Early Postnatal Lipopolysaccharide Exposure Leads to Enhanced Neurogenesis and Impaired Communicative Functions in Rats

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Abstract

Perinatal infection is a well-identified risk factor for a number of neurodevelopmental disorders, including brain white matter injury (WMI) and Autism Spectrum Disorders (ASD). The underlying mechanisms by which early life inflammatory events cause aberrant neural, cytoarchitectural, and network organization, remain elusive. This study is aimed to investigate how systemic lipopolysaccharide (LPS)-induced neuroinflammation affects microglia phenotypes and early neural developmental events in rats. We show here that LPS exposure at early postnatal day 3 leads to a robust microglia activation which is characterized with mixed microglial proinflammatory (M1) and anti-inflammatory (M2) phenotypes. More specifically, we found that microglial M1 markers iNOS and MHC-II were induced at relatively low levels in a regionally restricted manner, whereas M2 markers CD206 and TGFβ were strongly upregulated in a sub-set of activated microglia in multiple white and gray matter structures. This unique microglial response was associated with a marked decrease in naturally occurring apoptosis, but an increase in cell proliferation in the subventricular zone (SVZ) and the dentate gyrus (DG) of hippocampus. LPS exposure also leads to a significant increase in oligodendrocyte lineage population without causing discernible hypermyelination. Moreover, LPS-exposed rats exhibited significant impairments in communicative and cognitive functions. These findings suggest a possible role of M2-like microglial activation in abnormal neural development that may underlie ASD-like behavioral impairments.

Introduction

Very low birth weight infants (VLBW, <1500 g) are at great risk for developing long-term neurological disabilities [1]. Decades ago, Periventricular Leukomalacia (PVL), which is a necrotic form of brain white matter injury, was the predominant cause of neurological morbidity in this group. Thanks to markedly improved neonatal care, the survival rate of VLBW infants has
been greatly improved. Unfortunately, many of those survivors live with neurological disabili-
ties that manifest predominantly as non-motor related symptoms, ranging from sensory, cog-
nitive, attentional, language, executive, to behavioral impairments [2, 3]. Some of those
behavioral problems such as impairments in language, communication and social behaviors,
are among the core behavioral symptoms of Autism Spectrum Disorders (ASD), which is a per-
vasive neurodevelopment disorder with no clearly defined etiology and neuropathology. Epide-
miological studies suggest that the prevalence of ASD among VLBW infants is approximately
5-fold higher than term infants [4, 5]. Given that perinatal infection has been strongly linked to
the etiology of both WMI and ASD [6, 7], it is possible that there may be shared underlying
pathophysiology between these two groups, at least in a subset of patients.

The diffuse WMI, which is characterized by microscopic damage to developing oligoden-
drocytes (OLs) and axons in the white matter track, is now more common than PVL. Although
the diffuse WMI might be chiefly responsible for neurological/neurobehavioral deficits in
affected VLBW infants [8], MRI confirmed cases account for only one-third of all neurologi-
cally impaired patients [9, 10], leaving the majority of neurologically/behaviorally impaired
very premature infants without clear evidence of brain injury. This suggests that abnormal
growth in gray matter structures may also contribute to neurological morbidity of those surviv-
ing infants, especially concerning cognitive and neurobehavioral impairments. Recently, the
term “encephalopathy of prematurity” has been introduced to highlight the importance of
other brain areas beyond the white matter [8].

