MATERNAL IMMUNE ACTIVATION:

EVALUATING IMMUNE RESPONSES TO LIPOPOLYSACCHARIDE

IN PREGNANT RATS, THEIR FETUSES,

AND NON-PREGNANT FEMALE COUNTERPARTS

by

Elina Rodriguez

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Masters of Science in Neuroscience

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ABSTRACT

In this project, we examined the innate immune response involving the production and expression of immune-related genes and proteins, specifically for cytokines, that were induced using a model of Maternal Immune Activation (MIA). Correlations between these factors were examined in dam and offspring, as well as comparisons between non-pregnant and pregnant female rats serve as the focus of this research. All female rats received injections of LPS (i.p., 50μg/ml/kg) or sterile saline, with emphasis on E(15) injections for pregnant dams. At either 2, 4, or 24 hours post-injection, brain and peripheral tissues were collected from female rats and fetuses. Quantitative, real-time polymerase chain reaction (qRT-PCR) was used to examine differences in gene expression between LPS and saline groups, pregnant and non-pregnant rats across the various tissues. Pearson correlations were run as well, examining potential correlations in immune-related gene expression between dams and their litter (1 male, 1 female per litter). Fetal sex was evaluated in correlations to observe any sex-specific correlations in immune responses between dam and fetus.

Chapter 1

BACKGROUND

The connection between the immune system and brain development and function is the foundation of Dr. Jaclyn Schwarz' lab at the University of Delaware. When understanding the context and causes of neurodevelopmental disorders, much attention has been directed to postnatal influences, yet there is evidence to suggest the gestational period is especially vulnerable. In one hospital study, Swedish researchers found that patient diagnosis of infection during pregnancy had a 30% increased risk of ASD diagnosis in offspring (Lee, 2014). With the prevalence of neurodevelopmental disorders being over 10% of children affected in the United States alone, it is important to consider all forms of possible exposures and the age of fetal development when determining one's relative risk of developing neurodevelopmental disorders. Moreover, there is a unique sensitivity in males compared to females, such that males are more likely to be diagnosed with many neurodevelopmental disorders such as autism, schizophrenia, and general learning disabilities than females (Boska, 2010). These can likely be attributed to differential sensitivity during gestation, when the brain begins the process of sexual differentiation, and is thus undergoing different developmental processes that are sex-dependent (Nelson, 2017). General approaches to understanding the cause of psychopathology often look to specific genes that can be inherited by offspring; however very few genes explicitly predict the risk of any one neurodevelopmental disorder. Thus, it is further suspected that certain environmental exposures during development exacerbate the risk and predisposition for the onset of neurodevelopmental disorders. As such, it is especially important to understand the role of the environment, particularly the embryonic environment, as a conduit to neural dysfunction which carries on to postnatal development and adulthood.

There is particular interest, more recently, in the immune system and its function as it relates to the underlying cause of various psychological pathologies. Increased inflammation in the periphery and brain is associated with major depressive disorder, schizophrenia, and anxiety (Yuan, 2019). The increased risk of early-life neurodevelopmental disorders is thought to result from early-life immune dysfunction or immune activation, whose sources can derive from environmental toxins, viruses, and bacteria. Neurons are very sensitive to immune molecules, affecting domains such as learning and memory, sociability, and other behaviors (Trofimov, 2017). Even more, the field of psychoneuroimmunology has proposed a working hypothesis whereby immune activation early in life can alter the nervous system semi-permanently through alterations in microglial function, neuronal function, and life-long gene expression (Wang, 2013).

As the field has developed various rodent models of neurodevelopmental disorders, there has been a particular focus on perinatal immune activation as a risk factor for maladaptive behavior in rodents. Behavioral abnormalities observed through these models may not be congruent with disorders outlined in the DSM-V but can serve as mirrors into the expression of adverse human behavior. As mentioned before, there is an entire perspective left unexplored, an incubation period during which the nervous system and immune system alike are both still developing during prenatal gestation. Maternal immune activation or MIA models focus on this period of prenatal gestation by intentionally activating the mother's immune system during late pregnancy to examine the potential adverse effects in offspring (Estes, 2016). In this study, we utilized a maternal immune challenge of lipopolysaccharide (LPS) – a gram-negative cell-wall component of *E.coli* bacteria – that was administered to pregnant rats on embryonic day 15 (E15). This type of immune challenge is ideal because the dosage can be controlled, and it doesn't result in overt, long-term infection like the bacteria itself would (Bordeleau, 2020). Moreover, there are many aspects of the immune system that are similar in both rodents and humans, as activation of the immune system induces a well-conserved concert of immune molecules and cellular functions (Bruce, 2019). Thus, utilizing rodent models of MIA allows for a controlled experimental design that allows us to explore the link between immune activation and the vulnerability to neurodevelopmental disorders.

Pregnancy itself is a unique period of the lifespan, associated with robust changes in maternal physiology. In order to prevent fetal rejection, immune function in pregnant humans and rats alike is significantly diminished (Kareva, 2020). While this altered immune function, which can be referred to as "immunosuppression", is important for maintenance of the pregnancy; it is also the case that pregnancy-induced immunosuppression generates an enhanced vulnerability to infections, particularly in later phases of pregnancy (Abu-Raya, 2020). For this reason, it is imperative that we understand pregnancy itself as an important factor in models of MIA. Surprisingly, the models of MIA assume immune activation in pregnant females is similar to that of non-pregnant females or even males; and this is not true. Moreover, the position current MIA research takes is that the diffusion of maternal-derived immune molecules affects the developing fetus, it is important to consider the impact pregnancy can have on immune responses. Considering and measuring the immune-response within a cohort of non-pregnant rats is especially important in this case. Pregnancy already alters maternal immune function. A partially compromised system therefore would be assumed to generate a unique response compared to non-pregnant rats.

Moreover, our lab contends that the shared blood flow and placental tissue are potential ways in which immune activation in pregnant females can be transferred, mirrored, or even enhanced in the offspring, thereby having access to the fetus and the fetal brain. Though blood supply is shared between the fetus and mother, the placenta acts as a barrier in many ways, but it also has its own immune cells (Faas, 2018). Virtually no studies have quantified the relationship between immune-related gene expression in the fetuses, the placentae, and the pregnant moms. This then presents uncertainty in claiming the maternally-derived immune molecules as a *sole* factor in the etiology of developmental disorders in the offspring. Moreover, there may be individual differences in the mother or her offspring (e.g. genetic or otherwise) that have a role in susceptibility to potential immune dysregulation and later neurological consequences. Thus, we are interested in exploring immune activation in dams, and within each placenta and into the fetus to see how these responses either correlate within litters or possibly different across each fetus. As mentioned earlier, the realm of psychopathology related to sickness during gestation is particularly impactful on males, with higher rates of diagnosis for many neurodevelopmental disorders compared to females. Thus when exploring immune action *within* litters, we also need to be considerate of the sex-dependent relationships that might help explain this phenomenon.

Following immune activation, the release of immune-related molecules called cytokines can help modulate the severity and duration of the infection in the brain and periphery. In the periphery, these molecules are released from a variety of immune cells including macrophages, white blood cells (leukocytes or eosinophils), and T cells; whereas in the brain they are produced and released largely by microglia, the resident macrophages of the brain. Cytokines are necessary components of the immune response because of their modulatory role in communication with neurons in the brain and with other immune cells in the periphery (Ramesh, 2013). There are two classes of these cytokines; pro-inflammatory and anti-inflammatory. Pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and IL-6, work towards upregulation of immune reactions including increased proliferation, activation, targeting, and differentiation of immune cells (Zhang, 2007).

These cytokines hold very vast and unique roles in the overall health of individuals. IL-6 expression is used in clinical capacities as a marker for acute and chronic inflammation (Gabay, 2006). Enhanced IL-6 expression has been linked to mood disorders, particularly depression. In humans, a positive correlation was found between IL-6 levels in serum at age 9 to mood, concentration difficulties, fatigue and sleep disturbances at age 18 (Khandaker, 2014). Knockout IL-6 mice were observed to be resistant to stress-inducing situations, such as excessive darkness cycles (Monje, 2011). The M1 form of microglia, which is considered to be the pro-inflammatory type, is linked to IL-1β release (as well as IL-6 release) (Cai, 2022). IL-1β during immune responses also has implications in modulating the activity of other glial cells (astrocytes) or interneurons, which favor prolonged immune activity in ways of potentiating NMDA receptors or activating astrocytes (Gajtkó, 2020). Excessive IL-1β production in the hippocampus is associated with immature neuron formation and altered functionality of hippocampal cells leading to lapses in memory formation later in life (Wu, 2013).

