (serotype 0127:B8, 1 μg/mL). After 24 h, the culture supernatant was collected, and the cells were subjected to immunofluorescence.

2.3.1. Immunofluorescence

The mixed glial cells were plated in 6-well plates on coverslips coated with poly-L-lysine. After stimulation with LPS for 24 h, the coverslips were rinsed 3 times with PBS (37 °C) to remove the

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