Since perinatal infection/inflammation has been identified as a major risk factor for WMI
by a large body of epidemiological studies [6, 11, 12], a number of infection/inflammation-
based animal models (mostly in rodents) have been developed to study the underlying mecha-
nisms of this disorder. The most commonly used approach is to treat animals with bacterial
endotoxin, lipopolysaccharide (LPS), for initiating neuroinflammatory response via either
intracerebral or systemic applications. In our previous studies, we have demonstrated that
intracerebral injection of LPS to postnatal rats induces typical neuropathological features of
PVL, including periventricular white matter lesion, ventriculomegaly, and myelination impair-
ments [13–15]. At the cellular level, intracerebral LPS injection induces a robust microglia acti-
vation and subsequent proinflammatory cytokine release, which are associated with death of
OL progenitor cells, disturbances in OL development, as well as axonal injury [13, 14, 16]. The
limitation of this model, however, is that the route of LPS administration is less clinically rele-
vant given that majority of perinatal infection are maternal or systemic in origin. The decreased
incidence of PVL (less than 5% nowadays) and increased encephalopathy of immaturity
including diffuse WMI [17], calls for developing more clinically relevant animal models to
study the mechanisms underlying aberrant brain development especially those pertinent to
cognitive and behavioral impairments. Therefore, the current study is aimed to test the hypo-
thesis that systemic LPS exposure during early postnatal period may lead to a less severe forms
of WMI (e.g. diffuse WMI) and/or abnormal gray matter growth, reflecting cognitive and behav-
ioral deficits in rats. Interestingly, our data show that systemic LPS does not induce neural
injury, but instead leads to a hypertrophic effect on neural development. Moreover, neurode-
volutional abnormalities are associated with ASD-like neurobehavioral dysfunctions in rats.

Material and Methods

Ethics Statement

This study was conducted in strict accordance with the National Institutes of Health Guide for
the Care and Use of Laboratory Animals. The study was approved by the Institutional Animal
Care and Use Committee at the University of Mississippi Medical Center. All efforts were made to minimize the discomfort and stress of animals.

**Chemicals and Reagents**

Unless otherwise stated, all chemicals used in this study were purchased from Sigma (St. Louis, MO, USA). The sources of kits, antibodies, and other reagents are listed below: Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) kit: Millipore (Billerica, MA, USA). Western blot reagents (Life Technologies, Grand Island, NY, USA). ECL select kit: GE healthcare (Piscataway, NJ, USA). Antibodies used are list in Table 1:

**Animal Treatments**

Time-pregnant Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN). Animals arrived in the Laboratory Animal Facility (LAF) on day 18 of gestation and gave birth on day 22. Both male and female offspring were used in this study.

The day of birth was defined as postnatal day 0 (P0). On P3, pups (both males and females) were intraperitoneally (i.p.) injected with LPS (from E. coli, serotype O55:B5, Sigma-Aldrich, MO) at 1 mg/kg body weight. This dose of LPS is equivalent to what we have used in the intracerebral LPS model [13]. Control rats received the same volume of sterile saline solution (100 μl). After injection, pups were returned to their nursing dam. Numbers of animals per litter were adjusted to 10. Rat pups were weaned on P21.

**TUNEL and Immunohistochemistry**

On P6, P12 and P21, rats were transcardially perfused with normal saline followed by 4% paraformaldehyde (PFA). Rat brains were post-fixed in 4% PFA for 48 h, followed by incubation in sucrose solutions (sequentially in 10%, 20%, and 30%, each for at least 24 h) for cytoprotection. Free-floating coronal brain sections (40 μm) were prepared using a freezing microtome (Leica, SM 2000R, Wetzlar, Germany). TUNEL staining was performed following manufacturer’s instruction, with the exception that the sections were pre-treated with 0.5% triton (in PBS) for 1 h at RT to facilitate TDT enzyme penetrating into nuclei. For immunostaining, sections were

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**Table 1. Summary of antibodies used in this study.**

| Antibodies                                      | Source         | Cat#   | Target(s) of labeling               |
|-------------------------------------------------|----------------|--------|------------------------------------|
| Myelin basic protein (MBP)                      | Millipore      | MAB381 | myelin                             |
| PDGF receptor-α (PDGFR)                         | Santa Cruz     | Sc-9974| OL progenitor cells (OPC)          |
| OL transcription factor 2 (Olig2)               | Millipore      | AB9610 | total OL lineage (nucleus)         |
| Adenomatous Polyposis Coli (APC)                | Millipore      | OP80   | mature OLs (cell body)             |
| CD11b (OX-42)                                   | Millipore      | CBL1512| microglia/macrophage               |
| ED1                                             | Millipore      | MAB1435| activated microglia/macrophage      |
| Ionized calcium binding adaptor molecule 1 (Iba1)| Wako Lab      | 019–1974| microglia/macrophage               |
| Inducible nitric oxide synthase (iNOS)          | Chemicon       | ABS382 | M1 marker                          |
| Major histocompatibility complex-II (MHC-II)    | UBiological    | M3887–10B| M1 marker                         |
| CD206                                           | Abcam          | Ab8918 | M2 marker                          |
| Transforming growth factor beta (TGFβ)          | Abcam          | Ab66043| M2 marker                          |
| pSmad3                                          | Cell Signaling | 12747  | total Smad3                        |
| Smad 3                                          | Cell Signaling | 12747  | total Smad3                        |
| Caspase-3 (cleaved)                             | Cell Signaling | 9664   | active form of caspase-3           |
| Doublecortin (Dcx)                              | Cell Signaling | 4604   | neuroblasts                        |