In contrast, anti-inflammatory cytokines, such as IL-10, work to suppress and tightly regulate immune responses in order to prevent significant tissue damage IL-10's effects can mostly be seen during the *resolution phase* of an immune response (Serhan, 2007). Unregulated proinflammatory cytokine expression can result in cell-death during immune responses, so increasing levels of IL-10 is necessary to mitigate severity of apoptosis following immune activation (Iyer, 2012).

Concurrent expression of these cytokines across time is what dictates the innate immune response, which can be unique depending upon the immune challenge (viral vs. bacterial), the age of the individual, the sex of the individual, and the severity of challenge (Hall, 2023). These dynamics of pro- and anti-inflammatory molecules also fluctuate as functions of time, as the immune system continuously attempts to regulate itself to reach homeostatic levels again following the initial immune challenge.

The profile of certain cytokines can undergo changes during pregnancy. For instance, some studies found reduced IL-6 expression throughout pregnancy with enhanced expression nearing partition (Vilotić, 2022). IL-6 can serve as a biomarker for diseases during pregnancy. Gestational diabetes is linked to enhanced IL-6 expression found in maternal serum. Excessive IL-6 found in amniotic fluid has been associated with Chorioamnionitis, which is an infection or inflammation of the placenta or amniotic fluid (Amirian, 2020). It is to say at minimum that IL-6 activity and functionality is not confined to introduction to pathogens, but rather is currently playing adaptive roles during gestation. Non-pregnant and male rats do not experience these pregnancy-induced changes in immune function, and could be considered or compared against when implementing MIA models to better understand how the immune system changes during pregnancy.

The immune response is a self-regulating system that is meant to be robust enough to fight infection, but not over-activated or prolonged. Various immune-related genes and proteins can exist in different compositions throughout the duration of a response. This composition and duration in the expression of immune related molecules can be tissue specific, such that immune molecules derived from the adult, pregnant dam may not be similarly represented in the shared placental or immature fetal compartments. Yet at the same token, the shared circulatory system between mother and offspring can facilitate the migration of immune-generated cytokines into these tissues (Dawe, 2007). If the innate immune system on the maternal end is suppressed as a function of pregnancy, we expect this would influence the level and duration of cytokine transport through shared compartments to some extent. Furthermore, as the maternal immune molecules interact with other immune cells in the placenta or microglia in the fetal brain this can subsequently impact the expression of immune molecules directly in those tissues. Neurons, especially developing neurons, are exquisitely sensitive to these immune molecules and microglial activation (Bilbo, 2012), and thus MIA can impact the development of neural circuits and synaptic pruning, a process that also relies on microglia, in the developing fetal brain (Coiro, 2015). With these neural consequences, long-term neuronal abnormalities can persist and manifest into behavioral and cognitive deficits later in life.

Though we have an understanding of the variables at play during immune activation, and their modulation of neuronal function, the specific expression of these immune molecules, between both mother and offspring will help reveal a better understanding of the process by which MIA impacts the developing fetal brain and may identify additional risk factors in the genesis of neurodevelopmental disorders.In addition, we need to understand in what ways does pregnancy itself change the immunological makeup of women, and further how this translates following infection.

The current experiments utilized a model of maternal immune activation which introduces an immune challenge of lipopolysaccharide (LPS) – a gram-negative cell-wall component of *E.coli* bacteria – to pregnant rats on embryonic day 15 (E15). This agent is a toll-like receptor (TLR)-4 agonist, and has pronounced effects on the immune system (Bucknor, 2022). LPS usage mimics bacterial infection responses, typically marked by enhanced expression of cytokines such as IL-1β and IL-6 (Bucknor, 2022). Additionally, a cohort of non-pregnant female rats was also injected with LPS, which allowed us to better understand the impact that pregnancy has on the immune response. Peripheral and neural tissues were collected from fetuses, placentae, and dams, in addition to the non-pregnant females at 2, 4, or 24 hours post-LPS administration. The goal of these experiments was to understand the immune response, across the maternal-fetal interface, and to identify the various immune factors that could potentially disrupt neuronal function in the fetus, with a special emphasis on the maladaptive roles of cytokine expression at critical points during development.

In other MIA paradigms, consequences are often observed in post-natal days following injection, with emphasis on social behaviors and microglial form (Bucknor, 2022). However, the immune consequences of MIA are rarely observed within the initial onset following injection. Moreso, some models may only observe one time point, which does not properly represent the cycle of immune responses (Baines, 2020). Utilizing various time points across experimental groups, including 2hr, 4hr, and 24hr post-injection, a timeline for cytokine expression at the transcriptional level can be extrapolated. As mentioned previously, the immune response is dynamic, and expressions of different genes can vary over time, and in relation to each other. We can also use timelines to build correlations between mothers and their litters in regards to immune-related gene expression across time.

Two hypotheses can be derived from our primary research goals. One is that pregnancy induces significant immunosuppression such that pregnant dams would have significantly attenuated cytokine expression across various tissues compared to non-pregnant females following LPS-induced immune activation. When observing cytokine expression between dams and non-pregnant females, dams should have a significantly lower amount of cytokines in response to immune activation. Our second hypothesis is that correlations between dams and their offspring should persist due to placental diffusion of cytokines. Specifically, we expect the levels of pro-inflammatory cytokines to correlate positively across maternal tissues and fetal, which defends the ongoing theory that diffusion of these cytokines between mother and offspring generate the maladaptive neuronal alterations leading to later NDD diagnosis. These correlations should mainly be driven by males, given their increased likelihood of NDD diagnosis later in life.

Maternal health and physiology in general are glossed over when considering risk factors for fetal health. As result, current scopes of treatment for pathologies like NDDs often direct itself on postnatal risks. If risk factors can be introduced as early as prenatal development, interventions and greater emphasis should be placed on maternal health overall. Our goal is to increase our understanding of factors involved in maternal immune activation and its contribution to behavioral and cognitive abnormalities in animal models can be translated to humans. These findings may one day help to mitigate or intervene in these at-risk pregnancies associated with infection, immune activation, or dysregulation by potentially reducing specific cytokine enhanced expression and mitigating the cascade effects on developing neurons that might increase the risk of neurodevelopmental disorders in these affected offspring.

Chapter 2

METHODS

Seventy-four adult female rats (36 in the non-pregnant group and 38 in the pregnant group), ordered from Charles River Laboratories (Wilmington, MA) were utilized for the duration of the experiment. At the University of Delaware, male and female rats were paired together for breeding for no more than five days. Embryonic day one (E1) was determined when a sperm plug was present in the cage, upon which female rats were separated and housed individually. Based on E1, we calculated the anticipated date of birth, (P)0, and E15 for treatment conditions. Experimental procedures rely on the precise timing of both P0 and E15. Cage changes occurred on days not overlapping with E15 treatment (E9 and E13 for example) to prevent presence of internal stressors or anxiety. Adult female rats that were pair-housed and never bred were assigned to the non-pregnant group. Female rats that were bred but did not become pregnant were also categorized as non-pregnant rats, and were housed individually.

Under standard laboratory conditions, where temperatures did not exceed 22 °C, rats were housed in clear, polyethylene cages (45 cm × 20.5 cm × 24 cm). Each cage was provided with a tunnel, crinkle paper for nesting, a popsicle stick, and a nesting square. Standard laboratory food and tap water were available *ad libitum*. Colony rooms were maintained on a 12-hour light, 12 hour dark cycle, with the light cycle beginning at 7:00 am. All experimental procedures outlined in the document were performed during the established light cycle. Sentinel rats were housed in the colony room, examined for rat diseases, and held negative results. All experiments were approved in Animal Use Protocol 1388 by the University of Delaware Institutional Animal Care and Use Committee (IACUC).

Sprague-Dawley female rats received an intraperitoneal (i.p.) injection with lipopolysaccharide (50 μg/kg/ml) or saline (1 ml/kg). Pregnant rats were injected on embryonic day (E)15 and non-pregnant rats were injected on concurrent days. A saline-soaked cotton swab was used to perform vaginal swabs on non-pregnant females at the time of injection and time of euthanasia. Cells were transferred onto microscope slides to later determine the phase of estrous. All injections were performed to enter the lower right quadrant of the abdomen, to prevent injection into the internal organs. Syringes were aspirated to ensure no injection had entered organs or blood vessels.

Rats were euthanized at 2, 4, or 24 hours post-injection. Female rats were anesthetized with administration of Barbiturate euthanasia solution via i.p. injection. Euthanasia was not considered complete until failed motor response was observed for a toe-pinch test. Cardiac puncture was performed via insertion of a 26-gauge needle into the left ventricle of the heart to withdraw blood, performed first to collect a serum sample from every female.

