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Research article

Moderate prenatal alcohol exposure suppresses the TLR4-mediated innate immune response in the hippocampus of young rats

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ABSTRACT

Prenatal alcohol exposure (PAE) could lead to developmental disorders of the central nervous system (CNS) and mental retardation. Toll-like receptor (TLR) 4 plays an important role in PAE-induced neurodevelopmental defects. However, how PAE affects TLR4 response in the brain remains controversial. Using a moderate PAE model by feeding pregnant rats with liquid ethanol diet, we investigated the TLR4-mediated response to intraventricular injection of lipopolysaccharide (LPS) in the hippocampus of PEA rats at postnatal day (PND) 30. The results showed that PAE significantly up-regulated the expression of Toll-Interleukin-1 Receptor (TIR)-domain-containing adaptor protein inducing interferon (IFN)-β (TRIF), TNF-α, and IL-1β in the rat hippocampus in the absence of LPS, indicated by western blot assay. LPS treatment dramatically up-regulated the expressions of TLR4 and its downstream molecules in the hippocampus of paired-food and control groups. But no such significant changes of those molecules were found in the hippocampus of PAE animals. Moreover, the LPS stimulation even down-regulated the levels of TLR4 and TRIF in the PAE group. These data suggest that the relatively moderate level of PAE may lead to a mild neuroinflammation and a suppression of TLR4-mediated response to LPS in the hippocampus of young rats. As innate immunity plays crucial roles in CNS development, moderate PAE-induced suppression of TLR4-mediated response may serve as a new candidate mechanism of CNS developmental defects.

1. Introduction

Maternal consumption of alcohol during pregnancy may result in a wide range of adverse effects in the developing fetus. As ethanol can pass through the maternal-fetal blood barrier [1], prenatal alcohol exposure (PAE) may lead to fetal alcohol syndrome (FAS) diagnosed by the presence of specific facial features, growth retardation and the evidence of the central nervous system (CNS) dysfunction [2–4], which is the most prevalent non-inheritable cause of intellectual disability in children. Studies have shown that the ethanol uptake induces neurodegeneration which is associated with microglia activation [5–7], and Toll like receptor (TLR) 4 response plays crucial roles in this activation [8].

TLR4, a member of TLR family which is known as pattern recognition receptors, recognizes both endogenous molecular patterns and pathogen associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS), a component of the cell wall of Gram-negative bacteria [9]. TLR4 activation triggers the myeloid differentiation factor 88 (MyD88)-dependent and toll/interleukin-l receptor-domain-containing adapter-inducing interferon-β (TRIF)-dependent (MyD88-independent) pathway [10,11]. MyD88, tumor necrosis factor receptor-associated factor 6 (TRAF6) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p50 are the molecules of the MyD88-dependent pathway [12] which leads to the transcriptional activation of numerous pro-inflammatory genes including TNF-α and IL-1β [13,14]. Then the inflammatory reaction is initiated to eliminate the

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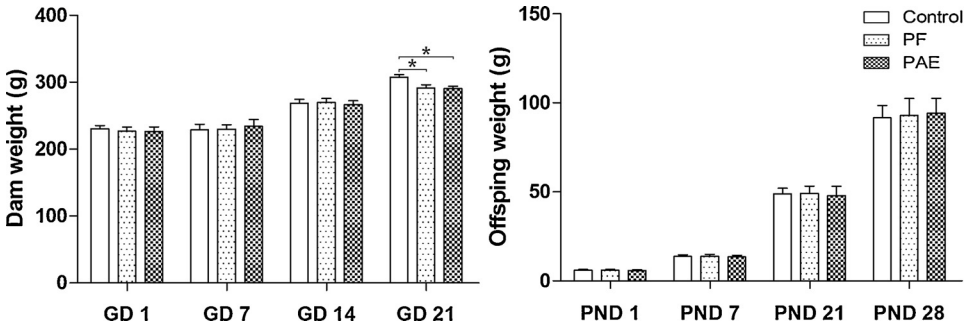

Fig. 1. Body weight of the pregnant dams and the offspring. One-way ANOVA analysis was used. The results are shown as the mean ± SD, n = 8. Significant differences: *P < 0.05 compared to control group.

foreign pathogens.

It has been documented that TLR4 response plays important roles in the brain of PAE animals and may lead to neurodevelopmental defects through ethanol-induced cytokine/chemokine productions [15]. It also has been demonstrated that acute ethanol administration induces microglial activation in vitro through TLR4 [8]. However, whether TLR4 response in the brain could be affected by PAE is not well understood.