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first washed with PBS, blocked with 10% normal goat serum (Millipore) in PBS for 1 h at room
temperature (RT), and then incubated with primary antibodies overnight at 4°C. The next day,
sections were washed with PBS and then incubated with secondary antibodies conjugated with
Alex fluo488 (1:300) or 555 (1:2000) at RT for 1 h. Sections were then washed and mounted on
slides. DAPI (100 nM) was included in the mounting medium for counter-staining. Sections
were viewed under a fluorescence microscope (Nikon NIE, Nikon Instruments Inc., Melville,
NY, USA) and images were acquired by Nikon Nis Element software.

Cell Counting
All cell counting was conducted by ImageJ software using the automatic cell counting function,
as described previously [15, 18]. Since TUNEL+ cells were found in highest density in the cau-
date putamen (CPu), they were counted in this region. Three images were captured for a single
section using 25× objective by a monoclonal digital camera, and 3 consecutive sections were
included in final analysis representing as a single brain sample. Iba1+ cells were counted in the
hippocampus in a similar manner. For PDGFR+, APC+ and Olig2+ cell counting, 5 adjacent
images at the corpus callosum were captured under 40x objective. Ki67+ cells were counted in
the dentate gyrus (DG), while the areas occupied by ki67 immunostaining in the subventricular
(SVZ) were determined by the ImageJ software. A total of 8 animals were included in each
treatment.

Immunoblotting
On P6, rats were sacrificed for dissecting brain tissue. Total proteins were extracted using the
tissue lysis buffer (Invitrogen) supplemented with protease inhibitor cocktails (Sigma). Total
protein contents in the lysate were determined by the BCA method, and were subsequently
adjusted at 1 mg/ml. Samples were denatured and subjected to SDS-PAGE, and proteins were
transferred to nitrocellulose membranes. For immunoblotting, the membranes were first
blocked with 5% non-fat milk/1% BSA in PBS for 2 hr at RT and then incubated with anti-
TGFβ or pSmad antibodies overnight at 4°C. Following washing, membranes were incubated
with horseradish peroxidase-conjugated second antibody, and signals were detected using the
ECL select system. After blotting for each of the target proteins, the membranes were stripped
and re-probed for alpha-tubulin or non-phosphorylated Smad3 as the loading controls. Images
were acquired by ChemiDoc MP Imaging system and data were analyzed by Image Lab soft-
ware (Bio-Rad).

Behavioral Tests
Behavioral tests were conducted in the Animal Behavior Core. The experimenter conducting
the behavioral tests was unaware of the treatment conditions.

Ultrasonic Vocalization
This test is based on the findings that rats communicate vocally to conspecifics using ultrasonic
vocalizations in the range between 10–100 kHz [19]. For rat pups, brief isolation from their
dams caused them to emit a characteristic 35–40 KHz calls, and this behavior can be used to
assess the development of basic social communication [20]. Briefly, rat pups at P10 were sepa-
rated from their dams to a holding room. The vocalizations were recorded using the Metris
Sonotrack USV detection system (Hoofddorp, the Netherlands). Pups were recorded individu-
ally for 120 sec and then returned to their dam. Data were analyzed using the Sonotrack system
(Metris).
Novel Object Recognition Test

This test is used to evaluate exploratory and cognitive functions in rodents. Animals were placed in a locomotor activity monitoring chamber (Automex, Columbus Instruments, Columbus, OH) with the size of 42×42 cm, and their activity was monitored by infrared sensors that divided the chamber into 16 zones. On P40, animals were allowed to explore the chamber for 20 min, and a novel object (white, Nalgene-covered block) was then placed in a randomly chosen corner and the rats were monitored for another 10 min. Exploration of the four corner zones (entries in square, and duration in square) was compared between the first 10 min exploration epoch (baseline) and the third 10 min exploration epoch (Novel object). Data were analyzed by one-way ANOVA.