Rats were perfused with saline (0.9%) to flush out peripheral immune cells from female rats before tissue collection. In pregnant dams, uterine horns were removed prior to perfusion and were soaked in phosphate-buffered saline (PBS) solution three times to ensure the removal of excess blood and cytokines from maternal circulation. Hypothalamus samples were derived via dissection from one half of the brain and spleen samples were collected from medial regions of the organ. Fetal tissue; such as amniotic fluid, brains, and placenta were derived from the dams uterine horn. Fetal whole brains were excised precisely under a dissecting microscope (Swift S41-20). Following tissue collection, all samples were placed on dry ice to maintain tissue integrity. At the close of the procedure, samples were transferred and stored in a -80°C freezer for future processing and analysis.

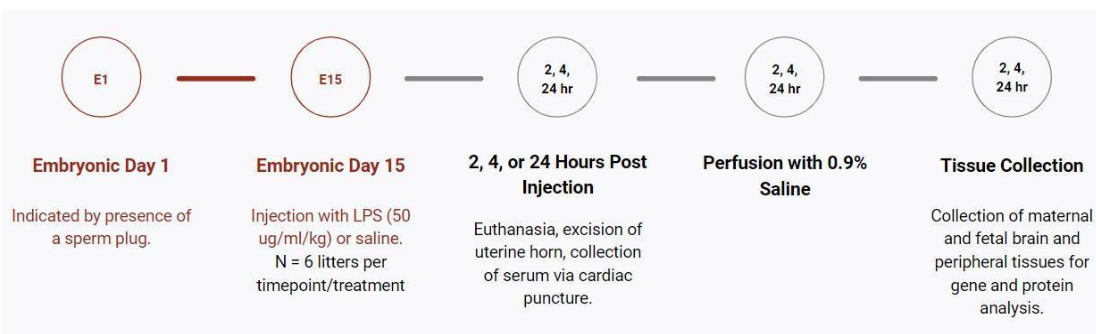

Figure 1: Timeline of experimental procedures done on animals starting at embryonic day 1.

RNA was extracted from tissues from both fetal and adult female specimen; including hypothalamus, spleen, fetal brain, and fetal placenta by using Isol-RNA

Lysis Reagent (Cat. No. 15596018). In a 1:8 ratio, tissues were homogenized with Isol-RNA Reagent with a Biospec Tissue Tearor. Cross-contamination was controlled with a series of deionized water rinses between sample homogenization. RNA pellets derived from the aqueous phase of samples were washed twice with 75% ethanol and dried until physically translucent. Reconstitution of RNA pellets were contingent on pellet size; with most samples reconstituted in 8μl of RNase-free water. In cases where pellets were not observable to researchers, tubes were reconstituted with 6μl of RNase-free water. Samples were stored in a -80°C freezer until they were thawed to determine the RNA concentration per sample. All samples were analyzed for RNA concentration under a Nanodrop (Thermo Fisher Scientific). Individual samples were further diluted with RNase-free water to ensure a final RNA concentration ranging from 750 to 1000 ng/μl.

To determine the sex of fetal samplesfor sex-based statistical analysis, fetal tail DNA was extracted. The Qiagen QIAamp Mini Kit was used (Cat. No. 51304) to first lyse samples with proteinase K enzyme and subsequently rinse cellular products with a series of wash buffers to isolate DNA from cells. As with the other samples, DNA concentrations were determined with use of a Nanodrop. All DNA extracts were standardized to be equivalent to the sample with the lowest concentration extracted initially, via dilution with RNAse-free water.

Complementary DNA (cDNA) synthesis was performed using the QuantiTect Reverse Transcription kit (Qiagen, Cat. No. 205314). Extracted RNA was thawed and subjected to a genomic DNA elimination reaction. cDNA was made using this extracted RNA as a template in a reverse-transcription reaction. The resulting cDNA aliquots were stored at -20°C for subsequent PCR reactions.

Using the PerfeCTa SYBR FastMix kit (QuantaBio, Cat. No.101414-272) in 10µl reactions on a CFX96 TOUCH real time PCR machine, relative gene expression was measured. 18S, IL-6, IL-1β, IL-10, and TLR4 were run in all samples. Additional genes were run for the fetal brain (RPLP1, GAPDH). IL-6, RPLP1, were analyzed using a QuantiTect® Primer Assay (IL-6: QT00182896, RPLP1: QT00365561) and diluted according to protocol. All other primers were ordered through Integrated DNA

Technologies and diluted to a final concentration of 0.13 µM (IL-1β, 18S, IL-10, GAPDH; see **Table 1** for a list of primer sequences). Hypothalamus and spleen utilized 18S as the housekeeping gene, which did not significantly differ between groups. For the fetal whole brain, we tried several housekeeping genes (18S, RPLP1, and GAPDH) that showed variability and instability in expression within and between groups, so instead geometric mean between these three genes was used, which did not differ significantly between E15 groups, timepoint, nor sex.

Table 1: Rat primers used for quantitative real-time PCR.

Gene of Interest	**Primer Sequences**
18s	Forward: ATGGTAGTCGCCGTGCCTA Reverse: CTGCTGCCTTCCTTGGATG
GAPDH	Forward: GTTTGTGATGGGTGTGAACC Reverse: TCTTCTGAGTGGCAGTGATG
IL-1β	Forward: GAAGTCAAGACCAAAGTGG Reverse: TGAAGTCAACTATGTCCCG
IL-10	Forward: TAAGGGTTACTTGGGTTGCC Reverse: TATCCAGAGGGTCTTCAGC
BDNF	Forward: ATCCCATGGGTTACACGAAGGAAG Reverse: AGTAAGGGCCCGAACATACGATTG
IL-6	Forward: GACAACTTTGGCATTGTGG Reverse: ATGCAGGGATGATGTTCTG

SRY	Forward: GGATGACTGTACGAAAGCCACAC Reverse: TTTGTCCAGTGGCTGTAGCGGT

Samples were numbered, blinded to group, and run in triplicate on real-time PCR plates. For each reaction, the average quantitative threshold amplification cycle number (Cq) value was determined from each triplicate, and the 2-ΔΔCq method was used to calculate the relative gene expression for each gene of interest relative to a reference sample. The sample with the highest ΔCq (housekeeping Cq minus gene of interest Cq) was chosen as the reference sample for each target gene.

To determine the sex of fetuses according to their expression of the male-specific SRY gene in tail samples, qRT-PCR was performed according to the procedure described above. RPLP1 was utilized as the housekeeping gene and did not significantly differ between E15 groups, timepoint, nor sex. RPLP1 was analyzed using a QuantiTect® Primer Assay (RPLP1: QT00365561), and the SRY primer was ordered from Integrated DNA Technologies and diluted to a final concentration of 0.13 µM (see **Table 1** for a list of primer sequences). As described above, the 2-ΔΔCq method was used to calculate the relative gene expression for the gene of interest relative to a reference sample. Results that were under 5 for the relative-fold gene expression of Sry indicated the fetus was female, whereas results greater than 400 indicated the fetus was male. One male and one female fetus was selected from each litter for further analysis of gene expression in their brains and placentas, using RNA extraction and PCR as described above.

Following the collection of maternal serum and amniotic fluid, ELISAs were run to measure IL-6 protein expression for pregnant dams and their litter. No other gene was analyzed using ELISAs in this experiment.

All statistical analyses were performed on IBM® SPSS® Statistics 29. For pregnant versus non-pregnant comparisons, a three-way ANOVA was used to measure significant differences in relative gene expression for each gene of interest with immune activation treatment (LPS vs. saline), time point of injection (2, 4, or 24 hours), and pregnancy status (pregnant vs. non-pregnant) as between-subject factors in the analysis.

A three-way ANOVA was used to measure significant differences in relative gene expression for each gene of interest in all fetal tissue type collected, with E15 condition (LPS vs. saline), time point of injection (2, 4, or 24 hours), and sex (male vs female) as between-subject factors in the analysis. In the context of this experiment, we will not be examining these differences. Rather, we collect data regarding each tissue's immune-related gene expression for future correlational analysis. This experiment does not aim to use fetal tissue samples or maternal tissue to examine sex-based, E15 treatment, or timepoint factors in immune responses.

Using VassarStats, correlation regression analysis was performed between subsections of maternal and fetal tissue. We examined maternal serum levels of IL-6 protein expression and compared against IL-6 expression levels in amniotic fluid. We also performed correlation analysis between IL-6 maternal serum levels and IL-6 and IL-1β gene expression in fetal placenta and brain. Inter-fetal tissues were correlated as well, looking at cytokine expressions within one tissue type (IL-6 and IL-1β in placenta, IL-6 and IL-1β in fetal brain). Outliers were determined using Graphpad, which determines outliers using the Grubbs test for outliers. Pups were collapsed across sex and examined by per condition group (2hr LPS, 2hr Saline, etc.). In our data sheets, outliers were also examined within sex as well; however, we did not remove more than one statistical outlier from any individual group. To correct for the multiple comparisons associated with the various correlations across tissues, p-values were adjusted to $p \leq 0.005$.

