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MOUSE MODEL OF INTRAUTERINE INFLAMMATION: SEX-SPECIFIC DIFFERENCES IN LONG-TERM NEUROLOGIC AND IMMUNE SEQUELAE

Tahani Dada^{1,*}, Jason M. Rosenzweig^{1,*}, Mofeedah Al Shammary¹, Wance Firdaus¹, Shorouq Al Rebh¹, Talaibek Borbiev¹, Aylin Tekes², Jiangyang Zhang², Eman Alqahtani², Susumu Mori², Mikhail V. Pletnikov³, Michael V. Johnston⁴, and Irina Burd^{1,4,5,†}

¹Integrated Research Center for Fetal Medicine, Department of Gynecology and Obstetrics, Johns Hopkins University School of Medicine, Baltimore, MD

²The Russell H. Morgan, Department of Radiology and Radiological Science, Johns Hopkins University School of Medicine, Baltimore, MD

³Department of Psychiatry, Johns Hopkins University School of Medicine, Baltimore, MD

⁴Department of Neuroscience, Kennedy Krieger Institute, Baltimore, MD

⁵Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD

Abstract

Preterm infants, especially those that are exposed to prenatal intrauterine infection or inflammation, are at a major risk for adverse neurological outcomes, including cognitive, motor and behavioral disabilities. We have previously shown in a mouse model that there is an acute fetal brain insult associated with intrauterine inflammation. The objectives of this study were: 1) to elucidate long-term (into adolescence and adulthood) neurological outcomes by assessing neurobehavioral development, MRI, immunohistochemistry and flow cytometry of cells of immune origin and 2) to determine whether there are any sex-specific differences in brain development associated with intrauterine inflammation. Our results have shown that prenatal exposure appeared to lead to changes in MRI and behavior patterns throughout the neonatal period and during adulthood. Furthermore, we observed chronic brain inflammation in the offspring, with persistence of microglial activation and increased numbers of macrophages in the brain, ultimately resulting in neuronal loss. Moreover, our study highlights the sex-specific differences in long-term sequelae. This study, while extending the growing literature of adverse neurologic outcomes following exposure to inflammation during early development, presents novel findings in the context of intrauterine inflammation.

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[†]Corresponding Author: Irina Burd, M.D., Ph.D., Integrated Research Center for Fetal Medicine, Department of Gynecology and Obstetrics, Johns Hopkins University Hospital, Phipps 228, 600 North Wolfe Street, Baltimore, MD 21287 iburd@jhmi.edu.

*These authors contributed equally to this work.

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Keywords

intrauterine inflammation; preterm birth; mouse model; lipopolysaccharide; brain damage; MRI and behavior

1. INTRODUCTION

Recent annual summary of vital statistics shows that the preterm birth rate (<37 weeks) is 11.7% in the United States, with more than 500,000 infants born preterm each year (Hamilton et al., 2013). Preterm children exposed to intrauterine infection or inflammation are at a greater risk for a spectrum of long-term adverse neurologic disorders (Wu and Colford, 2000; Yoon et al., 2000), emotional disorders and even autism (Johnson et al., 2010a; Johnson et al., 2010b).

Mechanisms of inflammation-induced fetal brain injury are most likely determined by proinflammatory chemokine and cytokine signaling (Hsiao and Patterson, 2011; Hsiao et al., 2012; Lieblein-Boff et al., 2013; Smith et al., 2007; Williamson et al., 2011). Intrauterine infectious pathogens activate toll-like receptors on the surface of cells in the decidua and placental membranes, resulting in the production of proinflammatory cytokines that can cross compromised blood-brain barrier into the fetal brain where they induce activation of microglia, which contribute to glutamate excitotoxicity and production of reactive oxygen species. Altogether, this chain of events leads to cerebral inflammatory response and subsequent brain injury. While chorioamnionitis and *in utero* inflammation as a host immune response may underlie a whole spectrum of long-term effects on the nervous system of neonates, fetal inflammatory responses, which might be initiated by intrauterine inflammation, are now thought to have a stronger association with adverse effects in the neurodevelopment of offspring (Ashdown et al., 2006; Beloosesky et al., 2006; Cai et al., 2003; Dammann and O'Shea, 2008; de Vries, 2009; Hagberg and Mallard, 2005; Malaeb and Dammann, 2009).