Data Analysis

Data were analyzed by unpaired t-test or one-way ANOVA (Novel object recognition test) using Sigma Plot software (version 12). All data were presented as Mean±SEM. A value of p<0.05 was considered statistically significant.

Results

Systemic LPS Exposure Leads to a Robust Microglial Activation Characterized with Both M1 and M2-Like Polarizations

First we examined general morphological characteristics of microglia 3 days (on P6) following LPS treatment. As shown in Fig 1, LPS treatment led to a marked increase in Iba1+ microglial population as well as morphological transformation. Although the effect of LPS on microglia activation appeared to be global, there was a regional heterogeneity. Areas with the most significant changes include the white matter tracks (such as corpus callosum, cingulum, internal capsule, fornix, and the fimbria of hippocampus), the periventricular areas (SVZ and intrastriatal VZ), and the hippocampal formation, which was clearly noted at low magnification (Fig 1B). In the control, typical microglia in the cortex exhibited smaller and elongated cell bodies with a few long processes (Fig 1G), although they appeared to be much less ramified compared to typical microglia in the adult animals. A small population of amoeboid-like microglia was observed in major white matters such as the cingulum (Fig 1I), and fimbria of hippocampus (Fig 1K). In contrast, most Iba+ microglia in LPS treated rats exhibited activated morphology, characterized with larger amoeboid-like, or rod-shaped somata (Fig 1D, 1F, 1H, 1J and 1L). A subset of Iba1+ cells appeared to have numerous thin processes/filopodia (arrow heads, Fig 1H & 1J). This type of activated microglia morphology was not typically noted in the adult brain, suggesting they might be functionally distinct from the classically activated phenotype. Cell counting in the hippocampus area showed that the overall number of Iba+ cells was more than doubled by LPS treatment (Fig 1M). Although the functional diversity of activated microglia has been increasingly recognized in a number of neurodegenerative disorders [21], much less is known for the developing brain. To assess functional states of activated microglia in the LPS-treated rat brain, several M1 and M2 markers were double-immunolabeled with pan-microglia markers CD11b or Iba1 at P6. As shown in Fig 2, the classically activated M1 markers were only marginally detected in the LPS group, since either their expression levels or the number of positively labeled microglia were relatively low. For instance, iNOS+ cells were noted only in the meninges between the corpus callosum and the septum or the cortex (Fig 2D, arrow heads), while a small number of MHC-II+ cells were detected in the SVZ and meninges (Fig 2E-2I), but not other brain regions. The M1 markers were not detected in any of the controls. As a general marker for activated microglia, ED1+ was expressed by a subset of Iba1+ cells in...
the periventricular areas and major white matter tracks such as the internal capsule (Fig 2A–2C). Those amoeboid-like cells, however, were also noticed in the control, suggesting they are development-specific rather than an indication of activation.

In contrast to M1 markers, several M2 markers were detected at relatively higher levels. For the controls, TGFβ immunostaining was mainly detected in neurons (data not shown), with a few scattered TGFβ+/CD11b+ cells noted in the white matter such as the corpus callosum (Fig 3A–3D, arrow). LPS induced a strong TGFβ expression in CD11b+ amoeboid-shaped microglia, which were clearly visible in subcortical white matter under low magnification (Fig 3E–3H). At a higher magnification, most TGFβ cells were found to be co-localized with CD11b+ cells (Fig 3I–3K). Similarly, numerous CD206+/CD11b+ microglia were observed in both the white and the gray matter (Fig 3L–3N, shown in the thalamus). Immunoblotting showed that TGFβ and its downstream signaling protein phosphorylated Smad3 (pSmad3) were significantly increased by LPS treatment (Fig 3O & 3P).
These M1 or M2 markers were no longer detectable on P21. However, based on morphological criteria, it appears that microglia from the LPS treated rats were not fully resolved at this stage. For example, the majority of control microglia within the cerebral cortex adopted ramified morphology, which is characterized with smaller soma and numerous, longer processes, as compared to microglia on P6. In contrast, significant more microglia in the LPS-exposed rats still exhibited larger cell bodies with less and shorter processes in similar regions. The difference in morphological characteristics were more pronounced in the white matter tracks and hippocampus (S1 Fig).

