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Short communication

Increased cell-mediated immunity in male mice offspring exposed to maternal immune activation during late gestation

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ABSTRACT

Early life experiences, particularly during the gestational period, are homeostatic determinants for an individual's brain development. However, recent data suggest that the immune response of the offspring is also affected by events during the gestational period. Here, we evaluated the impact of prenatal immune activation on the innate and adaptive immune responses of adult offspring. Pregnant Swiss mice received saline or lipopolysaccharide (LPS) on gestational day 17. In adulthood, male offspring were analyzed using 2 experimental techniques: *in vitro* analysis of cytokine production and immune cell activity and development of the delayed-type hypersensitivity (DTH) responses of ovalbumin-sensitized mice. We analyzed Th1/Th2/Th17 cytokine production *in vitro*, neutrophil and dendritic cell function, and the DTH response. Offspring from LPS-treated dams displayed increased cell-mediated immunity as indicated by increased IL-12 production by cultured antigen-presenting cells and an enhanced DTH response as well as impaired production of the regulatory cytokine IL-10. This study provides new insights regarding the influence of immune activation during late gestation on the immunological homeostasis of offspring, particularly on Th1 immunity.

1. Introduction

The occurrence of maternal infection during pregnancy is considered an environmental risk factor for the emergence of many disorders in later life including autism [1,2], schizophrenia [3], anxiety [4] and depression [5]. Studies from our lab have recently shown that prenatal immune stimulation with lipopolysaccharide (LPS) has significant impacts on behavior and neurochemistry in later life in animal models [6,7]. Briefly, LPS is recognized by toll-like receptors (TLR) 2 and 4 [8] on immune cells and stimulates the release of inflammatory cytokines (interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)), which, in turn, affect placental function and fetal development.

Despite the recent body of evidence indicating that brain abnormalities in the offspring may be a consequence of maternal immune activation, the influence of this maternal phenomenon on immune function is still poorly understood. It should be noted that both animal experiments concerning prenatal inflammation and clinical studies have indicated immune dysregulation in autistic subjects, including defects in regulatory T cells [9] and increased IL-17 production [10], supporting the hypothesis of a close relationship between prenatal immune activation, adult immune dysfunction and the emergence of psychiatric disorders.

Changes induced by prenatal immune activation seem to indicate the delayed development of innate immunity and increased serum concentrations of IL-2, IL-6 and TNF in offspring [11]. Recently, it has been shown that prenatal immune activation induced by the viral mimetic polyriboinosinic–polyribocytidylic acid (Poly I:C) induces preferential development of Th17 cells in offspring [12,13], demonstrating that maturation and polarization of the adaptive immune response may be influenced by the gestational environment.

Based on previous findings, we infer that elevated levels of maternal cytokines play a major role during the development of different critical systems in the fetus such as the peripheral immune system. Thus, the aim of the present study was to investigate the impact of prenatal immune activation induced by LPS during late gestation on the immune function of adult offspring. Specifically, cytokine production by cultured splenocytes, neutrophil and dendritic cell activity, and delayed-type hypersensitivity responses were analyzed.

2. Methods

2.1. Animals

Pregnant albino Swiss mice from our colony, weighing 45–60 g each, were used in this study (gestational day [GD] 0: presence of a vaginal plug). Dams were housed in pairs in standard polypropylene cages at a controlled temperature (22 ± 2 °C) and humidity level (65–70%) with artificial lighting (12 h light/12 h dark cycle) in a non-SPF environment 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, and experiments were performed

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according to Ethical Committee of the School of Veterinary Medicine, University of São Paulo (protocol # 1683/2009, FMVZ-USP).

2.2. Prenatal treatment

LPS derived from *Escherichia coli* serotype 0127:B8 (Sigma, St. Louis, MO, USA) was freshly dissolved in 0.9% sterile saline and administered intraperitoneally (i.p.) to pregnant mice at a dose of 120 μg/kg on gestational day (GD) 17. This 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 [7,14–16]. In addition, GD 17 in mice is equivalent to week 20 of pregnancy in humans, a period during which the pluripotent stem cells migrate and proliferate in the fetal bone marrow, which controls hematopoiesis that was initially regulated by the fetal liver, spleen and thymus [17].

These dams were allowed to give birth and nurture their offspring normally and an average of 7 litters per treatment was used to obtain male offspring for the study. No cross-fostering was performed because cross-fostering *per se* alters the behavioral and corticosterone responses to LPS [18]. After weaning on postnatal day 21, the pups were distributed into different groups based on their prenatal treatment. No more than one pup from the same litter was paced into the same cage or same group.

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 prenatal treatments at any time points.

2.3. Experimental procedure

2.3.1. Experiment 1: Blood and tissue collection

On the day of the experiment, 70-day-old animals (n = 10/group) were decapitated, and an aliquot of blood from each mouse was placed into a tube containing liquid heparin for neutrophil activity analysis. Spleens were surgically removed and analyzed following the culture procedures described below. These procedures were performed twice with an analysis of half of the samples each time to reach a total of 10 animals per group.

