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Effects of Maternal Exposure to LPS on the Inflammatory Response in the Offspring

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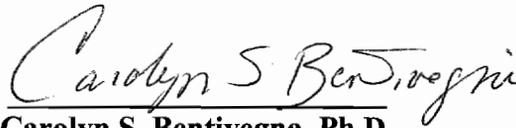**Effects of maternal exposure to LPS on the inflammatory response in the offspring**

Natalie La Sala

Submitted in partial fulfillment of the requirements for the degree of Master of Science in
Biology from the Department of Biological Sciences at Seton Hall University
April 16, 2008

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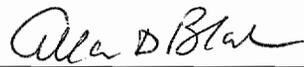
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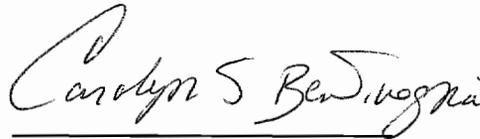
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Acknowledgements

Table of Contents

List of Figures

List of Tables

Abstract

To gain better understanding of the effects that maternal infection has on the offspring's inflammatory response, pups born to LPS or saline-treated dams were subsequently challenged with LPS or saline and the production of pro-inflammatory cytokines in the serum and in the brain was examined. Pregnant rats were randomly divided into two treatment groups with one group receiving one intraperitoneal (i.p.) injection of (500µg/kg) LPS and the other group one i.p. injection of saline on day 18 of pregnancy. All surviving offspring were subsequently challenged with either saline or LPS on postnatal day 21 (P21), and their serum and brain tissues were collected 2 h later. We found that the serum levels of pro-inflammatory cytokines, namely tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, were significantly inhibited in the offspring born to LPS-treated dams as compared to the offspring born to saline-treated dams following stimulation with LPS. Furthermore, the mRNA expression of TNF-α, IL-1β, and IL-6 in the brain of pups born to LPS-treated dams was also decreased as compared to the pups born to saline-treated dams following LPS stimulation on P21. In addition, to better assess the effects of maternal inflammation on the offspring's neuroinflammatory response, we examined leukocyte inflammation in the brain of P21 pups using myeloperoxidase (MPO) immunohistochemistry. Our preliminary data demonstrate that the infiltration of leukocytes may be decreased in the brain of P21 pups born to LPS-treated dams as compared to those born to saline-treated dams. Our data suggests that maternal infection suppresses the offspring's inflammatory response to LPS.

Introduction

The innate immune system serves as the first line of host defense against the harmful effects of invading infectious pathogens (Blatteis et al. 2005). A key component of this natural response is the production of inflammatory mediators (Glezer, Simard and Rivest 2007). Lipopolysaccharide (LPS, endotoxin), the primary component of the cell wall of Gram-negative bacteria, is a prime target for recognition by the innate immune system and is responsible for most of the inflammatory effects of infections from gram-negative bacteria (Bell and Hallenbeck 2002). For this reason, LPS is widely used in investigations of bacterial infection-induced inflammatory response (Saluk-Juszczak and Wachowicz 2005).

LPS is recognized by the innate immune system because it has typical pathogen-associated molecular pattern that is recognized by Toll-like receptor-4 (TRL-4) on many cells including monocytes and macrophages (Dantzer 2004). LPS interacts with a circulating LPS-binding protein and CD14, a glycosylphospatiylinositol-linked cell surface glycoprotein, to transfer LPS to TLR-4. This transfer marks the start of intracellular signaling, translocation of nuclear proteins, and transcriptional activation of genes associated with inflammatory processes (Beutler 2000).

It has been well studied that peripheral exposure to LPS in mammals causes macrophages and monocytes to release cytokines such as, tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6 and chemokines (Kawai et al. 2001). These pro-inflammatory cytokines induce adhesion molecules on the walls of vascular endothelial cells to adhere neutrophils, monocytes, and lymphocytes before undergoing diapedesis and moving out of the vessel lumen (Dinarello 2000). These endothelial cells allow

1

granulocytes to exit from the blood and these phagocytic cells can then move down chemokine gradients to the site of infection. There they can destroy as many bacteria as they can come in contact with and keep the infection under control (Jack 2000).

TNF-α and IL-1β are of primary importance in the initiation and propagation of the acute phase of the inflammatory response (Blond et al. 2002). TNF-α is a cytokine responsible for many of the systemic effects of infection including, septic shock, and is associated with chronic infection in mammals. A systemic infusion of TNF-α results in fever, fatigue, and an elevation of plasma glucocorticoids and catecholamines, all of which are mediated by the central nervous system (CNS). TNF-α has direct effects on central nervous stem neurons deep within the hypothalamus that are involved in producing the febrile response (Breder et al. 1994). After LPS is administered to rats, TNF-α is the first cytokine normally detected at the site of inflammation in the plasma, reaching maximum levels as soon as one hour after the injection (Fortier, Luheshi and Boksa 2007), where as IL-6 peaks 2-4 hours after treatment (Luheshi et al. 1997).

IL-1β is also one of the earliest expressed pro-inflammatory cytokines. This cytokine is a related gene product of the larger precursor, IL-1. A serine protease named, IL-1β converting enzyme (ICE), cleaves IL-1 to give rise to IL-1β. IL-1 is synthesized by many cell types but particularly by activated monocytes and macrophages (Hamblin 1993). IL-1β enables an organism to respond quickly to infection by creating a cascade of reactions that leads to inflammation (reviewed in (Bird et al. 2002; Dinarello 1997; Huising et al. 2004)). Many of the effector roles of IL-1β are mediated through the up- or down-regulation of expression of other cytokines and chemokines (Dinarello 1997).

2

IL-6 is a pleiotropic cytokine involved in the regulation of inflammatory and immunologic responses (Kishimoto et al. 1995). IL-6 is secreted by a broad range of cells including B cells, T cells, fibroblasts, monocytes, endothelial cells, microglia, and astrocytes (Benveniste 1997). IL-6 has been found in the cerebrospinal fluid of patients with multiple sclerosis, and other demyelization disorders, due to inflamed or virally-infected microglia and astrocytes. IL-6 facilitates the upregulation of IL-1β, TNF-α, and other acute phase reactants (Chiang et al. 1994); and therefore, it is responsible for promoting a classic inflammatory environment.