Our prior studies utilized an established mouse model of localized inflammation (Burd et al., 2012; Elovitz et al., 2003) by an intrauterine injection of lipopolysaccharide (LPS). In contrast to models of systemic inflammation induced by poly[I:C] and LPS administered intraperitoneally or models of neonatal inflammation, this model mimics a local inflammatory response in the uterus in the absence of overt infection symptoms in the dam, similar to the most common clinical scenario of preterm birth in humans. Our previous studies indicate that intrauterine inflammation results in acute perinatal brain injury as evidenced by altered neuronal morphology, upregulation of pro-inflammatory cytokines and neurotoxicity, which seems to contribute to irreversible neuronal damage (Burd et al., 2009; Burd et al., 2010a; Burd et al., 2011). However, less is known if this inflammation-induced brain injury could have chronic inflammatory impact and adverse neurological sequelae.

Hence, for the current study, we hypothesize that intrauterine inflammation-induced fetal brain injury has long-term effect on murine brain development into adulthood. Also, these studies sought to assess whether these long-term neurodevelopment outcomes are different between sexes.

2. MATERIALS AND METHODS

2.1. Mouse model of intrauterine inflammation

All animal care and treatment procedures were approved by the Institutional Animal Care and Use Committee. Animals were handled according to the National Institutes of Health guidelines. An established model of intrauterine inflammation was utilized for these studies (Burd et al., 2009; Burd et al., 2010a; Burd et al., 2010b; Burd et al., 2011; Burd et al., 2012; Elovitz et al., 2003; Elovitz et al., 2011). Timed pregnant CD-1 outbred mouse strain was obtained from Charles River Laboratories (Wilmington, MA).

Intrauterine injections of LPS (from *Escherichia coli*, 055: B5, Sigma-Aldrich, St Louis, MO) at a dose of 50µg in 100 µL of phosphate-buffered saline (PBS) were administered on embryonic day 17 (E17) of a 19-day gestation in four independent experiments. Control dams for these experiments received the same volume of intrauterine injection of vehicle. In total, 11 dams were injected with PBS with all litters surviving and 43 dams were injected with LPS with 16 litters surviving. For survival surgery, pregnant mice were placed under a mask that kept a continuous flow of isoflurane/oxygen for adequate anesthesia. The mini-laparotomy was then performed in the lower abdomen, which was closed with suture and staples, and the dams were recovered in individual cages. Live pups were separated by sex for immunohistochemistry, behavioral, magnetic resonance imaging (MRI) studies as well as flow cytometry.

2.2. Behavioral evaluation

A developmental milestone scoring system (Hill et al., 2007) was used with modifications to evaluate pups. Ambulation and pivoting behavior was determined by the ability to move out of a circle 13 cm in diameter. The negative geotaxis test measured the ability to turn 180° when placed head down on a 45° inclined plane. The cliff aversion test measured the ability to turn and crawl away from an edge and the surface righting test determined the ability to right itself after being placed on its back. Open field evaluation, negative geotaxis, cliff aversion, and surface righting were performed on PND 5, 9 and 13 to assess preweaning neurodevelopment.

For the adult behavioral assessment, novelty-induced activity was evaluated in open field test for 30 minutes on PND 60 as previously described (Ayhan et al., 2011).

2.3. MRI

At PND 14 and PND 60, *in vivo* imaging was performed on a horizontal 11.7 Tesla MR scanner (Bruker Biospin, Billerica, MA, USA) with a triple-axis gradient (maximum gradient strength = 74 Gauss/cm). The radio frequency pulses were transmitted through a quadrature volume excitation coil (70 mm diameter, Bruker Biospin, Billerica, MA, USA) and signal was acquired using a four-channel phased array receive-only coil. During imaging, mice were anesthetized with isoflurane (1%) together with air and oxygen mixed at 3:1 ratio via a vaporizer and positioned in an animal holder (Bruker Biospin, Billerica, MA, USA). Respiration was monitored via a pressure sensor (SAII, Stony Brook, NY, USA) and maintained at 40–60 breaths per minute. After imaging, animals recovered within 5 minutes.