LPS Exposure Markedly Suppresses Programmed Cell Death

Apoptotic or programmed cell death (PCD) plays a critical role during normal neurodevelopment by serving as a refining mechanism to regulate neuronal [22] and oligodendroglial [23] numbers, while neuronal death induced by pathological insults such as hypoxia-ischemia or inflammation also manifested as a form of apoptosis. Therefore, we next investigated whether LPS exposure could increase PCD. In agreement with early reports that PCD peaks in the first postnatal week of rats [24], extensive apoptotic cell death was detected in the brain of control rats on P6. As shown in Fig 4, a large number of TUNEL+ cells were observed in the subcortical gray matter, including the CPu, septum, amygdala, thalamus, hypothalamus, etc., whereas fewer TUNEL+ cells were found in the cortex. Unexpectedly, LPS treatment led to a marked decrease in TUNEL+ cells. Cell counting in the CPu demonstrated that the number of TUNEL+ cells was reduced more than 3-fold by LPS exposure (Fig 4G). To verify the specificity of TUNEL for PCD, immunostaining of cleaved caspase-3 was conducted. Consistent with TUNEL data, the number of caspase-3+ cells was also significantly lower in LPS treatment (Fig 4H).
LPS Exposure Leads to Over-Production of OLs without Affecting Myelination

Next we assessed OLs and myelination on P21. Mature OLs, total OL lineage cells, and myelin were identified by their respective markers APC, Olig2, and MBP, respectively. Compared to the control (Fig 5A–5D), LPS treatment led to a significant increase in both APC+ mature OLs and Olig2+ total OL lineage cells (Fig 5E–5H), which was mostly prominent in the white matter areas (e.g., the corpus callosum, the cingulum, fimbria of hippocampus, fornix, internal and external capsules, etc). Cell counting in the corpus callosum revealed a 1.4- and 1.5-fold increase in APC+ (Fig 5K) and Olig2 (Fig 5L) cells, respectively. The significant increase of in APC+ mature OLs, however, did not lead to any discernable changes in myelination. MBP Immunostaining revealed rather similar pattern and intensity of myelination between LPS-treated and the control rats (S2 Fig).

LPS Exposure Triggers a Prolonged Cell Proliferation in the SVZ and DG

The over-production of OL lineage cells as well as reduction of apoptotic cell death suggested that there might be an over-expression of growth-promoting factors following LPS exposure, raising the question whether there was an increase in neurogenesis. Therefore, we next
examined cell proliferation by ki67 immunostaining on P21. As shown in Fig 6, in the control, ki67+ cells were primarily identified in the SVZ and DG, although scattered ki67+ cells were...
also presented in other brain regions especially the white matter. LPS treatment led to a marked increase in ki67+ cell density in both the SVZ (Fig 6A–6D) and DG (Fig 6G–6J). The intensively immunostained ki67+ cells in the SVZ tended to expand both laterally and ventrally, leading to a significant increase in the area with ki67 immunoreactivity (Fig 7F). In the DG, some ki67+ cells appeared to form clusters (arrows in Fig 6J). Double immunostaining showed that ki67+ cells in the SVZ of LPS treated animals partially overlapped with DCX, a marker for neuroblasts or immature neurons (Fig 6K–6M).