Models of peripheral immune challenge have shown cytokine induction in the brain (Turrin et al. 2001), specifically increases in the mRNA levels of IL-1β, IL-6, and TNF-α (Eriksson et al. 1999; Luheshi et al. 1997; Quan, et al. 1999). Whereas studies have shown peripheral LPS to increase these pro-inflammatory cytokine in the brain, the mechanisms by which brain cytokines are regulated during infections are still disputed. Currently, research suggests the following possible models: (i) transport of cytokines across the blood-brain barrier and/or circumventricular organs (CVOs) or direct activation of CVO neurons (Banks et al.1995; Maness et al. 1998); (ii) intermediate soluble factors, such as prostaglandins or cytokines themselves, that are produced and released within the CNS, and/or from endothelial cells of the cerbrovasculature and/or from CVOs, in response to LPS (Day and Akil 1996; Herkenham, et al. 1998; Maness et al. 1998); (iii) neural afferent signaling inducing CNS cytokine production (Goehler et al. 1999; Maier et al. 1998). Although the exact mechanisms of cytokine regulation in the brain are under debate, data show that peripheral injection of LPS to increase in mRNA

3

levels of pro-inflammatory cytokines causing neuroinflammation in the brains of rodents (Quan, et al. 1999).

The neuroinflammatory response may be due to both the central nervous system (CNS) and systemic immunoactivation (Bullock and Nathoo 2005; Marshall 2000; Morganti-Kossmann et al. 2007). For example, during neuroinflammation, the peripheral immune cells, such as neutrophils, are recruited to the brain and produce more inflammatory mediators (Montero-Menei et al. 1994). These neutrophils, among other polymorphonuclear leukocytes (PMNL), contain abundant amounts of the enzyme myeloperoxidase (MPO). MPO, a hydrogen peroxide (H_2O_2) oxidoreductase (Xia and Zweier 1997), is therefore often used as a marker of PMNL infiltration (Grattendick et al. 2002).

While it is clear that exposure to LPS stimulates the expression of pro-inflammatory cytokines in the peripheral system and in the brain of the adult rat, few studies have investigated how maternal LPS exposure affects the offspring's inflammatory response. For example, the ability of an adult rodent to coordinate an LPS-induced inflammatory response is critical to protect the animal against infection. However, to the fetus, maternal LPS-induced inflammatory response leads to increased intra-uterine fetal death (IUFD), preterm delivery, neonatal morbidity, and intrauterine growth retardation (Bakos et al. 2004; Buhimschi, et al. 2003; Xu et al. 2006). A study by Eklind and coworkers (2006) indicates that peripheral maternal rat exposure to LPS causes not only a high frequency of developmental abnormalities, but neurological defects, such as schizophrenia, cerebral palsy, or Parkinson's in the offspring (Ashdown et al. 2006; Bakos et al. 2004; Cai et al. 2000). Furthermore, excessive secretion of pro-

4

inflammatory cytokines is known to be toxic to the developing fetal brain (Bell et al. 2004; Dammann and Leviton 2000). Cytokines have a significant influence on the proliferation, survival, differentiation and phenotypic maintenance of the embryo in general and in the developing nervous system specifically (Jonakait 2007). Therefore, the immature brain may be more susceptible to inflammatory mediators than the adult brain.

The purpose of this study was to investigate the effects of maternal infection on the offspring's inflammatory response at the systemic level as well as in the brain, and therefore the experiments were designed as follows: (i) pups born to LPS-or saline treated dams were stimulated with LPS or saline to determine the expression of Il-1β, IL-6, and TNF-α levels in the serum, and mRNA levels of these cytokines in the brain ; (ii) pups born to LPS- or saline treated dams were stimulated with LPS or saline and monocyte/neutrophil infiltration was monitored by MPO immunohistochemistry. We hypothesize that maternal infection affects the offspring's inflammatory response to subsequent challenge with LPS.

Materials and Methods

Animals

Ten male and twenty-three female Sprague-Dawley® were purchased from Harlan Inc. (Indianapolis, IN). All animals were housed in a humidity and temperature controlled environment with a 12 hour light:dark cycle. Rats were provided standard rat diet and water ad libitum, and were allowed to adapt to their environment for at least one week prior to any experimental treatment. All animal studies and animal breeding were performed according to the Institutional Animal Care and Use Committee (IACUC) at Seton Hall University.

For mating to occur breeding colonies were established such that 3 nulliparous females were housed with one stud male each night in abovementioned housing conditions. The following morning conception was confirmed by daily visual inspection for a vaginal plug. The presence of a vaginal plug was designated as day 0 of pregnancy. On this day the female rats were moved to a separate cage under abovementioned housing conditions. When necessary, the litter size per dam was culled to 10 pups after birth.

Prenatal administration of LPS

LPS (*Salmonella enterica* serovar Typhimurium; Sigma, St. Louis, MO) was dissolved in sterile pyrogen-free saline. The pregnant rats were randomly assigned to either a LPS group or a saline control group. Due to the high rate of fetal reabsorption following LPS administration, the LPS group had twice as many animals as the saline control group. On day 18 of pregnancy, dams in the LPS group received one

6

intraperitoneal (i.p.) injection of 500µg / kg LPS, while dams in the saline control group received one i.p. injection of saline.

Post-natal administration of LPS

Pups from dams belonging to the LPS group and saline control group matured in a humidity and temperature controlled environment with a 12 hour light:dark cycle. Rats were provided standard rat diet and water ad libitum, and were allowed to adapt to their environment until postnatal day 21 (p21). On this day pups were randomly grouped into a LPS group or a saline control group. The pups in the LPS group were administered a single i.p. injection of 250µg / kg LPS, while the pups in the saline control group were administered a single i.p. injection of saline. Both groups were either sacrificed or perfused 2 hours later.

After sacrificing by decapitation, blood was collected and left clotting on ice for a minimum of 20 minutes, before being rimmed and centrifuged at 3000 rpm for 15 minutes at 4°C. The serum supernatant was aliquoted from each blood sample and stored at -80°C until cytokine assays were performed. Brains were dissected from each pup and sliced into left and right halves. The left half of brain was used immediately for total RNA extractions. The right half of brain was stored at – 80°C for further processing and analysis. For perfusion, the pups were anaesthetized with euthasol, tanscardially perfused with 10 mL of phosphate buffered saline (PBS) at pH 7.4, followed by 10 mL of 4% paraformalydehyde (PFA) in PBS. The brain were then removed from the skull and immersed in the same PFA fixative for 2 hours, then transferred to a 30% sucrose

7

solution. After equilibration in the sucrose solution, the brains were stored at -80°C until coronal sectioning.

Enzyme-linked immunosorbent assay (ELISA)

Serum levels of TNF-α, IL-1ß, and IL-6 were quantified using ELISA kits purchased from Invitrogen (Grand Island, NY), and ELISA assays were performed according to the manufacturer's protocol.