Chapter 3

RESULTS

3.1 Female Spleen

IL-6. ANOVA revealed significant main effects of immune activation [$F(1,62) = 13.92$, $p < .001$, $\eta_p^2 = .183$] such that LPS increased IL-6 expression compared to saline. There was a significant effect of time point [$F(2,62) = 4.149$, $p = .02$, $\eta_p^2 = .118$], such that 4hr treatment groups had increased IL-6 expression compared to 2hr and 24hr. There was also a significant effect of pregnancy [$F(1,62) = 12.557$, $p < .001$, $\eta_p^2 = .168$], whereby IL-6 expression was higher for pregnant females than non-pregnant females. There was a significant 2-way interaction between immune activation and pregnancy condition [$F(1,62) = 10.63$, $p = .002$, $\eta_p^2 = .146$]; see **Figure 2**. Pregnant dams had elevated IL-6 expression in the LPS group compared to pregnant dams that received saline $p < .001$). Furthermore, LPS elevated IL-6 expression for pregnant females significantly more than non-pregnant females ($p < .001$). There was a significant 2-way interaction between time point and immune activation [$F(2,62) = 10.63$, $p = .02$, $\eta_p^2 = .119$]. In the 4hr time point, LPS treated females had elevated IL-6 expression compared to saline treated females ($p <.001$).

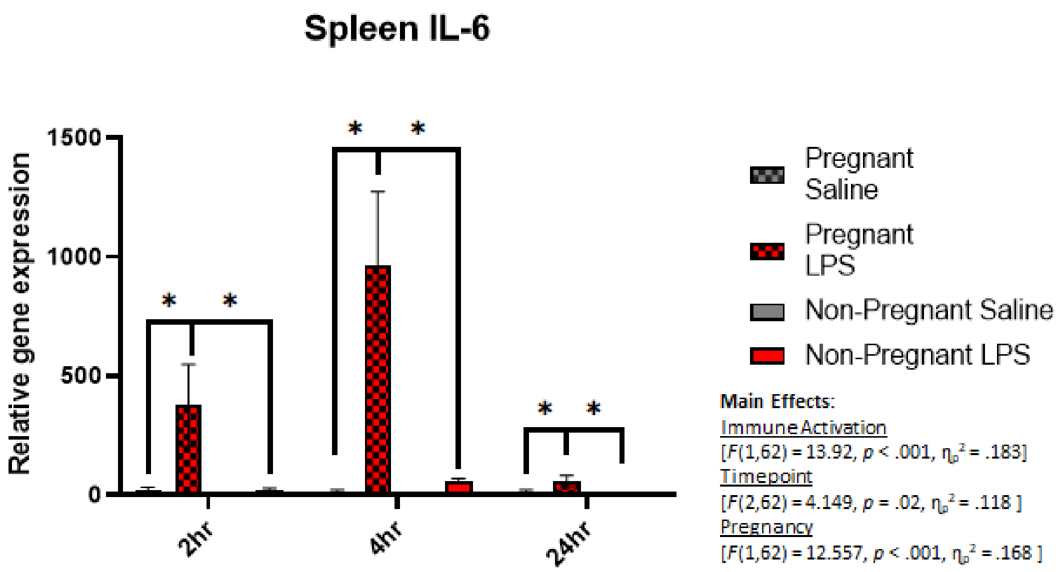

Figure 2: Relative gene expression IL-6 in female rat spleen. *Post-Hoc pairwise comparisons for E15 condition x pregnant interaction, p < .05. Data represented as mean ± SEM. N=5-8/group.

IL-10. See **Figure 3**. ANOVA revealed significant main effects of immune activation [$F(1,60) = 10.11$, $p = .002$, $\eta_p^2 = .144$] such that LPS increased IL-10 expression compared to saline, specifically when comparing 4hr post-injection against 24hr. There was also a main effect of pregnancy [$F(1,60) = 6.26$, $p = .015$, $\eta_p^2 = .094$] such that pregnant females had elevated IL-10 expression compared to non-pregnant females. There was a significant 2-way interaction between immune activation and pregnancy [$F(1,60) = 4.76$, $p = .033$, $\eta_p^2 = .073$]. Dams had elevated IL-10 expression in the LPS group compared to dams that received saline ($p < .001$). Furthermore, LPS elevated IL-10 expression for pregnant females significantly more than non-pregnant females ($p < .001$).

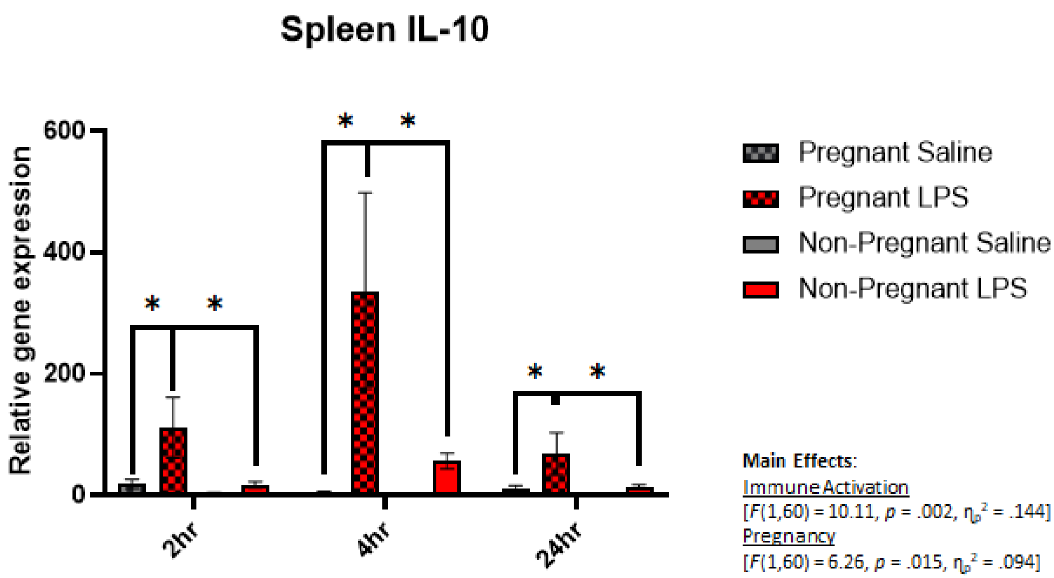

Figure 3: Relative gene expression IL-10 in female rat spleen. *Post-hoc pairwise comparisons for E15 condition x pregnant interactions. Data represented as mean ± SEM. N=5-7/group.

IL-1β. See **Figure 4**. ANOVA revealed significant main effects of immune activation [$F(1,61) = 9.90$, $p = .003$, $\eta_p^2 = .140$] such that LPS increased IL-1β expression compared to saline, regardless of pregnancy condition and time point.

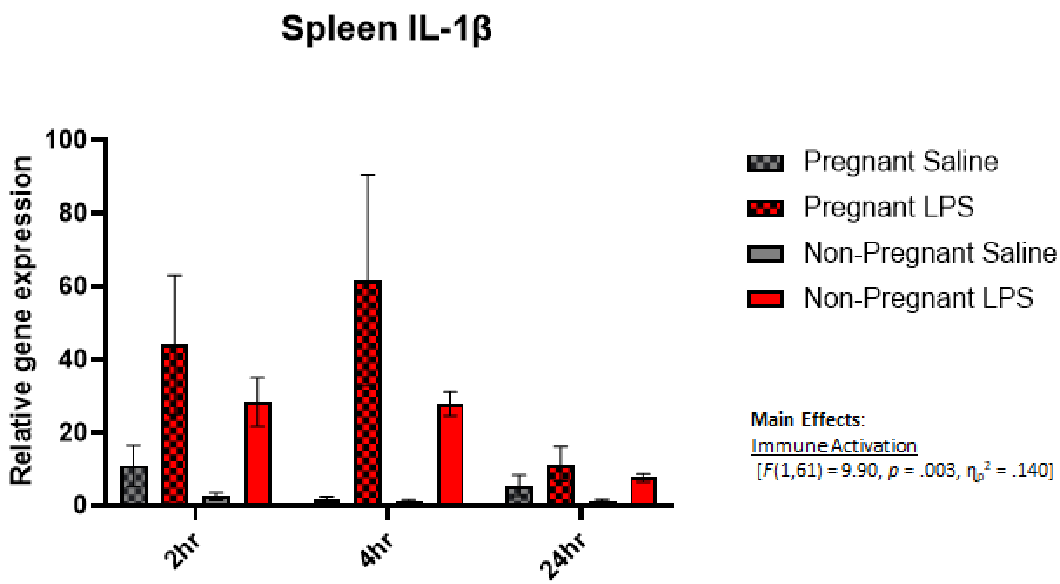

Figure 4 Relative gene expression IL-1β in female rat spleen. *Main effect of immune condition, p < .05. Data represented as mean ± SEM. N=5-8/group.