2.3.2. Oxidative burst and phagocytosis by circulating neutrophils

Briefly, 100 μL of whole blood (2×10^5 cells/100 μL) was mixed with 200 μL of dichlorofluorescin (DCFH 0.3 mM) in phosphate-buffered saline (PBS) and 100 μL of either SAPI (Propidium iodide-labeled *Staphylococcus aureus*, 2.4×10^9 bacteria/ml) or phorbol myristate acetate (PMA/100 ng) in different tubes. Samples were incubated at 37 °C for 30 min. Phagocytosis reactions were stopped by adding 2 ml of cold EDTA solution (3 mM). After centrifugation (250 *g* for 10 min), the erythrocytes were lysed with 0.2% NaCl (2 ml per tube) for 20 s. Immediately thereafter, a 1.6% sterile NaCl solution (2 ml) was added to restore isotonicity. These procedures were repeated twice for complete erythrocyte lysis. Samples were then centrifuged (250 *g* for 10 min), and the cell pellets were resuspended in 200 μL of cold PBS.

The samples were then analyzed by flow cytometry (FACS Calibur, BD Biosciences, USA), collecting 5000 events in the gate being analyzed. For the acquisition and analysis of the results, we used the CellQuest Pro (BD Biosciences) and FlowJo (Tree Star) software programs. Direct measurements of the green-channel (DCFH) and red-channel (SAPI) mean fluorescence levels were recorded to indicate oxidative burst and phagocytosis, respectively, as described elsewhere [19]. Quantification of oxidative burst was estimated as the geometric mean of the DCFH fluorescence/cell. The intensity of phagocytosis was expressed as the geometric mean of the SAPI fluorescence that was ingested by the neutrophils. Percent phagocytosis (percent of neutrophils ingesting bacteria) was expressed by the number of neutrophils with red fluorescence divided by the total number of cells, multiplied by 100.

2.3.3. Spleen cell culture

Each spleen was collected, mechanically dissociated and homogenized in 5 ml of sterile RPMI-1640. This suspension was centrifuged at 450 *g* for 5 min and resuspended in 9 ml of sterile ammonium chloride to lyse erythrocytes, following which the solution was centrifuged again. The cells were then resuspended in 5 ml of complete RPMI medium containing 5% fetal calf serum and incubated in sterile 6-well plates at 37 °C (5% CO_2) for 2 h.

Non-adherent cells (lymphocytes) and adherent cells (antigen-presenting cells — APCs) were then separated, adjusted to a concentration of 1×10^6 cells/ml and incubated in RPMI (5% FCS) for 24 h in the presence of different stimuli. Non-adherent cells were stimulated with concanavalin-A (Con-A, 10 μg/ml), while the adherent cells were stimulated with LPS (1 μg/ml) or LPS plus IFN-γ (50 U/ml). After 24 h of incubation, cultured cells were collected and centrifuged at 450 *g* for 5 min and the supernatants were then frozen at −80 °C until the analysis.

2.3.4. Quantification of cytokines

Culture supernatants were analyzed for the concentrations of IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17 and IL-10 by flow cytometry using the Cytometric Bead Array (CBA) Th1/Th2/Th17 kit (BD Biosciences, USA) according to the manufacturer's instructions. Additionally, the IL-12 analysis was performed by ELISA (ElisaMax Biolegend, CA, USA) according to the manufacturer's instructions.

2.3.5. Phenotyping of dendritic cells

Adherent cells stimulated with LPS or LPS + IFN-γ were resuspended and centrifuged at 450 *g* for 5 min. The remaining cells were labeled with the following antibodies: CD80-PE, CD86-FITC, IA^b-PE and CD11c-FITC (BD Pharmingen, USA). After incubation for 60 min at room temperature in the dark, cells were washed twice in PBS (100 μl/tube) and resuspended in 150 μL of PBS. Then, the samples were analyzed by flow cytometry (FACS Calibur, BD Biosciences, USA), collecting 5000 events for the analysis gate. For the acquisition and analysis of the results, we used CellQuest Pro (BD Biosciences) and FlowJo (Tree Star). Due to the differences in flow cytometer compensation between experiments, all of the results were normalized to percentages relative to the control group (saline) for presentation and interpretation.

2.3.6. Experiment 2: Delayed-type hypersensitivity (DTH)

Another set of twenty 70-day-old male mice (n = 10/group) was used to induce a DTH response to ovalbumin (OVA grade V, Sigma-Aldrich®, Inc., St. Louis, MO, USA) by employing a protocol described elsewhere [20]. Briefly, animals were injected with 50 μg of OVA emulsified in 100 μL of Complete Freund's adjuvant (CFA, Sigma-Aldrich®) subcutaneously (s.c.) at the base of the tail. Seven days later, the animals were challenged s.c. in the left hind paw with 20 μL of a 4% heat-aggregated OVA solution in saline. Paw thickness was measured at 1, 4, 6, 24 and 48 h after challenge. The net increase in paw thickness was calculated by subtracting the initial thickness.

2.4. Statistical analysis

The data were tested for normality using the Kolmogorov–Smirnov test. Parametric data were analyzed with an unpaired Student's t-test or a repeated measures ANOVA followed by Bonferroni's test and non-parametric data were analyzed with a Mann–Whitney U-test. The results are expressed as the mean ± SEM and the level of statistical significance was set at $p \leq 0.05$.