Tissue homogenization and Total RNA extraction

Total RNA from the collected brain tissues was homogenized in TRIzol reagent (Invitrogen, Grand Island, NY) and total RNA was isolated according to according to Life Technologies (Gaithersburg, MD) protocol. Briefly, to separate homogenized samples into RNA, DNA, and protein phases, chloroform was added to the samples followed by centrifugation at 4 °C. After transferring RNA phase to fresh tube, isopropanol was added, followed by centrifugation to precipitate the RNA. 75% ethanol was used to rinse the RNA pellet before it was air-dried. The dried RNA pellet was then redissolved in RNase free water and stored at – 80 °C for RT-PCR assays. RNA concentration was determined by the use of spectrophotometry at an absorbance of 260 and 280 nm. RNA integrity was evaluated by agarose gel electrophoresis and ethidium bromide staining.

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay

8

2 µg of total RNA was reverse transcribed to synthesize cDNA using oligo

(dT)12-18 primer and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase

(Invitrogen, Grand Island, NY). PCR amplification was carried out using sense and

antisense primers specific for rat ß-actin, IL-1ßm IL-6, and TNF-α (synthesized by Fisher

Scientific, Springfield, NJ). RT-PCR reactions were performed in a total volume of 50 µl

containing, 2µl of cDNA, 1X PCR buffer, 0.2 mM of dNTPs, 0.2 µl of each sense and

antisense primer, and 1 unit of Taq DNA polymerase (Applied Biosystems, Foster City,

CA). All reactions were reverse tanscribed at 94 °C for 5 minutes, followed by suitable

cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, an extension at 72 °C

for 30 s, and another 7-minute extension step at 72 °C was included after the final cycle.

A 2.0% agarose gel was used to visualize PCR products. Levels of ß-actin (control) was

used to normalize the relative intensities of IL-1ß, IL-6, and TNF-α.

Immunohistochemsitry

Coronal sections were cut from mounted frozen brain tissue surrounded by

embedding medium on a frozen stage. The stage was then attached to the cryostat with

consideration given to the angle of the blade to the specimen. The cryostat was set at a

box temperature of ~-25° C and upon the slice of each brain section, the coronal sections

were immediately placed into a well-plate containing 50% glycol and 50% PBS and

stored at -20 °C prior to staining.

Immunohistochemistry was performed on free-floating sections. All sections were

stained at the same time, using the same solutions of antibodies. This guaranteed that

incubation times and washes were the same for each brain. The standard protocol is as

follows: All sections were washed in Tris-buffered saline with 0.1% Tween-20

(1XTBST-20 0.1%). Antigen retrieval was performed by incubating sections in boiling

10mM citrate buffer for a total of at least 6 minutes. After the sections returned to room

temperature, the sections were thoroughly washed and incubated in 5% normal horse

serum blocking solution (2% horse serum, 1% BSA, 0.1% cold fish skin gelatin, 0.1%

Triton X-100, 0.05% Tween-20, 0.05% sodium azide, 0.01M PBS, pH 7.2) at 4 °C for 1

hour. Next the sections were incubated in 1.0µg/ml myeloperoxidase antibody at

appropriate (20X) dilution, in 1% normal horse serum in TBS, at 4 °C for 16 hours while

gently rocking. The sections were thoroughly washed again and incubated with diluted

biotinylated secondary antibody, in 1% normal horse serum in TBS, at room temperature

for 1.5 hours. The sections were washed and incubated with VECTASTAIN®ABC-AP

reagent for 1 hour at room temperature with gentle rocking. After another wash, the

sections were incubated in peroxidase substrate solution approximately 10-20 minutes

until dark brown color develops. The sections were thoroughly washed for the last time,

rinsed in tap water, dehydrated, and mounted on Superfrost slides. Each batch of

immunohistochemical staining included positive control tissue, and a negative control

sample in which the primary antibody was omitted.

Statistical analysis

All data are expressed as means ±SD. Two-way analysis of variance (ANOVA)

was used to analyze the RT-PCR and ELISA data. Maternal LPS treatment and post-

natal LPS challenges served as between-subject factors. A Bonferroni post-test was

performed if overall treatment effects were found statistically significant, and physical

10

parameters of the pups were computed by Student's t-test (GraphPad Prism Software,

San Diego, CA). Only results with p≤0.05 were considered statistically significant.

Results

Pregnancy outcome following maternal exposure to LPS

A total of twenty-two dams were administered a single i.p injection of 500 µg /kg LPS on day 18 of pregnancy. Of these dams, eleven experienced fetal reabsorption, two delivered stillborn pups, and the remaining nine delivered 87 live and 4 stillborn pups. The eleven saline-treated control dams delivered 145 live pups. A significant difference in the length of pregnancy occurred between the dams of the two treatment groups. Saline-treated dams gave birth after an average of 21.8 days of pregnancy, while LPS-treated dams gave birth after an average of only 20.7 days of pregnancy. In addition, pups born to LPS-treated dams took an average of two days longer to develop a hair coat than the pups born to saline-treated dams.

Table 1. Physical and numeral parameters of the offspring born to dams treated with saline or LPS on day 18 of pregnancy.

Maternal Treatment	Saline	LPS	Student's t-test p value
Duration of pregnancy	21.8± (21-23)	20.7±1.1(19-22)	0.013
# of live pups per dam	13.3±2.5 (9-16)	5.4±? (4.1-6.6)	0.0006
Hair coat (d)	6.3±0.8 (5-7)	8.3±1.8 (7-12)	0.01

LPS dams were administered an i.p. injection of 500µg/kg LPS on day 18 of pregnancy, and Saline were administered an i.p. injection of saline on day 18 of pregnancy. d, postnatal day in which hair first develops. Data were expressed as mean ±S.D.. In parentheses is the range of each parameter. *p<0.05 (Student's *t*-test)

Effects of maternal exposure of LPS on the serum levels of IL-1ß, IL-6, and TNF-α in the

offspring following LPS challenge on P21

Pups born to saline-treated and LPS-treated dams, who were subsequently

challenged with saline (S+S and L+S respectively), showed the lowest serum levels of

IL-1ß (Fig. 1), IL-6 (Fig. 2), and TNF-α (Fig.3). The pups born to saline-treated dams,

who were challenged with LPS (S+L), demonstrated a significant increase in serum

levels of IL-1ß (Fig. 1), IL-6 (Fig. 2), and TNF-α (Fig.3) as compared to S+S, L+S, and

L+L pups. The pups born to LPS-treated dams, who were challenged with LPS (L+L),

however, exhibited serum levels of IL-1ß (Fig. 1), IL-6 (Fig. 2), and TNF-α (Fig.3)

significantly lower than those of the S+L pups, but still greater the serum levels found in

the S+S and L+S pups. There was no difference in the population numbers for each

treatment group (n =6).