In this study, we investigated the effects of PAE on the TLR4 associated innate immune response in the hippocampus of young rats, using a moderate PAE model throughout the entire pregnancy. We observed TLR4-mediated innate immune response to LPS in the rat brains at postnatal day 30 (PND30) and found that PAE led to a higher expression of inflammatory cytokines in the rat hippocampus, but an attenuated TLR4-mediated proinflammatory response to LPS.

2. Materials and methods

2.1. PAE model

Adult virgin male (80-day-old, 280–320 g, n = 9) and female (60-day-old, 200–245 g, n = 18) Sprague-Dawley rats were purchased from the Animal Center of the Fourth Military Medical University. Male and female rats were separately housed on 12:12-hour light/dark cycle. The colony room temperature was held constant (21 ± 1 °C). Animals were given ad libitum access to water and standard rat chow during recovery from transferring and adaptation to the colony room. One week accommodating after their arrival, each male rat was randomly paired with two female rats in a polycarbonate cage (63 × 24 × 18 cm). Daily check was done for the presence of vaginal plugs which indicated day 1 of gestation (GD 0). The female rat at GD 0 was taken out and singly housed in clear polycarbonate cages (46 × 24 × 20 cm) lined with bedding. Those pregnant rats were weight-matched and randomly assigned to one of three groups: (a) PAE, liquid ethanol diet (5% w/v; 36% ethanol-derived calories; diet No. 710262, Dyets), ad libitum (n = 6 dams); (b) pair-fed (PF), liquid control diet (diet No. 710029; Dyets Inc., USA), with maltose-dextrin isocalorically substituted for ethanol, in the amount consumed by the weight-matched PAE rat, ad libitum (n = 6 dams); (c) control (C), lab chow, ad libitum (n = 6 dams). All dams had ad libitum access to water throughout gestation and lactation. Our model was established following a previous study in which the average blood concentration was 144.36 ± 31.32 mg/dl [16]. Experimental diets were provided to dams from GD1 until GD 21, and after this period, their food was replaced with standard laboratory chow ad libitum. The pregnant females were handled only at GD 1, 7, 14 and 21 for routine cage changing and weighing. There were 8–12 pups in one litter. All the pups were weighed at postnatal day (PND) 1, 7, 14, 21 and 28. The offspring were weaned at PND 21, then they were group-housed by litter, in the original room, with free access to a normal rat diet. All animal protocols met the standards recommended by the NIH Guide for Care and Use of Laboratory Animals.

2.2. Intraventricular injection

At PND 30, the PAE, PF and control pups (2 male and 2 female pups from 1 litter, 4 litters in total, n = 16 per group) were respectively and randomly assigned to the LPS and blank groups (1 male and 1 female pups from 1 litter, 4 litters in total, n = 8 per group). The pups in the LPS group were anaesthetized by sodium pentobarbital (40–50 mg/kg·bw, 1%, ip), and stereotaxic injections of LPS (5 μl, 1 mg/ml, *Escherichia coli* lipopolysaccharide serotype 0111: B4, Sigma-Aldrich, USA) were conducted referring to some rat brain atlas [17,18] and previous studies [19,20], using a stereotaxic device (Stoelting Lab Standard Stereotaxic Instrument with rat adaptor). For the blank group, the rats' brain did not receive any treatment. Twelve hours after LPS injections, animals were sacrificed by decapitation for western blot, or by perfusion with 4% paraformaldehyde for immunohistochemistry.

2.3. Western blot

Protein samples of the rat hippocampus (about 0.05 g each, n = 8 per group) were homogenized and analyzed. The protein concentration was determined using Bradford method, and equal amount of protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Millipore, USA). After being blocked with 5% nonfat milk, the membranes were incubated with different primary antibodies for approximately 16–24 h at 4 °C. Primary antibodies were as follows: TLR4 (1:500, rabbit anti rat, AP1504a, Abgent, USA), MyD88, (1:500, rabbit anti rat, IMG-2005-1, Imgenex, USA), TRAF6 (1:1000, rabbit anti rat, IMG5766, Imgenex, USA), TRIF (1:500, rabbit anti rat, IMG5031 A, Imgenex, USA), NF-κB p50 (1:2000, rabbit anti rat, 1559-1, Epitomics Inc., USA), phosphorylated I-κB (1:1000, rabbit anti rat, 5740-1, Epitomics Inc., USA), TNF-α, (1:1000, rat anti rat, ab6671, Abcam, UK), IL-1β (1:1000, goat anti rat, sc-1251, Senta Cruz biotechnology, USA), and β-actin (1:2000, rat anti rat, CW0096, Cwbiotec, China). The membranes were then washed with TBST buffer and incubated with the secondary antibody conjugated with horseradish peroxidase (1:2000, goat anti rat, CW0102; 1:2000, goat anti rabbit, CW0103; 1:2000, rabbit anti goat, CW0168, Cwbiotec, China) for 1 h at room temperature and visualized in ECL solution. The density of specific bands was measured with Image J (NIH, USA) software.