**Systemic LPS Exposure Results in Deficits in Communicative and Cognitive Functions**

The overall neurobiological features following systemic LPS exposure appeared to be non-destructive (e.g. no signs of cell and tissue damage), but rather growth-promoting (e.g. decreased PCD, increased proliferation) in nature. Given that disturbances of early cell developmental milestones, such as increased cell proliferation [25], increased cortical neuronal density [26], and abnormal patterns of neuronal migration [27, 28], have been reported in ASD brains by postmortem studies, we then conducted neurobehavioral assessments to test whether
ASD-like behaviors could be replicated in our animal model. Results of the ultrasonic vocalization test showed that the average as well as the maximum durations of calls emitted by pups were significantly reduced by LPS treatment (Fig 7A). The novel object recognition test showed that LPS-treated rats spent significantly shorter time duration in the target corner but more in the opposite corner, compared to control rats (Fig 7B). If the time duration for the target vs the opposite corner was compared for individual animals, control rats spent significantly longer time period in the target corner than in any other corners. In contrast, LPS-exposed rats spent significantly shorter time period in the target corners opposite to the novel object than in any other corners. This is also true for entries into corners with or without a novel object, i.e., LPS-treated rats had significantly fewer entries into the target corner than the control rats.

**Discussion**

The major finding of this study is that early postnatal exposure to systemic LPS leads to a robust microglia activation characterized with mixed M1 and M2 phenotypes. The unique pattern of microglia activation is associated with a seemingly growth-promoting effect on brain development and ASD-like neurobehavioral abnormalities.
Previously, we have demonstrated that intracerebral injection of LPS to P5 rats resulted in a robust proinflammatory response in the brain, as indicated by a surge of proinflammatory cytokines including TNFα, IL1β, and IL-6 in the rat brain [13], while pro-inflammatory mediators such as IL1β and iNOS were found to be co-localized with activated microglia [13, 15, 29]. In contrast, the current study revealed a mixed, M2-biased microglia polarization following systemic LPS exposure. It has been reported that in the adult animals, either intracerebral or intravenous injection of LPS induce a predominantly M1-like microglia activation [30, 31]. Thus, it appears that the differential microglial responses in the intracerebral vs systemic LPS neonatal models are specific to the developing brain. Based on the literature and our own data, several potential mechanisms may underlie this difference. First, there is a large disparity of LPS availability in the brain parenchyma between these two models. Although LPS is lipophilic, only minimal amount of LPS could cross the blood brain barrier (BBB) [31, 32]. Second, the molecular mechanisms underlying LPS-induced microglia activation following central vs systemic exposure may also be different. Intracerebral LPS is likely to activate microglia directly by activating TLR4 receptor, which is highly expressed by microglia and endothelial cells of blood vessels [33]. In contrast, due to the restriction of BBB, systemic LPS may first activate endothelial cells to release proinflammatory cytokines, which then indirectly activate microglia, although TLR4 appears to be necessary for this action [34]. Finally, there may also be intrinsic differences between immature and mature microglia, in terms of phenotypic response to LPS challenge. Although there is no direct evidence to support this hypothesis, microglia activation

![Fig 7. Systemic LPS-exposed rats exhibit communicative and cognitive impairments.](image-url)

The ultrasonic vocalization test conducted on P10 shows that LPS-exposed rats emitted a significantly reduced average calls and max duration of calls as compared to control rats (A). Test for novel object recognition on P40 shows that LPS-exposed rats spent significant shorter time duration (B, left) and fewer entries in the target corner with a novel object. Conversely, LPS-treated rats spent significantly longer time duration and more entries in the opposite corner (B, right). * p<0.05 vs control. N = 8.

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is known to be highly context-dependent [35, 36], while the extracellular environment is very different between the developing and mature brain.