Figure 1. The serum levels of IL-1β in pups born to dams treated with a single i.p. injection of 500 μg/kg LPS or saline on day 18 of pregnancy, and subsequently challenged with one i.p injection of 250 μg/kg LPS or saline on postnatal day 21. Each treatment group (n= 6). S + S: saline + saline; S+L: saline + LPS; L+S: LPS + saline; L + L: LPS + LPS. *, $p<0.05$ vs S + S; #, $p<0.05$ vs L + S; &, $p<0.05$ vs S + L (two-way ANOVA followed by the Bonferroni post-test).

Serum level of IL-6 (pg/ml)

3500
3000
2500
2000
1500
1000
500
0

1801 pg/ml
*

395 pg/ml
* #, &

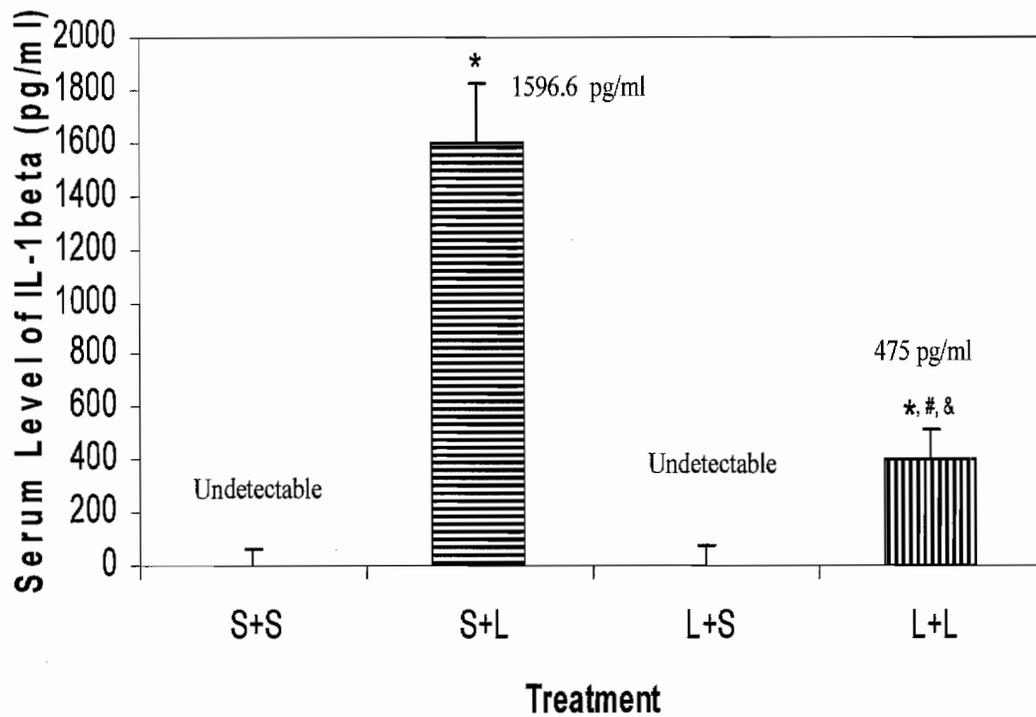Undetectable

Undetectable

S+S   S+L   L+S   L+L

Treatment

Figure 2. The serum levels of IL-6 in pups born to dams treated with a single i.p. injection of 500 µg/kg LPS or saline on day 18 of pregnancy, and subsequently challenged with one i.p inection of 250 µg/kg LPS or saline on postnatal day 21. Each treatment group (n=6). S + S: saline + saline; S+L: saline + LPS; L+S: LPS + saline; L + L: LPS + LPS. *, $p < 0.05$ vs S + S; #, $p < 0.05$ vs L + S; &, $p < 0.05$ vs S + L (two-way ANOVA followed by the Bonferroni post-test).