TLR4. **See Figure 5**. ANOVA revealed significant main effects of pregnancy [$F(1,62) = 6.688$, $p = .012$, $\eta_p^2 = .097$] such that pregnant female rats showed increased TLR4 gene expression compared to non-pregnant rats, regardless of immune treatment or timepoint.

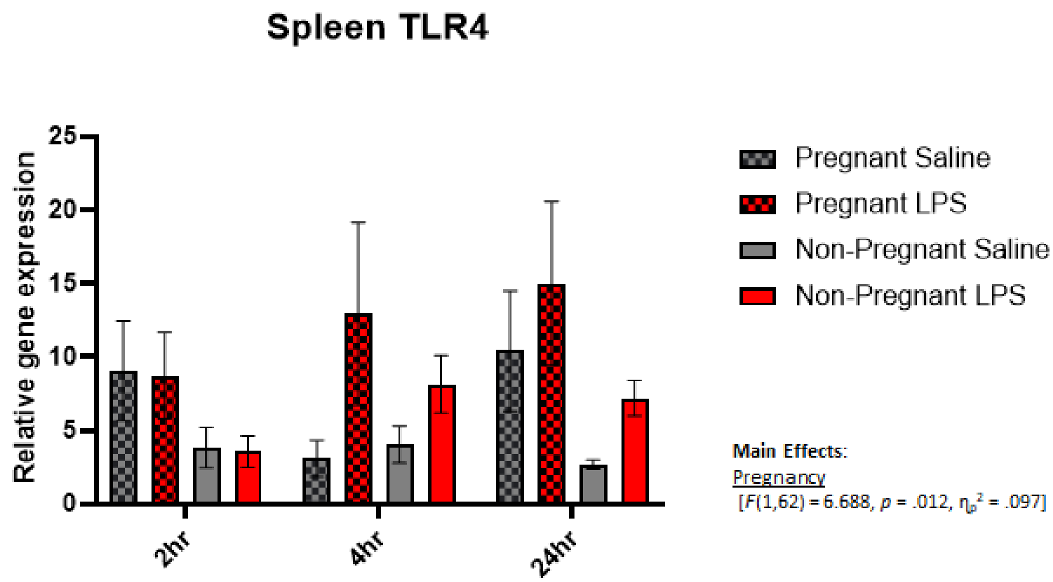

Figure 5: Relative gene expression TLR4 in female rat spleen. Main effect of pregnancy, p < .05. Data represented as mean ± SEM. N=5-7/group.

3.2 Female Hypothalamus

IL-6. ANOVA revealed a significant main effect of immune condition [$F(1,57) = 10.34$, $p = .002$, $\eta_p^2 = .154$] such that LPS increased IL-6 expression compared to saline. There was also a significant effect of time point [$F(2,57) = 3.89$, $p = .026$, $\eta_p^2 = .120$], whereby IL-6 expression was higher at 2hr as compared to 24hr ($p = .022$). Furthermore, there was a significant 2-way interaction between immune condition and timepoint [$F(2,57) = 4.00$, $p = .024$, $\eta_p^2 = .123$]. Females exposed to LPS had increased IL-6 expression compared to those exposed to saline ($p < .001$), regardless of pregnancy condition. Furthermore, LPS elevated IL-6 expression at 2hr compared to 24hr ($p < .001$), but not 4hr post-injection. There was a significant 3-way interaction between immune condition, timepoint, and pregnancy condition [$F(2,57) = 3.29$, $p = .044$, $\eta_p^2 = .104$] on IL-6 expression in the hypothalamus; see **Figure 6**. Pairwise comparisons revealed that for pregnant females, LPS increased IL-6 expression only at 2hr post-injection as compared to saline ($p < .001$). Furthermore, for pregnant females, LPS caused elevated IL-6 expression at 2hr compared to 4hr ($p = .002$) and 24hr ($p < .001$). IL-6 expression at 2hr was significantly higher in pregnant dams versus non-pregnant females (p < .001).

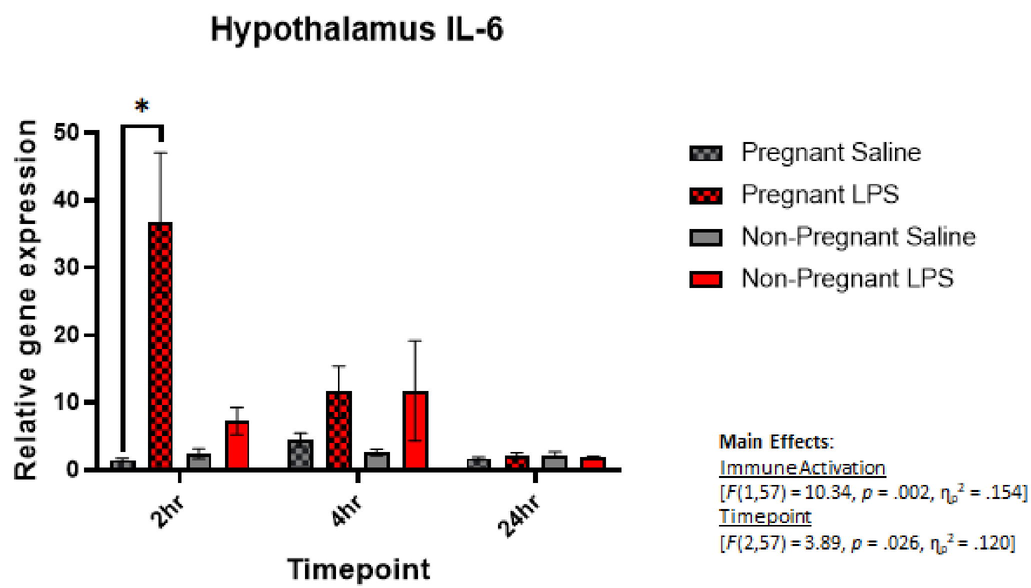

Figure 6: Relative gene expression IL-6 in female rat hypothalamus.*Pairwise comparisons; significant interaction between pregnancy x immune condition x time point. Data represented as mean ± SEM. N=5-8/group.

IL-10. See **Figure 7.** ANOVA revealed significant main effects of immune condition [$F(1,61) = 10.123$, $p = .002$, $\eta_p^2 = .142$] such that LPS-treated females had increased IL-10 expression compared to saline-treated females in hypothalamus. There was a main effect found for timepoint [$F(2,61) = 5.008$, $p = .010$, $\eta_p^2 = .141$], whereby at 24 hours post-injection, IL-10 expression was significantly less than that of 2 hour *(p* = .013) and 4 hour (*p* = .038).

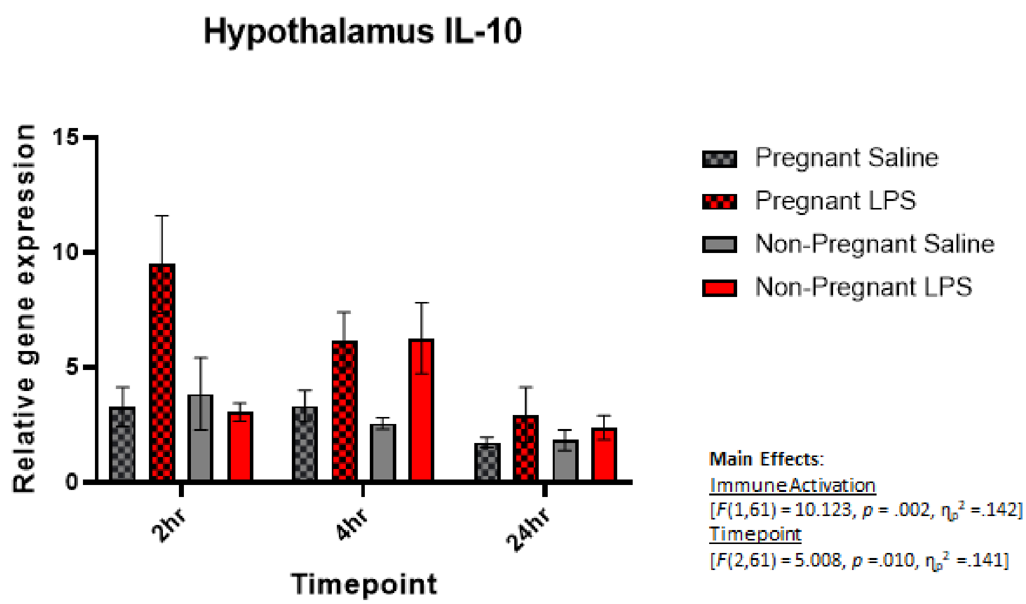

Figure 7: Relative gene expression IL-10 in female rat hypothalamus. Main effect of immune condition, p < .05. Main effect of time point, p < .05.Data represented as mean ± SEM. N=5-8/group.