Multi-slice T₂-weighted images (echo time (T_E) = 50 ms, repetition time (T_R) of 2000 ms) were first acquired with an in-plane resolution of 0.08 mm × 0.08 mm, a slice thickness of 0.5 mm, and an imaging time of 12 minutes. The images were reconstructed using the Paravision software. Volumes of major brain structures were obtained from the T₂-weighted images as described (Cheng et al., 2011). Following MRI, mice were sacrificed for immunohistochemical analysis.

2.4. Immunohistochemistry

For immunohistochemistry of brain cryosections, mice at post-natal day (PND) 14 and PND 60 from all experimental groups were euthanized with carbon dioxide and their brains were fixed overnight at room temperature in 4% paraformaldehyde. The brains were saturated with ascending amount of sucrose (10% – 20% – 30%, 12 hours each step), snap frozen in optimal cutting temperature (OCT) media and kept at –80°C until they were sectioned. Brains were cut using Leica CM1950 cryostat and mounted on positive charged slides (Fischer Scientific). For immunohistochemical staining, the slides were washed with PBS, which was followed by incubation in 1% hydrogen peroxide for 30 minutes to inactivate the endogenous peroxidase, and then incubated in PBS solution containing 0.05% Triton X-100 and 5% normal goat serum (Invitrogen, Carlsbad, CA) for 30 minutes. The brain tissue was incubated with the following antibodies: rabbit anti-Iba1 for microglia (Wako Chemicals, Richmond, VA), mouse anti-GFAP for astrocytes (Sigma-Aldrich, St Louis, MO), anti-NeuN for neurons (EMD Millipore, Billerica, MA), anti-Myelin basic protein (Abcam, Cambridge, MA). The slides were mounted with Prolong gold antifade reagent as mounting medium (Invitrogen, Carlsbad, CA) and viewed using a confocal Zeiss LSM-510 or Zeiss Axioplan 2 microscope.

2.5. Flow cytometry

To detect whether there was a chronic inflammatory state present in cortex, flow cytometry was performed at PND 120. Brain tissue was harvested from adult mice at PND120 and cortical tissue was dissociated by pressing through a 100 µm mesh cell strainer followed by enzymatic digestion with Liberase TM (Roche, Indianapolis, IN) for 20 minutes at room temperature. The cortical suspensions were washed in Hank's balanced salt solution (HBSS) and separated over a discontinuous density gradient of 70% and 35% Percoll (GE Healthcare, Piscataway, NJ). Mononuclear cells were collected from the interface of the 70% and 35% Percoll layers, rinsed with HBSS, and suspended in PBS with 2% fetal bovine serum (FBS). Mononuclear cells and compensation beads (eBioscience, San Diego, CA) were stained with anti-CD11b (rat monoclonal, BD Biosciences) and anti-CD45 (rat monoclonal, BD Biosciences) primary antibodies for 30 minutes at 4°C in the dark, then rinsed and resuspended in PBS + 2% FBS. Flow cytometry data was collected on a FacsCalibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo Software (TreeStar Inc., Ashland, OR).

2.6. Statistical Analysis

Statistical analysis was performed using SigmaStat 3.5 (Aspire Software International, Ashburn, VA). When tested, litters were assessed as an N of 1 with 11 PBS control litters and 16 LPS litters. Data were expressed as the mean standard deviation of the mean (SD) or as

the median where the data is not normally distributed. Statistical significance was defined as a two-sided $p < 0.05$. For statistical analysis of behavioral tests, MRI data, and flow cytometry data, Student t- test (for parametric data) or Mann-Whitney Rank Sum (for non-parametric data) was utilized. The data for open field test in adult mice were analyzed with repeated measures two-way ANOVA followed by post-hoc comparison tests when applicable.

3. RESULTS

3.1. Neurodevelopmental and behavioral assessments

To evaluate the functional consequences of intrauterine exposure to LPS in neonates, posture, locomotion and motor behaviors were observed in an open field test and reflexes and postural reactions were assessed by cliff aversion, surface righting, and negative geotaxis on PND 5, 9, and 13 (preweaning). LPS-exposed mice demonstrated significantly increased time of completion of behavioral tests during preweaning neurodevelopment (Figure 1). Specifically, the surface righting test demonstrated significant differences in LPS- and PBS-exposed groups on PND 5 and 9 ($p < 0.05$, Figure 1A). During the ambulation test, pups of LPS-exposed mice showed a statistically significant increased time of test completion on PND 9 ($p < 0.01$, Figure 1B). LPS-exposed pups showed a transient deficit in the cliff aversion test on PND 5 ($p < 0.001$, Figure 1C) that was not observed on PND 9. Finally, the negative geotaxis test showed statistically different results between LPS- and PBS-exposed groups on PND 5, 9, and 13 (PND 5, $p < 0.05$; PND 9 and 13, $p < 0.001$; Figure 1D).