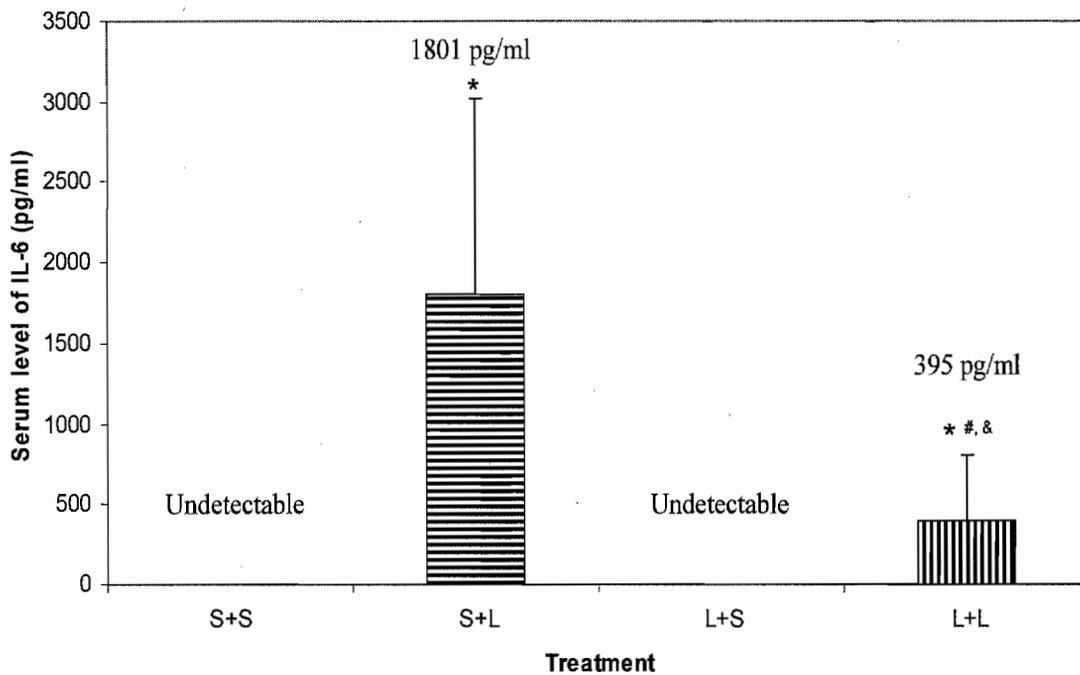
Figure 3. The serum levels of TNF-α in pups born to dams treated with a single i.p. injection of 500 μg/kg LPS or saline on day 18 of pregnancy, and subsequently challenged with one i.p inection of 250 μg/kg LPS or saline on postnatal day 21. Each treatment group (n=6). S + S: saline + saline; S+L: saline + LPS; L+S: LPS + saline; L + L: LPS + LPS. *, $p<0.05$ vs S + S; #, $p<0.05$ vs L + S; &, $p<0.05$ vs S + L (two-way ANOVA followed by the Bonferroni post-test).
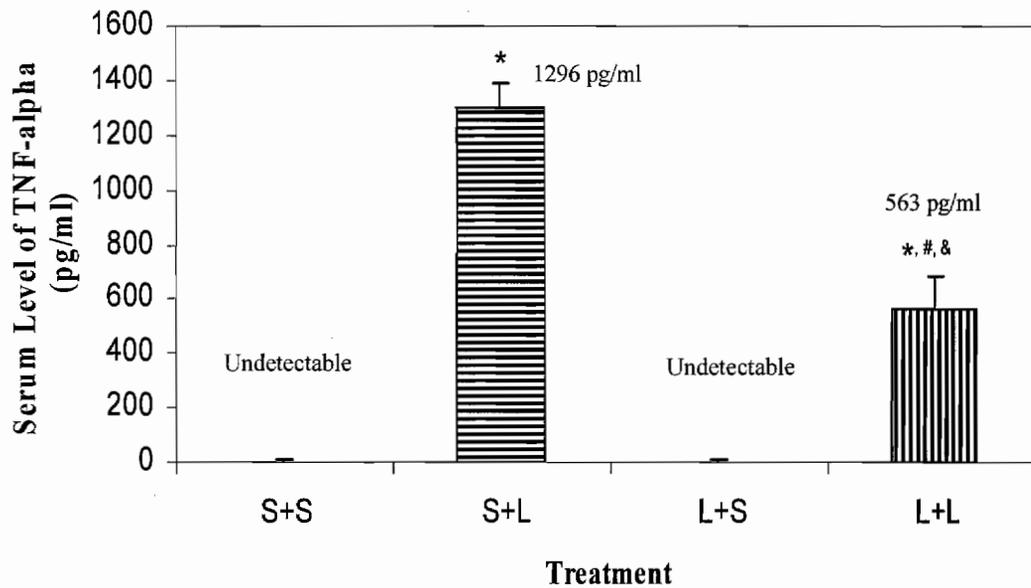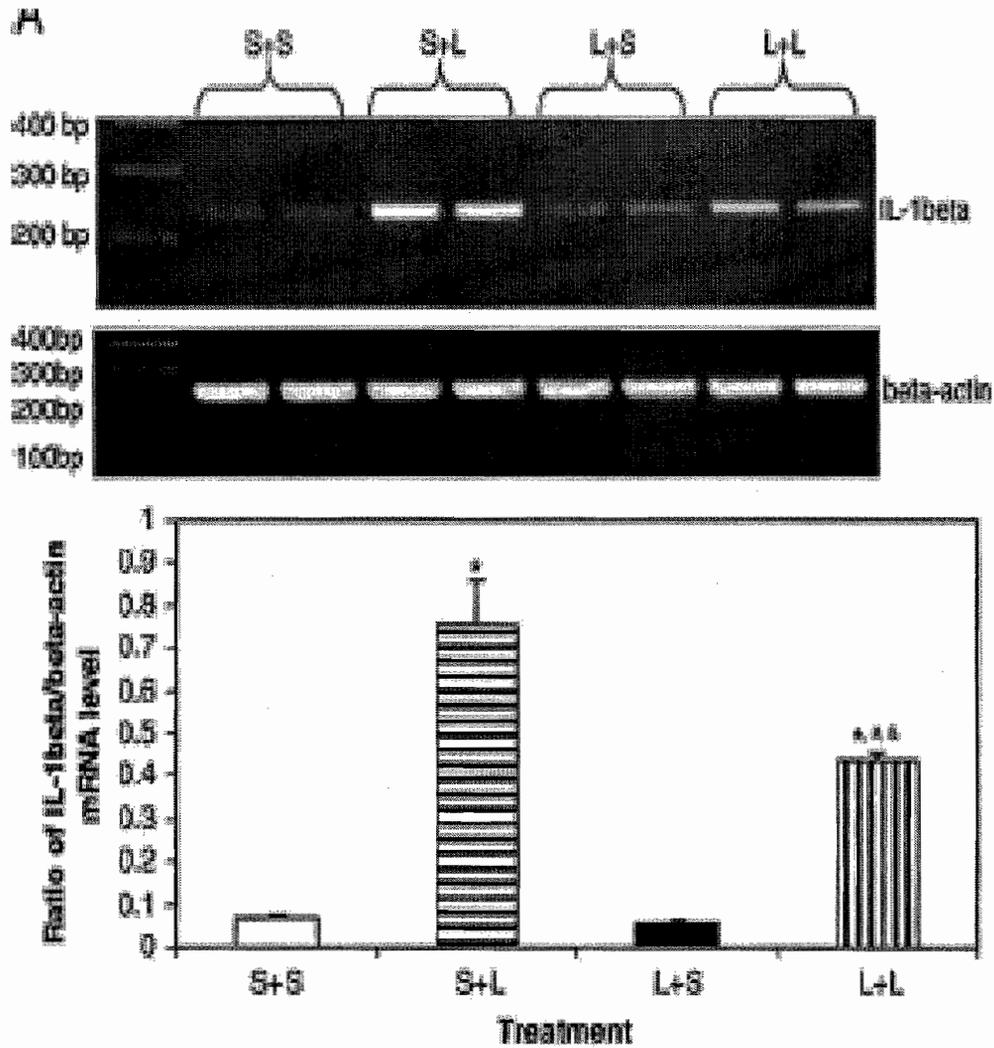
Figure 4. Expression of mRNA levels of IL-1β in the brain of pups born to dams that were administered a single i.p. injection of either 500 μg/kg LPS or saline on day 18 of pregnancy, and subsequently challenged with one i.p. injection of 250 μg/kg LPS or saline on post-natal day 21. The bar graph illustrates the mean + S.D. of the data shown. S + S: saline + saline; S+L: saline + LPS; L+S: LPS + saline; L + L: LPS + LPS. *, $p < 0.05$ vs S + S; #, $p < 0.05$ vs L + S; &, $p < 0.05$ vs S + L (two-way ANOVA followed by the Bonferroni post-test).