IL-1β. See **Figure 8.** ANOVA revealed significant main effects of immune condition [$F(1,61) = 16.451$, $p < .001$, $\eta_p^2 = .212$] such that LPS-treated females had increased IL-1β expression compared to saline-treated females in hypothalamus.

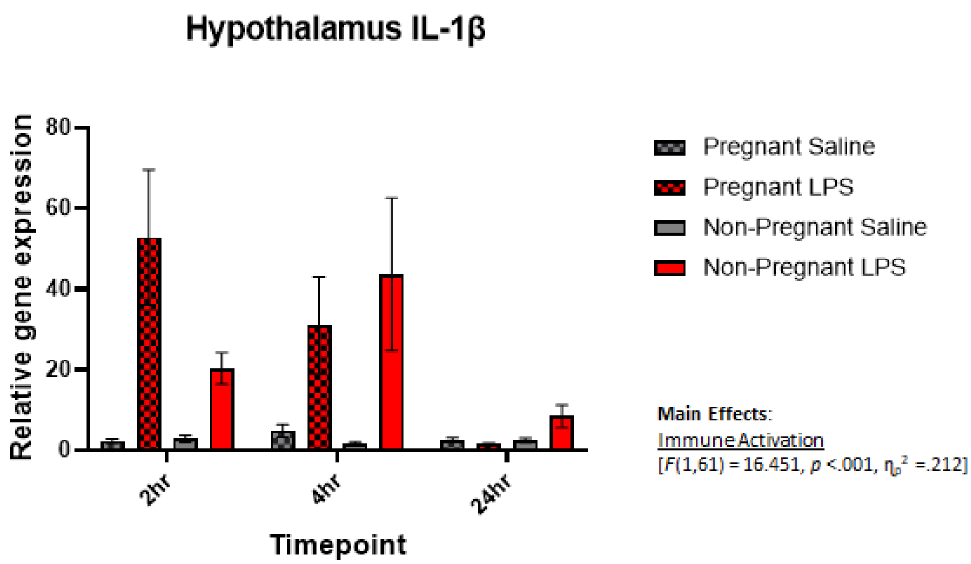

Figure 8: Relative gene expression IL-1β in female rat hypothalamus.Main effect of immune condition, $p < .05$. Data represented as mean ± SEM. N=4-8/group.

TLR4. See **Figure 9**. There was a significant 2-way interaction between time point and immune condition [$F(2,58) = 3.911$, $p = .026$, $\eta_p^2 = .119$]. Pairwise comparisons revealed that 2hr LPS females had elevated levels of TLR4 gene expression compared to 4hr LPS females ($p = .047$).

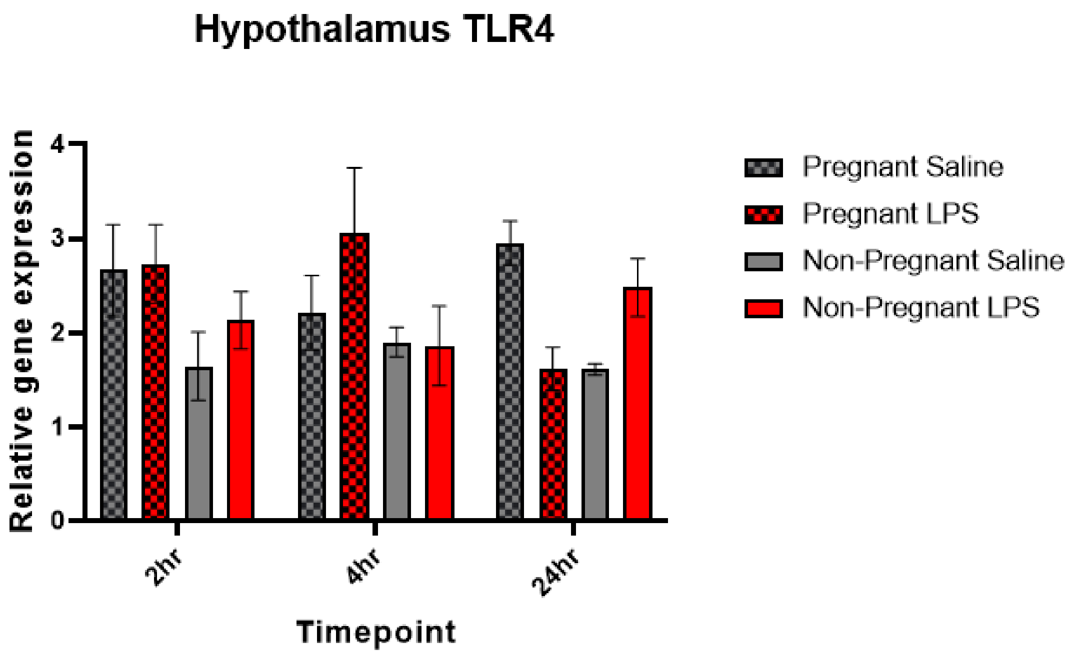

Figure 9: Relative gene expression TLR4 in female rat hypothalamus. Data represented as mean ± SEM. N=4-8/group.

3.3 Maternal and Fetal Cytokine Expression in LPS-treated Dams

ELISAs were run to measure IL-6 protein in amniotic fluid derived from pups and the serum of pregnant female rats. For LPS treated litters, we look for correlations between maternal serum and gene expression in fetal samples including; placenta, amniotic fluid, and fetal brain across the following genes: IL-6 and IL-1β. We also look for correlations between fetal samples for IL-6 and IL-1β, including placenta, fetal brain, and amniotic fluid. The pups and dams collected between timepoints are not the same, thus we did not perform correlations between samples across timepoints. We corrected for multiple comparisons by adjusting the P-value to $p \leq 0.005$ and highlighted those values in **Table 2**, showing all *p* values for fetal x fetal correlations.

Table 2: P values for fetal x fetal correlations. Pink text indicates the p value correlations found within female offspring. Blue text indicates the p value for correlations found within male offspring. Black text represents the p value found for correlations

	IL-6 Placenta (2 µg)	IL-6 Placenta (4 µg)	IL-6 Placenta (24 µg)	IL-1B Placenta (2 µg)	IL-1B Placenta (4 µg)	IL-1B Placenta (24 µg)	IL-6 Fetal Brain (2 µg)	IL-6 Fetal Brain (4 µg)	IL-6 Fetal Brain (24 µg)	IL-1B Fetal Brain (2 µg)	IL-1B Fetal Brain (4 µg)	IL-1B Fetal Brain (24 µg)
IL-6 Amniotic Fluid (2 µg)	.89, .25, .052			.34, .23 , .15			.92, .49, .42			.44, .67, .31		
IL-6 Amniotic Fluid (4 µg)		-, 0.02 , *0.0001			.95, 0.02, .08			.56, .65, .70			.80, .51, .92	
IL-6 Amniotic Fluid (24 µg)			.21, .45, .12			.07, .59 , .08			0.01, .43, .09			0.03, .15, 0.02
IL-6 Fetal Brain (2 µg)	.95, .53, .70			.46, .20, .18								
IL-6 Fetal Brain (4 µg)		.96, .51, .95			.79, .57, .51							
IL-6 Fetal Brain (24 µg)			.53, .17, .47			.08, .40, .67						
IL-1B Fetal Brain (2 µg)	.60, .30, .22			.09, 0.04, 0.0096			.65, .80, .59					
IL-1B Fetal Brain (4 µg)		.53, .43, .11			.18, .63, .17			.27, 0.06, .91				
IL-1B Fetal Brain (24 µg)			.08, .44, .11			.25, .36, .18			.13, 1, .44			

IL-6 Maternal Serum. A significant correlation was found between IL-6 maternal serum and IL-6 pup amniotic fluid at 24 hours following LPS injection (R^2= .435, *p* = .01). This correlation was driven mainly by female offspring (R^2= .656, *p*

=.027). LPS dams with higher IL-6 protein in serum were likely to have female offspring with higher IL-6 gene expression in amniotic fluid. When maternal serum IL-6 was correlated with IL-6 fetal brain gene expression, a significant correlation was detected at 2 hours post LPS injection (R^2 = .35, *p* = .007). At 2 hours, dams with higher IL-6 protein in serum were likely to have offspring with increased IL-6 gene expression in the fetal brain. When correlating IL-6 maternal serum with IL-1β fetal brain expression, a significant negative correlation was found at 24 hours post LPS-injection (R^2 = .834, *p* = .002). This correlation is mainly driven by females at 24hr (R^2 = .33, *p* = .01). Dams who had lower levels of IL-6 protein in their serum had female offspring with higher IL-1β gene expression in the fetal brain.