When investigating adverse neurodevelopmental consequences of early life neuroinflammation, most studies have focused on the role of proinflammatory response and/or M1-like microglial activation in mediating neural injury and functional deficits in animals. Our data revealed that systemic LPS exposure caused a hyperplastic, rather than injurious effect on neuronal cells, which is consistent with the observation that M2 rather than M1 microglia activation dominate the early neuroinflammatory response in this particular animal model. Although many environmental insults could trigger apoptotic cell death in the developing brain, PCD is a normal developmental program that plays a pivotal role in regulating neuronal [22] and oligodendroglial [23] numbers, since both cell types are generated in excess during early developmental period. Programmed neuronal death is primarily regulated by neurotrophic factors, whereas apoptosis of OPCs is regulated by competing survival factors especially platelet-derived growth factor-AA (PDGF-AA) [37]. A marked decrease of PCD in LPS-treated rat brain may be caused by excessive production of trophic factors, most likely produced by M2-like microglia. Accumulating evidence suggest that microglia with alternatively activated phenotype play a role in adult neurogenesis. For instance, it was recently reported that there is a distinctive population of activated microglia exhibiting alternative activated phenotype in the SVZ of adult mice, as indicated by their expression of M2 cytokine profiles. Deletion of microglia in the SVZ leads to a significant decrease in both survival and migration of neuroblasts, suggesting that these alternatively activated microglia might provide trophic support for neurons [38]. This finding is consistent with in vitro evidence that IL4-activated microglia enhance, whereas IFNγ-activated microglia suppress, neurogenesis and oligodendrogenesis [39]. In the current study, we clearly demonstrated a high level of TGFβ expression in activated Iba1+ microglia, suggesting that microglial M2-associated cytokines and/or growth factors may underlie the hypertrophic effect on neuronal progenitors and OL lineage cells.

In the adult animal, newly generated neuroblasts in the SVZ and DG migrate and integrate into local circuitry. Experimental studies suggest that adult neurogenesis plays a crucial role in development of certain brain functions. For example, neurogenesis in the DG plays a significant role in acquisition of certain contextual memory functions [40]. The proliferation of neuroblasts in the SVZ and DG also showed high plasticity, since it can be enhanced under both physiological (such as physical exercise, task learning, environmental enrichment, etc.) as well as pathological (seizures, stroke, etc.) conditions [41, 42]. The effect of neuroinflammation on neurogenesis remains controversial and contradictory results have been reported. For example, Chapman et al. [43] reported increased striatal neurogenesis in the adult rat following intrastriatal LPS injection. Dinel et al. [44] demonstrated that LPS exposure at P14 leads to neurobehavioral abnormalities including altered anxiety-like and depressive-like behaviors, without affecting hippocampal neurogenesis. However, when animals at adulthood were challenged with a second LPS, there was a significant decrease in hippocampal neurogenesis. It is worth noting that ages of animals, routes of LPS administration, and the time course between LPS treatment and neurogenesis assessment, are different between these studies and ours. Given that microglia respond to immune challenge in a highly context-dependent manner, the neurobiological mechanisms underlying LPS-mediated effect on neurogenesis reported in those studies might be very different. A more comparable experimental setting from this study is the work by Smith et al.[45], who reported that LPS exposure at P5 does not affect total numbers of Brdu+ cells in the hippocampus at P8, P21 and P74 in mice. However, both type 3 neuronal precursors and Brdu+/Dcx+ double-labeled cells were reduced, suggesting an inhibitory effect of LPS on proliferating neuroblasts. A possible contributing factor for the different effect of LPS on neurogenesis observed between our study and by Smith et al. might be different.
approaches used to label proliferating cells. In their study, Brdu was injected 24 h before ani-
mals were sacrificed (P21 and P74), thus Brdu positive cells represent all cells at S phase during
this narrow window. The ki67 labeled cells in our study comprise of all cells in the proliferating
cycle except the resting phase. It was demonstrated that the number of Ki67+ cells are about
50% higher than BrdU labeled cells [46]. In addition, different animal species (rats vs mice) and
the timing of LPS administration (P3 vs P5) may be other potential contributing factors. In
brief, the effect of neuroinflammation on developmental neurogenesis is inconclusive and
future studies are needed to clarify this issue.