18

Figure 5. Expression of mRNA levels of IL-6 in the brain of pups born to dams that were administered a single i.p. injection of either 500 μg/kg LPS or saline on day 18 of pregnancy, and subsequently challenged with one i.p. injection of 250 μg/kg LPS or saline on post-natal day 21. The bar graph illustrates the mean + S.D. of the data shown. S + S: saline + saline; S+L: saline + LPS; L+S: LPS + saline; L + L: LPS + LPS. *, $p < 0.05$ vs S + S; #, $p < 0.05$ vs L + S; &, $p < 0.05$ vs S + L (two-way ANOVA followed by the Bonferroni post-test).
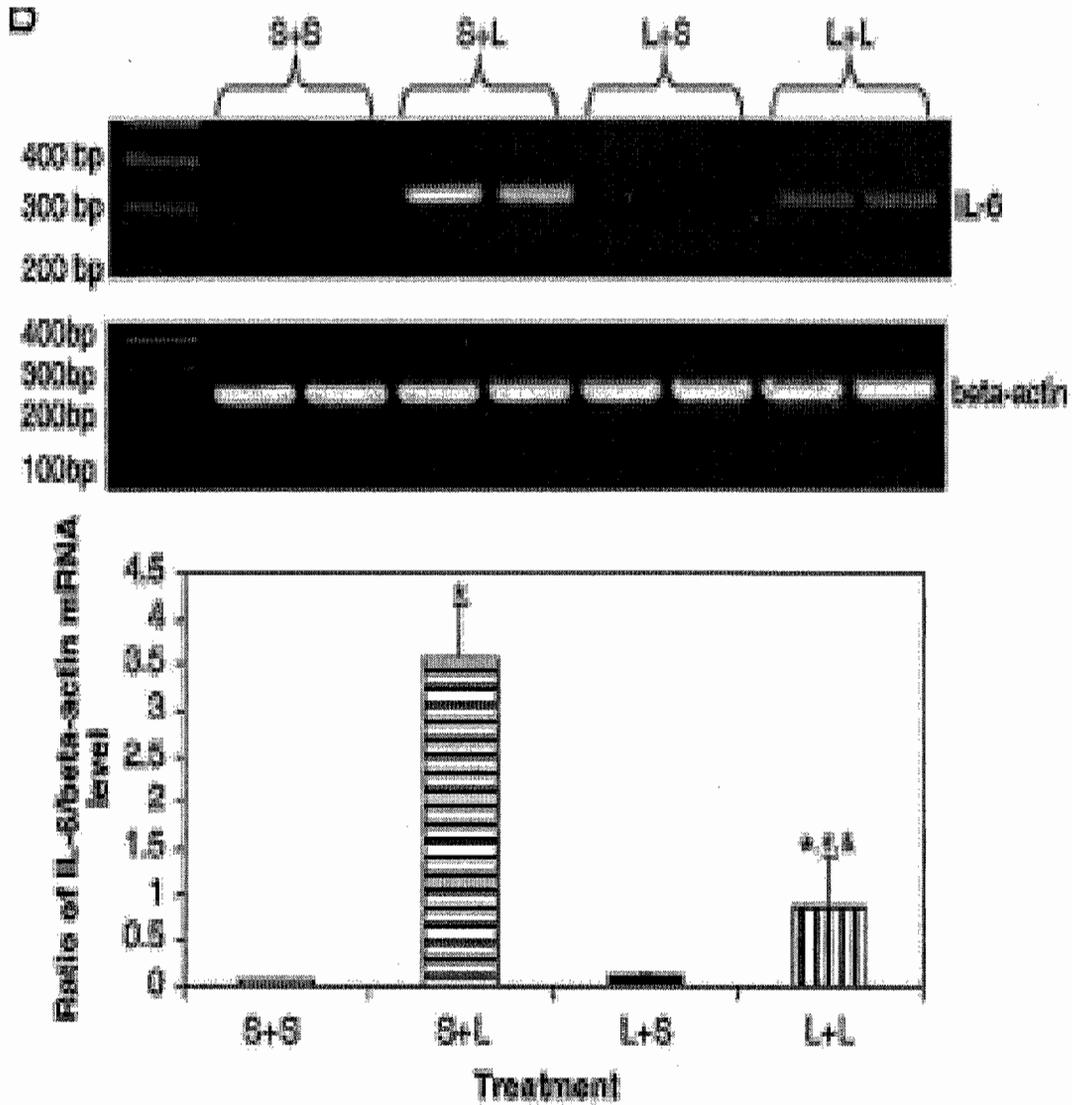
Effects of maternal exposure of LPS on the expression of IL-1β, and IL-6 in the brain of the offspring following LPS challenge on P21

Pups born to saline-treated and LPS-treated dams that were subsequently challenged with saline on P21 (S+S and L+S respectively), exhibited a nearly undetectable mRNA level of IL-6 (Fig. 5), and basal mRNA levels of IL-1ß (Fig. 4), as determined by RT-PCR. The pups born to a saline-treated dams that were challenged with LPS on P21 (S+L), as well as the pups born to LPS-treated dams that were challenged with LPS on P21 (L+L), demonstrate significantly higher mRNA levels of IL-1ß (Fig. 2A), IL-6 (Fig. 2B) in the brain than both the S+S and L+S pups. The mRNA levels of IL-1ß and IL-6 in the brain of pups born to LPS-treated dams that were also challenged with LPS at P21, are considerably lower than that in the S+L pups. There were no differences in the population numbers for each treatment group (n = 6).

Immunohistochemisty detection of myeloperoxidase in the brain (MPO)