IL-6 Amniotic Fluid A significant correlation was found at 4 hour post-LPS injection between IL-6 amniotic fluid and IL-6 pup placenta (R^2 = .843, *p* < .001). This correlation was driven mainly by male offspring (R^2 = .878, *p* =.019). Males with greater IL-6 protein in amniotic fluid were likely to have elevated IL-6 gene expression in pup placenta, see **Figure 10**. There was a significant correlation between IL-6 amniotic fluid and IL-1β pup placenta in male offspring at 4 hours post-LPS injection (R^2 = .078, *p* =.017), whereby males with elevated IL-6 protein in amniotic activity were likely to have elevated IL-1β gene expression in pup placenta. A significant correlation was found 24 hour post-LPS injection between IL-6 amniotic fluid and IL-1β gene expression in fetal brain (R^2= .378, *p* = .02). The correlation was mainly driven by females (R^2= .652, *p* = .03). A significant correlation was found 24 hour post-LPS injection between IL-6 amniotic fluid and IL-1β gene expression in fetal brain (R^2= .378, *p* = .02). The correlation was mainly driven by females (R^2= .652, *p* = .03).

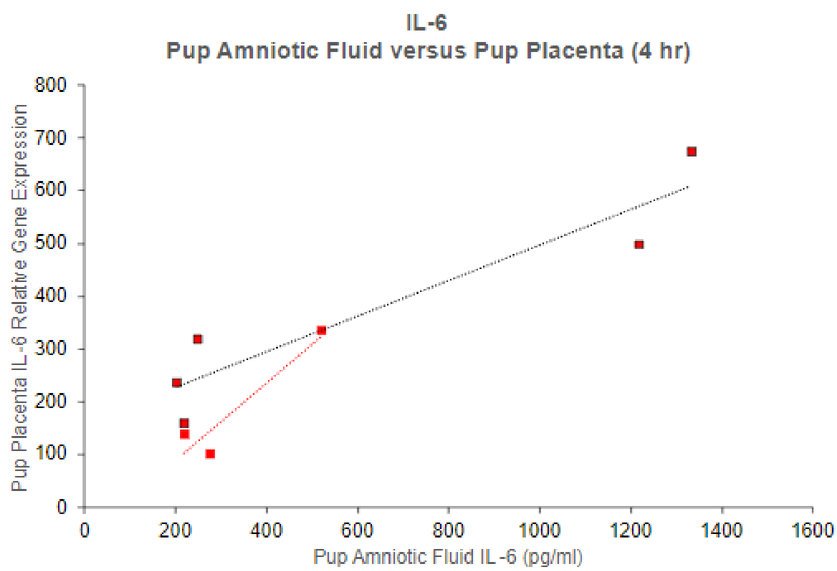

Figure 10: Correlation between IL-6 amniotic fluid and IL-6 pup placenta at 4 hours post LPS-injection. Red trend line shown for female offspring, black trend line shown for male offspring. * p < .001

IL-1β Pup Placenta. A significant correlation was detected when comparing pup placenta IL-1β gene expression to IL-1β gene expression in fetal brain at 2hr following

LPS injection (R^2 = .391, *p* = .01). This correlation was driven by males (R^2 = .597, *p* =.042). When male fetuses expressed low IL-1β gene expression in placenta, they were likely to have enhanced IL-1β gene expression in their brain.

Chapter 4

DISCUSSION

The current experiment examines the consequences of maternal immune activation via two perspectives. First, we sought to understand the inherent difference in immune-related gene expression following immune activation as a function of pregnancy. Second, this experiment aims to determine whether potential individual differences in how a dam responds to an immune challenge are subsequently transmitted to the fetuses and their cytokine expression, or whether the fetuses generate an immune response that is independent and does not correlate with the maternal immune response.

4.1 Adult Female Spleen

First, we analyzed gene expression in the female spleen as a marker of peripheral immune activation. We observed main effects of pregnancy for the expression of IL-10, IL-6, and TLR4. However, pregnant dams expressed significantly higher levels of cytokine mRNA compared to non-pregnant dams. In contrast, there were no significant effects of pregnancy on the expression of IL-1β in the female spleen. Leading up to parturition, there are several cytokines upregulated as the body prepares for the inflammatory event of birth (Jarmund, 2021). Other studies have found that cytokines like IL-6 and IL-1β are upregulated during implantation (Yockey, 2018). However, our current experiment frames itself during the second trimester of pregnancy. We would predict based on previous literature that cytokine expression would be attenuated at this stage of gestation, even in the face of an immune challenge. In contrast, we found that immune activation during pregnancy significantly exacerbates the overall immune response, particularly the expression of IL-6 and IL-10. Interestingly, TLR4 expression revealed no significant interaction of pregnancy and immune condition, rather, it was significantly upregulated in the spleen and the brain by pregnancy alone. Thus, TLR4, which binds LPS may be the starting point whereby immune function is exacerbated. TLR4 is widely expressed in innate immune cells including macrophages, neutrophils and eosinophils of the peripheral immune cells, and cells like the extravillous cytotrophoblasts and hofbauer cells of the placenta (Firmal, 2020). These cells are present in the placenta to aid in buffering against intrauterine infections (Firmal, 2020). As such, TLR4 upregulation can serve as an overall moderator of the immune function and fetal health, regardless of immune activation. LPS and immune activation can perhaps influence the translational aspect of TLR4, which can be explored in future studies.

4.2 Adult Female Hypothalamus

Second, we analyzed gene expression in the female hypothalamus as a marker of central immune activation. Surprisingly, pregnancy did not produce any significant findings across all four genes of interest. Main effects of immune activation were observed, where LPS treated females expressed higher levels of IL-6, IL-10, and IL-1β compared to that of saline treated females. mRNA levels of these cytokines did not differ greatly between dams and their non-pregnant counterparts, unlike the trends observed in female spleen. These findings continue to validate the use of LPS in MIA models, as the immunogen enhances cytokine expression.

It may be the case that pregnancy causes down regulation of cytokines in the brain. There are implications that pregnancy imparts a reduction of density of microglia in maternal brain regions such as the hypothalamus (Haim, 2017). Cytokines like IL-6 upregulate during pregnancy, but closer to parturition in the hypothalamus as well (Haim, 2017). At E15, we are looking at a window into gestation for fetal vulnerability not necessarily maternal. Whereas the maternal immune system may not privy to major changes, outcomes of activation at this point can disrupt fetal neuronal functioning.

It may even be possible by which the sensitivity to pregnancy for cytokines exists beyond the scope of our timeline. In one study, there were interactions between LPS-treatment and pregnancy in relation to pro-inflammatory cytokines, but these were found at different embryonic days of pregnancy (Sherer, 2017) as opposed to distinct timepoints post-injection. Interactions between pregnancy and immune condition were even found on postpartum days (Sherer, 2017). A comprehensive study could utilize both timepoints post-injection and select embryonic days to examine cytokine sensitivity to pregnancy and immune condition.

4.3 Maternal and Fetal Cytokine Correlations

In this study, we examined correlations for cytokines (gene expression or protein) in maternal and fetal tissue. We looked for correlations between maternal serum and fetal samples, as well as within samples of an individual fetus. Significant correlations were found between IL-6 protein in maternal serum and several fetal tissues; including IL-6 in amniotic fluid, IL-6 in fetal brain gene expression, and IL-1β fetal brain expression.

In some cases, like IL-6 amniotic fluid and IL-1β fetal brain expression, these correlations persist 24 hours after LPS administration. Although IL-6 protein in maternal serum resolves itself significantly compared to 2 hour or 4 hours, IL-6 and IL-1β gene expression remains rather elevated in offspring themselves, specifically in females. In human studies, 16-18 weeks in pregnancy, similar trends were observed (Chow, 2008) between maternal serum and amniotic fluid. It may be the case that amniotic fluid can serve as a biomarker for pathology as it consists primarily of fetal waste products (Chow, 2008). Though this may be true, it was unexpected that female offspring would drive this correlation. As mentioned earlier, males are more likely than females to be diagnosed with NDDs (May, 2019). It could then be assumed that male susceptibility could occur as early as gestational age. In mouse MIA models that observe adult behaviors of offspring, males are found to exhibit autism-like behaviors following mid-gestational LPS administration (Xuan, 2014). Perhaps female IL-6 protein and pro-inflammatory gene expression are more likely to be correlated with mom at the same time point (relative to the male pups) due to genetic sex differences in immune function that are the result of having XX vs XY chromosomes (Klein and Schwarz, 2018). This trend was not consistent looking at correlations between fetal tissue as well. Significant correlations were found between IL-6 amniotic fluid and IL-6 pup placenta, and IL-1β in pup placenta that was driven mainly by males. Males who had higher IL-6 protein in amniotic fluid also had elevated cytokine gene expression in pup placenta four hours following injection. Peripheral immune responses in this case have an overall rapid response rate, with the possibility that placental waste generated since injection enters amniotic fluid. This could reflect hypersensitivity or hyperactivity of immune systems within males specifically (Hunter, 2021). This could be one mechanism at which male susceptibility to NDDs occurs, but further examination of glial cell function and state can give greater insights into neuronal activity at this stage.