Open field test on adult animals (PND 60) showed a significantly greater peripheral activity in LPS-exposed group compared to control ($p < 0.05$), with the central activity and rearing being comparable between two groups (Figure 2). ANOVA of the peripheral activity revealed the significant group by time interaction, $F(5,167)=3.63$, $p=0.004$, indicating that LPS-exposed mice were active in the peripheral zone of the arena during the first 10 minutes that may be suggestive of enhanced anxiety and/or hyper-reactivity to novel environment.

3.2. MRI

There were significant differences in T2-weighted volumes of whole brain ($p < 0.01$) and neocortex ($p < 0.05$) in LPS-exposed group versus control group on PND 14 (Figure 3). On PND 60, though we could not detect any significant differences in whole brain, neocortex, thalamus, caudate putamen or ventricle volume between treated groups (Figure 4A), there was a significant difference in hippocampal volumes ($p < 0.05$; Figure 4B). Moreover, loss of hippocampal volumes in LPS-exposed group was sex-specific with males being more susceptible to loss of hippocampal volumes (Figure 4B).

3.3. Immunohistochemical evaluation

To determine whether motor impairments were correlated to brain injury such as neuronal loss, astrogliosis and microgliosis, we examined brain cryosections on PND 14 and 60 by immunohistochemistry using antibodies to GFAP (astrocytes), Iba-1 (microglia) and NeuN (neurons). A significant astrogliosis, as well as increased number and activation of microglia

and neuronal loss were observed in hippocampal sections of LPS-exposed group on PND 14 (data not shown) and PND 60 in comparison to PBS-exposed group (Figure 5).

3.4. Flow Cytometry Analysis

Analysis of brain tissue by flow cytometry was performed at PND 120 to assess the state of persistent inflammation in the adult brain of LPS exposed mice. We immunostained mononuclear isolates from cortical tissue with anti-CD11b and anti-CD45 antibodies and analyzed the cells by flow cytometry. After gating for monocytes and live cells based on forward scatter and side scatter, we grouped the cells as microglia ($CD11b^+CD45^{int}$) or macrophages and infiltrating cells ($CD11b^{lo}CD45^{hi}$) based on immunostaining (Figure 6A). Microglia represented similar proportions of the monocyte population regardless of animal sex or treatment (Figure 6B). While *in utero* LPS-exposed adult mice showed a significant increase in macrophage and infiltrating cells at PND 120 ($p < 0.05$), this difference was sex specific and affected male offspring ($p < 0.05$). Female mice showed no significant change in macrophage frequency (Figure 6C).

4. DISCUSSION

Intrauterine inflammation-associated preterm birth is linked to fetal inflammatory response syndrome and perinatal brain injury (de Vries, 2009; Glass et al., 2009; Kannan et al., 2012; Saadani-Makki et al., 2009). Using our established mouse model of intrauterine inflammation, we have previously demonstrated that *in utero* LPS administration at E15/E17 results in fetal brain injury as evidenced by upregulation of pro-inflammatory cytokines, altered neuronal morphology, and neurotoxicity (Burd et al., 2009; Burd et al., 2010a; Burd et al., 2011). In this study, based on histopathological data, neurodevelopmental and adult neurobehavioral studies, *in vivo* MRI results, and flow cytometry, we showed that exposure to *in utero* inflammation has long-term adverse neurologic effects and leads to chronic brain inflammation. In addition, our study revealed that there are long-term sex-specific differences in hippocampal volumes and cortical infiltrating monocytes, with males being more susceptible to these adverse effects.