Immunohistochemistry was performed on coronal brain sections of P21 offspring using anti-myeloperoxidase antibody. No MPO immunoreactivity was observed in the substania nigra (SN) of S+S rat pups (Fig. 6A). The S + L treated pups seemed to exhibit increased MPO immunoreactivity in the SN region as compared to the S+S group. Furthermore, the L+L pups appeared to exhibit less MPO immunoreactivity in the SN as compared to the S +L treated pups. In this study two P21 pup brains were stained for each of the treatment groups (n=2). Figure 1 illustrates one pup brain from each treatment group, other results not shown.
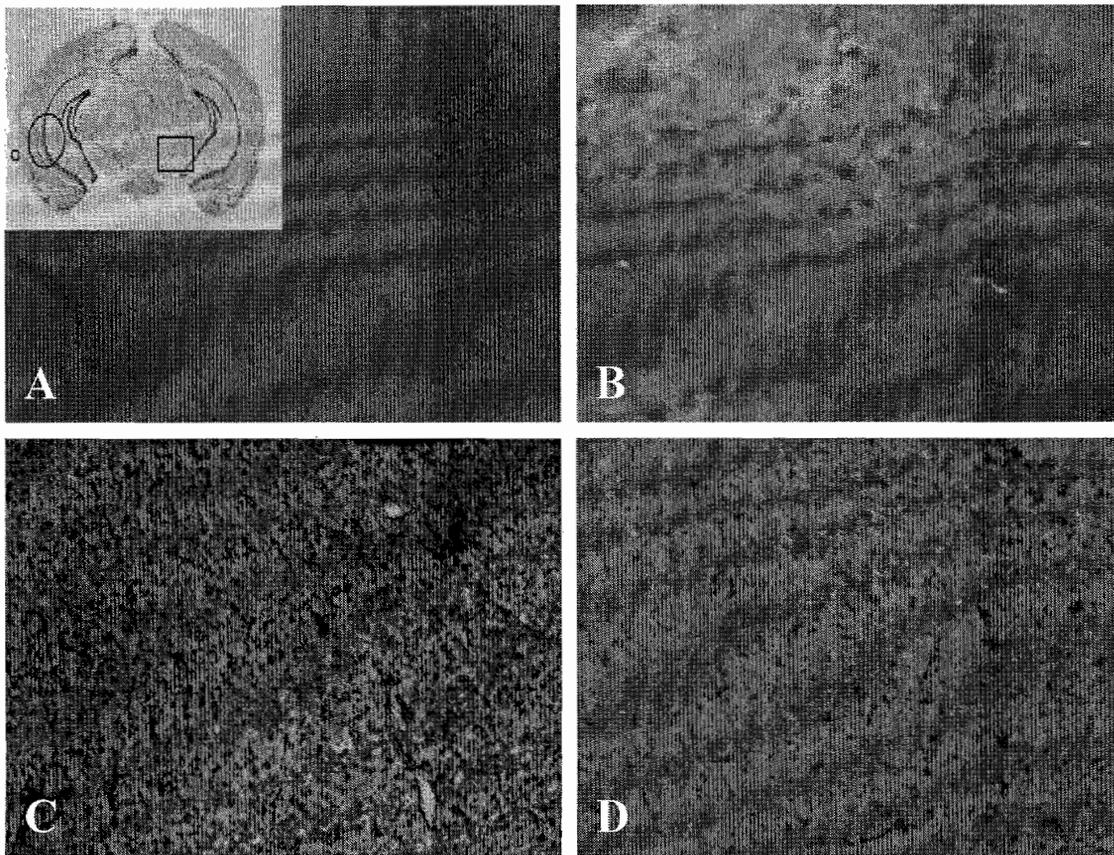
Figure 6. Preliminary photomicrographs indicates MPO signal in the SN of coronal mesencephalon sections of pups born to maternally exposed dams that received 500 μg/kg LPS-or saline on E18. These pups were subsequently challenged with one i.p. injection of 250 μg/kg LPS or saline on P21 and perfused 2 hrs later. (A) In the control, no MPO immunoreactivity is noticeable in brain parenchyma and (B) L + S treated pups imply minimal MPO immunoreactivity in brain parenchyma, while (D) L + L treated pups suggest reduced PMVL recruitment to the SN in the basil ganglia than (C) S + L treated pups, due to the increase in MPO immunoreactivity present in the parenchyma. PMVL, polymorphonuclear leukocytes; sn, substania nigra; Scale bar = 100μm.

Discussion

The major finding of this study was that pups born to LPS-treated dams, that were subsequently challenged on P21 with LPS, exhibited significantly inhibited serum levels of IL-1ß, IL-6, and TNF-α, as well as diminished mRNA levels of IL-1ß, and TNF-α. Our preliminary IHC data also illustrates that there may be attenuated quantities of PMNLs in the brains of pups born to LPS-treated dams that were challenged with LPS on P21. This suggests that maternal infection has a significant effect on the offspring's inflammatory response in the periphery. The results also indicate that day 18 of pregnancy represents a developmental period when maternal immune challenge has a considerable impact on the offspring's inflammatory response.

While the correlation between diminished mRNA levels of IL-1ß and TNF-α in the brain, and the inhibited levels of these same cytokines in the serum are not surprising, it must be noted that there is a "duality" taking place in the role of cytokines in inflammation and neuroinflammation. For example, although IL-6 is known both peripherally and centrally for its role in the acute phase reaction, it also has specific anti-iflammatory properties, such as the inhibition of TNF-α. Therefore, the decrease in serum levels of TNF-α, maybe dropping independently of mRNA expression (Morganti-Kossmann et al., 2007). This illustrates the complexity of the processes involved in the inflammatory response.

The mechanisms underlining the diminished production of TNF-α, IL-1ß, and IL-6 levels in the serum of L + L pups compared to the serum levels of S + L pups are currently debated. It is known that LPS does not enter the fetal circulation upon maternal exposure (Goto et al. 1994). This indicates the possible mechanisms for the effects of

22

maternal inflammation on the developing fetus may be related to the immune response of

the placenta, and the ability of inflammatory cytokines to pass through the placenta to

affect the fetus. However, literature is contradictory on whether maternal inflammatory

cytokines are transferred through the placenta to the fetus. For example, Urakubo and

coworkers (2001) and others (Beloosesky et al. 2006; Gayle et al. 2004) found an

increase in levels of TNF-α, IL-1ß, and IL-6 in the placenta upon maternal exposure to

LPS. However, other *in vitro* reports suggest there is minimal to no transfer of IL-1ß or

TNF-α, with a "modest passage" of IL-6 (Aaltonen et al. 2005) to the fetus. Furthermore,

when TNF-α is administered to uninfected pregnant rats, it was not found to cross the

placenta. A possible explanation for this is that cytokines are more likely to cross the

blood-placenta barrier in the setting of infection, as infection increases the permeability

between the blood and placenta. In all, our results indicate that maternally-derived

cytokines are activating the fetal immune cells resulting in the production of increased

levels of cytokines (Ashdown et al. 2006). These cytokines may significantly affect the

development of the fetal immune system (Pfeffer 2003; Sljivic et al. 2006), which could

explain the decreased inflammatory response seen in the LPS challenged offspring.