But the interactions between sex, cytokines, and time points were found to not fully support theories of female immunity. Though peripheral tissues in males are strongly correlated in pro-inflammatory cytokines, amniotic fluid is strongly associated with IL-1β in the female fetal brain. Though IL-6 protein resolves itself greatly at 24 hours compared to 2hr and 4hr, pro-inflammatory gene expression remains high in the fetal brain. This could be a mechanism for another pathology onset, distinct from neurodevelopmental disorders. Studies examining depression in humans for example, found that elevated prenatal IL-6 led to decreased hippocampal activity (Goldstein, 2021). Though we expected only male driven correlations within fetal tissue and between maternal and fetal tissue, findings with females can be used to model disorders where women are more likely to be diagnosed, like mood disorders (Albert, 2015).

4.4 Experimental Limitations

During the tissue collection process, up to 12 fetuses per litter were excised along the uterine horn. Collections began on the left side and transitioned to the right, with a significant amount of time between the first and last collection. It could be argued that right uterine collections may have undergone more tissue degradation during this timelapse, which affect subsequent RNA collection, cDNA synthesis, and PCR protocols. For this reason, to ensure mostly pure and stable tissue, samples collected earlier during the uterine horn extraction (left side of the uterine horn) were used for processing. It however should be noted that the placement of the pup along the uterine horn presents a unique experience of its own. Placements along the uterine horn may have introduced position-based exposure to maternal cytokine circulation, whether left or right or more lateral and medial to maternal midline. Previous studies find that uterine horn placement leads to differences in fetal weight, and fetal growth following exposure to endocrine-disrupting chemicals showed uterine horn effects (D'Errico, 2021) For future examinations, fetal tissue should include that from various positions along with uterine horn with respect to left and right.

In addition, the entire duration of the immune response following activation is not reflected in this experiment. Despite significant findings found at various time points, specifically 4 hours, what occurs between each time point, especially 4 and 24 hours after the immune challenge, is left undetermined. Based on such, we are unable to confidently conclude when immune activation to LPS exposure peaks and/or concludes in our pregnant, non-pregnant, and fetal tissues. There may be different increases and decreases in cytokine expression that change rapidly throughout the 24 hours. As such, including additional timepoints of examination can help clarify any such changes.

Given that the above data is representative of gene expression per gene of interest, it is unknown whether these changes in gene expression are subsequently translated into protein. Protocols such as ELISA provide tangible measures of protein expression in samples, but qRT-PCR only looks at relative gene expression. Measuring mRNA indicates that these cytokines are being expressed, yet we cannot conclude that these genes have translated into protein within both maternal and fetal circulation. Therefore, the relative level and timeline of cytokine expression may be different from its actual protein levels in the tissue. Future studies should examine each molecule of interest at both the protein and gene level to fully understand how they correlate in level and time. It may also be interesting to see differences in these correlations as functions of fetal sex and any other unique circumstances that may have occurred throughout the experiment, for example, in pups that were reabsorbed following immune activation, or the placement of each fetus along the uterine horn.

Individual differences across female rats may contribute to the innate immune response generated following immune activation, and their subsequent effect on the fetuses. These differences may include maternal age at conception, genetic heritability (that is, whether their own mother had unique sensitivities during pregnancy), gut biome (which can modulate immune responses), and stress-induced immune responses (Hall, 2023). Not only one of these factors, but interactions between them, can shape the immune response in an individual, creating a diaspora of various immune responses for the same immune challenge. For future studies, these factors should be measured and controlled for, to look further into different interactions playing into immune responses beyond pregnancy, immune activation, and time point. Alternatively, these various factors that create variability should be embraced in order to recapitulate variability in the human population. Not only should these factors be considered, but larger sample sizes could boost statistical power while considering the effect of these various factors.

When comparing pregnant and non-pregnant rats, we primarily used *active* pregnancy as the sole difference between groups. All of the female rats in this study did not experience prior pregnancies or reproductive efforts outside the scope of this experiment. It would be of interest to explore additional domains to pregnancy status; such as failed pregnancies or previous pregnancies. In this manner we can see whether pregnancies in the past sustain some immunological mark on the female rat, and if MIA models can reveal differences in prior pregnancies versus rats currently pregnant for the first time. Previous studies support that MIA sustains a critical alteration of neural functioning that persists into adulthood for fetuses (Cieślik, 2020), and so perhaps pregnancy overall may similarly sustain an altered immune profile in mothers as well. This is perhaps why risk factors for certain diseases can increase with multiple pregnancies (Dotters-Katz, 2015).

There are limitations present in the aim to compare pregnant and non-pregnant female rats following immune activation. We collected vaginal swabs from all non-pregnant rats rat the time of LPS injection and just prior to tissue collection. However, the results and statistical analysis did not take into account the phase of estrous each non-pregnant female was in at the time of injection or collection. Therefore, within-group differences could be further explored with respect to the estrous cycle. Differential expression and type of cells present during different estrous phases within non-pregnant rats can be somewhat related to the immune response observed.

Our experimental approach also used a number of correlations across various tissues, as such, p values were adjusted for multiple comparisons. That said, these data might be better analyzed with a more sophisticated regression analysis like Hierarchical Linear Modeling, which can be done in the future. Hierarchical analysis (HLM) - linear mixed effects analysis would consider litter as a factor, the maternal gene expression is not independent across the male and female in each litter. Thus with HLM modeling, perhaps additional significant correlations or trends in the data could be explored between dams and their pups.

4.5 Clinical implications and future research

Overall our findings suggest that there is shared expression of specific cytokines with moms and their pups following a maternal immune challenge, but more so within an individual pup and it's corresponding placenta. Cytokine expression is correlated within dams and their pups as well, though the mechanisms by which were not explored in the current experiment. These findings provide a framework for understanding how immune activation during gestation can occur, with the goal that we might one day decrease fetal immune responses and mitigate the onset of neuronal dysfunction following maternal illness. Future studies can perhaps uncover the underlying mechanisms of these correlations by observing specific cell activity within the maternal, fetal, and shared compartments within the placenta. If it is diffusion, and later cascade effects, between dam and pup that translate to these findings, what role do the resident immune cells in placental compartments play? It would be interesting as well to look for compensatory mechanisms, for example in the fetuses that survive the maternal immune activation vs those that do not, that might function as an evolutionary adaptation to ensure fetal vitality.

Contrary to our expectations, pregnancy had very minor effects on the immune responses measured in the current study. This however should not deter researchers from considering the impact of pregnancy on the immune response in models of MIA. Pregnancy overall has a host of physiological adaptations that are not covered in the scope of this experiment, including adaptive roles of pregnancy across all cytokines and cells in the immune system. In future studies, perhaps these cytokines can be measured and individual differences between dams and non-pregnant cannot only be measured, but controlled for in models of MIA. This experiment may not accurately represent the severity and scope of immunosuppression or immune-hypersensitivity that pregnancy may produce given that only 4 immune molecules were measured. Therefore, continued measures of such, and thorough analysis of different cytokines as well as protein levels can help unveil the full profile of female immune function during pregnancy.

Overall, these findings sought to understand the acute effects of immune activation during the prenatal period of fetal development, and do not attempt to connect these observations with behavioral deficits that may emerge in the offspring. One of the next steps would be to examine the specific neuronal correlates of these immune molecules by examining the expression of important neurotrophic and synaptic molecules? For instance, microglia were not measured in this experiment, but may be an important variable to consider in the context of long-term neuronal health and synapse formation. Astrocytes and T-cells should be considered as well. Though cytokines are generally known for their function in the immune response, many cytokines also have important roles in various aspects of neurodevelopment. As the MIA model becomes more of a tool to understand the etiology of neurodevelopmental disorders, the entire physiological profile of both dams and pups should continue to be explored. With these findings becoming more accessible and thorough, psychopathology can be better understood not only by postnatal exposure and risks, but gestational and congenital as well.

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