Compared to adult neurogenesis, the functional significance of enhanced neurogenesis/glio-
genesis during perinatal and neonatal periods, remains to be elucidated. From a developmental
standpoint, many early neural development programs are highly orchestrated in a spatiotem-
porally coordinated manner so that the right number of neurons and synapses, and the precise
circuitry and network can be established. Thus, one can readily speculate that any deviations
(either increase or decrease) of cell development programs can be detrimental to neural net-
work maturation, which may be reflected by altered neurobehavioral abnormalities. In fact,
studies suggest that many of the early cell development events are altered in ASD. For example,
accelerated brain overgrowth at 1–5 years of age is a defining neuroanatomical feature of ASD
[47], which might reflect overproduction of neural cells. This is supported by several postmor-
tem ASD studies reporting increased cortical neuron density [26, 48], cell proliferation [25],
abnormal pattern of neuronal migration [27], as well as altered GABAergic neurons [49] indic-
ative of abnormal neuronal differentiation. As mentioned earlier, reduced apoptosis might be a
result of excessive anti-inflammatory cytokines and/or growth factors released from M2 polar-
ized microglia. The observation that there was not only an increased cell proliferation in the
neurogenic niches but also overproduction of OL population in the white matter is in line with
this notion. Additional evidence to further support this hypothesis is that we also observed a
significant increase in OPCs on P12. OPCs are known to rely on growth factors especially the
platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) for survival
and proliferation [50].

The communicative and cognitive deficits in LPS-exposed rats suggest that early postnatal
systemic LPS exposure could lead to ASD-like symptoms. Currently, ASD diagnosis is based
purely on behavioral criteria, with language and communicative impairment at the core of
behavioral deficits. In addition, the majority of ASD patients are also cognitively impaired. In
animal studies, ASD-like behavioral impairments were most commonly replicated in maternal
LPS or poly I:C models [7]. To the best of our knowledge, this is the first study to demonstrate
that early postnatal LPS exposure leads to a M2-biased microglia polarization and ASD-like
behavioral impairments in animals. Although cell developmental abnormalities observed in
this animal model have also been reported in human ASD studies, the underlying mechanisms
remain elusive. Given that there is overwhelming evidence suggesting a dysregulated immune
system as well as microglia abnormalities in ASD [51], a linkage between dysregulated micro-
glia activation and early neural development is plausible. Critically, a recent human study
strongly suggests this possibility. In analyzing postmortem cortical tissue, Gupta et al. demon-
strated that many myelin related genes and microglial M2 genes were significantly augmented
in ASD patients compared to controls. Remarkably, the upregulated M2 microglia gene module
was negatively correlated with a differentially expressed neuronal gene module, suggesting a
causative role of dysregulated M2 microglia activation and aberrant neuronal development
[52].

Although we observed some similarities between the current animal model and ASD in
terms of cell development and behavioral phenotype, this postnatal LPS treatment is by no
means to be an ideal animal model for ASD. In fact, the maternal immune activation (MIA)
model is more commonly used in ASD study, due to both good face (behavioral phenotypes) and construct (similar cause) validity. Nevertheless, the current model still holds value in studying biological mechanisms underlying aberrant early cell development by inflammatory challenge, some of which may be shared across multiple neurodevelopmental disorders including ASD, schizophrenia, and depression.

Supporting Information

S1 Fig. Morphological profiles indicate that microglia in the LPS-exposed rats were not full resolved on P21. Microglia were immunostained with Iba1 antibody to reveal their morphology. Representative images taken from coronary sections at mid-hippocampal level show all layers of the cerebral cortex, white matter, and hippocampus of the control (panel A) and LPS-treated animals (D). Regardless of treatment, the morphology of microglia was quite heterogeneous across different brain regions, with more ramified cells seen in the cerebral cortex (showing layer 1, 4, and 6) while less-ramified cells seen in other regions such as the (WM), CA area and DG of hippocampus. However, when compared in a regional specific manner, it becomes apparent that microglia in the LPS group had fewer processes and bigger stomata, in comparison with the controls. Panel B&E show images taken from indicated regions (red arrows) of control or LPS group by 20× objectives. C&F further highlight individual cells from corresponding images in panel B&E, with higher magnifications.

(TIF)

S2 Fig. LPS-exposure led to a significant increase in OPC population on P12. OPCs were identified by PDGFR (A-D) and myelin was revealed by MBP immunostaining (F&G). LPS treatment caused a significant increase in OPC population (E), as shown in both the cortex (A&B) and corpus callosum (C-D). However, no discernable difference in myelination was noted between the control (F) and LPS (G) treatment. *p<0.01 vs controls (n = 4).

(TIF)

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