The exact mechanism(s) whereby TNF-α, IL-1ß, and IL-6 cytokines access the

fetal cerebral compartment to induce injury is not well understood either. Because TNF-

α, IL-1ß, and IL-6 fail to cross the fetal brain after maternal LPS injection late in

gestation (E18) (Ashdown et al. 2006) the rise in mRNA and serum levels observed there

(Bell et al.2004; Cai et al. 2000; Liverman et al. 2006) indicates that the fetal brain may

elaborate cytokines locally The embryonic brain, while lacking adult astrocytes, has

microglial cells capable of synthesizing cytokines locally (Jonakait 2007). The

23

inflammatory capabilities of microglia have been studied extensively in culture, and it has been shown that neonatal microglia can produce proinflammatory chemokines and cytokines that can be toxic to neurons and oligodendrocytes (Jonakait 1996; Merrill and Jonakait 1995). Once in the cerebral compartment proinflammatory cytokines can induce excitotoxicity by modifying the re-uptake of glutamate, stimulating the release of free radicals (Blumenthal 2004), and by increasing the risk of oxidative injury and additional inflammatory damage (Haynes et al. 2005). Overexpression of TNF-α and IL-1ß contributes to abnormal brain development and increased cell death in the developing brain (Cai et al. 2000; Dammann and Leviton 1997; Mehler and Kessler 1997). The neurotoxic nature of proinflammatory cytokines could be a possible explanation for the diminished mRNA levels of TNF-α, IL-1ß, and IL-6 exhibited by the L + L pups when compared to the S+L pups. As a result of maternal exposure to LPS, L + L pups may have had abnormal brain and neuronal development so much so their neuroinflammatory response is highly impaired when challenged with LPS.

In contrast, other research indicates that peripheral cytokines are transported across the blood-brain barrier (BBB) (Banks 2005; Baud et al. 1999) and damage the BBB so that permeability of the barrier is increased (Saija et al. 1995), and the expressions of other cytokines in the brain are induced (Laye et al. 1994). This would imply that the lower mRNA levels of cytokines found in the L+L pups was due to diminished levels of TNF-α, IL-1ß, and IL-6 in the serum of these offspring, than the levels of cytokines in the serum of S + L pups.

A potential explanation for the low levels of TNF-α, IL-1ß, and IL-6 in the serum of L + L pups may have something to do with LPS-binding protein (LBP). This serum

24

protein is able to extract LPS from micelles and present it to CD14, a cell surface glycoprotein(Jack 2000) to regulate the activation of immune response. LBP, *in vivo*, is believed to have a dual biological role. Initially, it may enable the host to quickly detect and respond to low concentrations of LPS, while later, during acute phase, it may cause suppression of LPS-dependent effects (Schumann et al. 1990). Although it was not monitored in this study, other studies have shown that LBP levels rise dramatically in the course of trauma. Perhaps trauma includes embryonic trauma sustained from maternal exposure to LPS. The rise in LBP levels is caused by transcriptional activation of the LBP gene mediated by IL-1 and IL-6 (alone or synergistically). In fact, an i.p. injection of high concentrations of LBP reduced the in vivo cytokine production induced by LPS (Lamping et al. 1998). Whether or not this explains the lower serum levels of TNF-α, IL-1ß, and IL-6 in the pups exposed, maternally and post-natally, to LPS remains unclear. Many more studies are necessary to elucidate the complexities of LPS mediated pathways.

Aside from inducing an inflammatory response, maternal exposure to LPS is known to result in changes such as; fever, in utero hypoxia, hypotension, and oxidative stress in both the mother and fetus (Cambonie et al. 2004; Coumans et al. 2005; Dalitz and Harding). In our study, as well as others (Menon et al. 2006; Xu et al. 2006), we observed preterm birth and growth retardation in pups maternally exposed to LPS as well (results not shown). Therefore, the deleterious effects of LPS on the mother and fetus may also be responsible to the L + L treated pup's lessened inflammatory response.

Despite the consistently lower birth weights found in offspring from LPS-treated dams, these pups followed the same postnatal growth trend as the offspring from saline-

treated dams. By visual inspection, the amount of milk in the stomach of the pups born to LPS-treated dams was similar to that in the pups born to saline-treated dams. For these reason, we do not suspect LPS-treated dams were any less nurturing than the saline-treated dams to their offspring. To prevent complications in differences in maternal fostering, all litters were culled to 10 pups when necessary.

Previous research has proven myeloperoxidase (MPO) to be a valuable marker of inflammatory cell infiltration in the brain (Barone et al. 1991; Jiang et al. 1995). Our preliminary MPO immunohistostaining data indicated fewer PMNLs in the brains of the L + L treated pups compared to the S + L treated pups. It has been implied that IL-1β expression in the brain is associated with neutrophil recruitment, while TNF-α expression is related to the recruitment of mononuclear cells (Blond et al. 2002). It is interesting to note that the elevated mRNA levels of IL-1β and TNF-α in the brains of S+L treated pups correlate with the stronger MPO signal seen by immunostaining. While the decreased mRNA levels of IL-1β and TNF-α in the brains of L + L treated pups, correlate with the weaker/fewer MPO signal found in the coronal sections of brain. More extensive immunohistochemistry studies need to be performed to confirm this preliminary finding. Additional and improved photomicrography is required for better analysis of MPO immunoreactivity in order to adequately distinguish MPO signal from the background.

Our result suggests that maternal immune challenge on day 18 of pregnancy represents a critical period that strongly influences the offspring's immune response. It is known that maternal infection affects offspring differently depending on the on the gestational stage at which the immune agent was administered (Meyer et al. 2005)(Fortier, Luheshi and Boksa 2007). For example, infections in pregnant woman

26

specifically during their 2nd trimester leads to increased risk of schizophrenia, and other neurodevelopmental disorders in humans (Brown et al. 2000; O'Callaghan et al. 1994; Suviasaari et al. 1999). Day 18 of pregnancy in the rat falls between days 12-22, which represent the general equivalent to the 2nd human trimester, based on criteria such as neurotransmitter expression, synaptogenesis, and CNS electrical activity (romijin, Hofman and Gramsbergen 1991). The attenuated serum and mRNA levels of pro-inflammatory cytokines found in the blood and brains of L + L treated pups indicate that this period in gestation affects the offspring's inflammatory response if challenged to a maternal immune stimulus. Whether or not the vulnerability of this period of pregnancy is due to the variation in the maternal immune response during gestation, the neurodevelopmental stage of the fetus, or even a conjunction of both, is something that remains to be discovered (Fortier et al. 2004).

In conclusion, our results indicated that maternal exposure to LPS on day 18 of pregnancy significantly attenuates the production of pro-inflammatory cytokines, IL-1ß, IL-6, and TNF-α in the blood, as well as notably reducing the induction of mRNA levels of TNF-α, IL-1ß, and IL-6 in the brain of pups challenged on P21 with LPS as compared to pups born to saline-treated dams or LPS-treated dams who were challenged on P21 with saline. Through the use of immunohistochemistry, our preliminary data imply that there might be decreased recruitment of immune cells in the brains of pups maternally exposed and subsequently challenged to LPS, compared to the brains of pups born to saline-treated dams. It is clear that systemically administered LPS induces complex biochemical alterations and inflammation in the adult CNS, yet as our data shows, much remains unknown of the response in the immature brain.

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