Although there is association between preterm birth, chorioamnionitis, and perinatal brain injury, less is known about long-term neurologic sequelae with exposure to intrauterine inflammation. Rodent models of systemic maternal immune response during pregnancy have shown altered cytokine expression in placenta as well as changes in behavior of adult offspring (Hsiao and Patterson, 2011; Hsiao et al., 2012; Smith et al., 2007). Similarly, neonatal inflammation models demonstrated activation of neuroinflammation and behavioral deficits in the effected rodents (Lieblein-Boff et al., 2013; Williamson et al., 2011). Our studies build on this literature and examine the effects following intrauterine exposure to inflammation.

Prior studies, using a rabbit model, show that prenatal administration of intrauterine LPS resulted in behavioral abnormalities similar to movement disorders observed in cerebral palsy (Balakrishnan et al., 2013; Saadani-Makki et al., 2009). These studies focused on short-term behavioral and neurological outcomes and did not examine sex-based differences in animal response. Our preweaning neurodevelopmental and neurobehavioral results

confirm the adverse neurological outcomes and extend the observations to adult offspring. Our results demonstrate that, in our experimental mouse model of exposure to *in utero* inflammation, there are significant motor capacity losses marked by decreased spontaneous movements with altered motor coordination. Even though we have noticed that some motor deficits could change with age during neurodevelopment, it is obvious that LPS *in utero* administration has adverse neurologic sequelae far into adulthood. In addition, increased activity in the peripheral zone of open field observed in adult mice suggests elevated anxiety and/or hyper-reactivity to novelty consistent with human studies (Kapellou et al., 2006; Linnet et al., 2006; Schendel and Bhasin, 2008).

The hippocampus is responsible for coordinating learning and memory, and, unlike other brain structures, is believed to be able to produce new neurons, since it contains a population of neural stem cells (Williamson and Bilbo, 2013). In a rat model of brain inflammation, inhibition of inflammation restored hippocampal neurogenesis (Monje et al., 2003). Our study has found that, based on *in vivo* MRI results of PND 60 mice, there is significant loss of hippocampal volumes between LPS- and PBS-exposed groups, as well as between LPS-exposed males and LPS-exposed females. In this context, our study provides evidence that inflammation induced by intrauterine LPS administration leads to neuronal loss and brain injury with long-lasting effects on hippocampal volume. Neurotoxicity from acute inflammation may cause primary insults leading to reduced hippocampal volume, while chronic inflammation suppresses neurogenesis and repair in the brain resulting in long-term neurologic sequelae. The presence of persistent neurologic changes suggests other cognitive deficits may exist that our tests did not measure. Further studies will address this issue as well as testing for hippocampal-specific behavioral changes.

In this study, we detected a significant astrogliosis, as well as microglial activation and neuronal loss in LPS-exposed adult mice. We also observed that, at PND 120, male mice exposed to inflammation *in utero* exhibit a significant increase in macrophages and infiltrating monocytes in cortex, suggesting that chronic neuroinflammation may play a role in the propagated adverse outcomes. Microglia-mediated immune response is a double-edged sword, simultaneously beneficial and detrimental (Kempermann and Neumann, 2003), because excessive release of proinflammatory cytokines and reactive oxygen species by microglia leads to glutamate excitotoxicity and brain injury. Our study supports the idea that fetal inflammatory syndrome and subsequent microglia activation may be key events leading to adverse neurologic sequelae in offspring.

Chromosomal sex is known to be an important determinant in outcomes following acute neuronal injury (Bramlett and Dietrich, 2001; O'Connor et al., 2003; Wagner et al., 2004). Sex specificity studies of perinatal brain injury, both *in vivo* and *in vitro*, suggest that mechanisms of ischemic cell death and pathophysiology of injury are not identical between males and females (Johnston and Hagberg, 2007; Sharma et al., 2011). Our study indicates that the impact of LPS-induced intrauterine inflammation has sex-specific effects. There have been reports of sex differences in microglial colonization and function in rodent development that begin at or shortly after birth but are absent at E17 (Schwarz et al., 2012). Glial function is increasingly understood to play a prominent role in brain development and learning by influencing synaptic plasticity and our findings of sex-specific differences of

microglial populations are consistent with previous studies (Tremblay et al., 2010; Wake et al., 2009). Given that sex differences in glia are not evident at E17 suggests a mechanism of propagated insult whereby the acute neuronal injury continues until such a time that sex differences can influence outcomes. Elucidating the mechanism of sex-specific differences in neurotoxicity will be important for stratification for future therapeutic interventions.

In conclusion, this study provides evidence that in a mouse model of LPS-induced intrauterine inflammation, fetal brain injury has long-term adverse neurologic and immune sequelae. Some of these adverse outcomes were sex-specific, with male offspring being affected more than females. In addition, we have found decreases in hippocampal volume, which may result from inhibition of neurogenesis due to chronic neuroinflammation. Unraveling the sex-based mechanisms underlying the responses will provide critical knowledge for targeted intervention and development of novel therapeutics.

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Figure 1. *In utero* exposure to LPS resulted in decreased motor activity and increased time to perform tests in neonates

Surface righting (**A**), ambulation (**B**), cliff aversion (**C**) and negative geotaxis (**D**) tests were performed to assess neurodevelopment in normal saline PBS- and LPS-exposed pups. LPS-exposed pups had significantly slower completion times for (**A**) surface righting at PND 5 and PND9 ($p < 0.05$), (**B**) ambulation at PND9 ($p < 0.01$), (**C**) cliff aversion at PND5 ($p < 0.001$), and (**D**) negative geotaxis at PND5 ($p < 0.05$), and PND9 and PND 13 ($p < 0.001$). Data bars represent means $\pm$ SEM. $n = 11$ PBS litters; $n = 16$ LPS litters; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Figure 2. LPS exposure *in utero* resulted in increased peripheral activity in the open field test at PND 60

Adult mice were evaluated in the open field test to assess novelty-induced activity. Rearing and central activity behavior was comparable between the PBS- and LPS-exposed mice. Mice exposed to LPS *in utero* had significantly higher peripheral activity during the first 10 minutes of the test versus PBS. * $p < 0.05$

Figure 3. LPS exposure *in utero* produced increased volumes of the whole brain and neocortex T₂-weighted MRI images were acquired for PBS- and LPS-exposed mice on PND14 and whole brain, neocortex and ventricular volumes (not shown) were determined. **(A)** LPS-exposed pups had significant increases in whole brain volume ($p=0.0049$) and neocortex volume ($p=0.036$). **(B)** Representative T₂-weighted coronal MRI images of PBS- and LPS-exposed mouse brains on PND14 are shown, depicting edema in LPS-exposed group. $n = 11$ PBS litters; $n = 16$ LPS litters. * $p<0.05$; ** $p<0.01$

Figure 4. LPS exposure *in utero* produced long-term decreases in hippocampal volume
(A) *In utero* exposure to inflammation did not change volumes of whole brain, caudate putamen, neocortex, thalamus or ventricles ($p > 0.05$). PBS-exposed animals (control) had significantly higher hippocampal brain volumes **(B)** than those with LPS exposure *in utero* ($p < 0.05$). Furthermore, *in utero* exposure to inflammation affected hippocampal volume of adult males ($p < 0.01$) more than females ($p < 0.05$). **(C)** Representative brain MR imaging of male offspring. $n = 6$ PBS litters, $n = 9$ LPS litters. * $p < 0.05$; ** $p < 0.01$

Figure 5. LPS exposure *in utero* generated long-term changes in neuronal and glial appearance in hippocampus on PND 60

Microglial activation (amoeboid shape), astrogliosis and decreased neuronal staining were observed in hippocampal brain cryosections at PND 60 (Iba-1 – microglia, GFAP – astrocytes, NeuN – neurons) in the LPS-exposed group as compared to PBS-exposed group. Scale bar = 20 μ m.

Figure 6. *In utero* exposure to inflammation leads to persistent elevation of infiltrating macrophages in male mice on PND 120

(A) Microglia and monocytes were isolated from the brains of PND120 mice, stained for CD11b and CD45, and analyzed by flow cytometry to discriminate microglia from macrophages. (B) Inflammation-exposed mice showed no change in microglial frequency. (C) While a significant increase in macrophage frequency was observed in LPS-exposed adult offspring as compared to PBS-exposed offspring ($p=0.024$, $n=10$ PBS, 12 LPS), the difference was only observed in male mice ($p=0.019$, $n=4$ PBS females, 5 LPS females, 8 PBS males, 5 LPS males). * $p<0.05$; ** $p<0.01$