2.4. Immunohistochemistry

The brains of the perfused animals (for the LPS or blank group, 1 male and 1 female pups from 1 litter, 2 litters in total, n = 4 per group) were removed. After post-fixed by 4% paraformaldehyde and sunk in 20% sucrose solution, the brains were cut coronally with a cryostat.

The sections with dentate gyrus (DGs) were selected for immunostaining. The primary antibodies were TRAF6, (1:100, rabbit anti rat, IMG5766, Imgenex, USA) or TRIF, (1:100, rabbit anti rat, IMG5031 A, Imgenex, USA) with NF-κB p50, (1:100, rat anti rat, 6956,

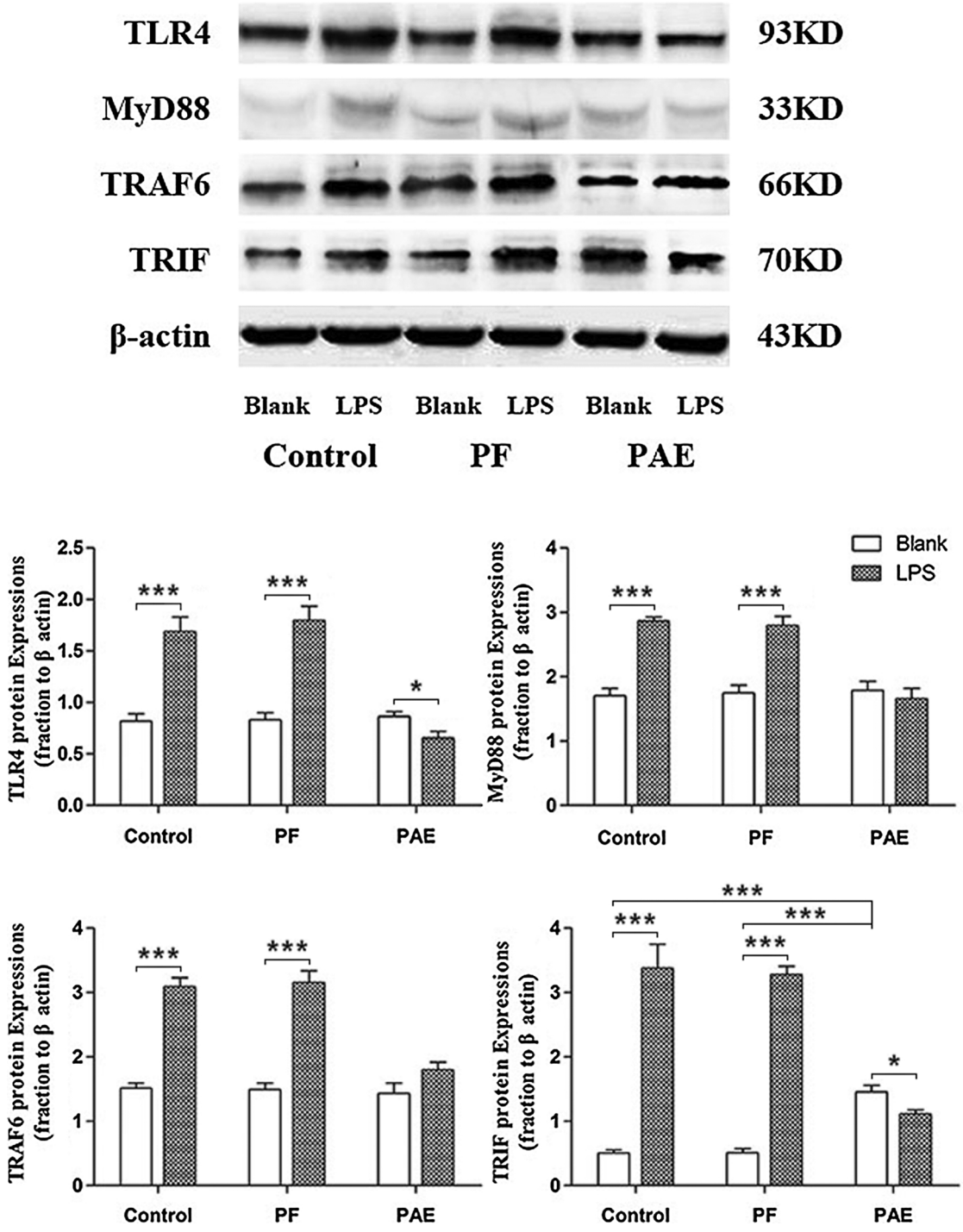

Fig. 2. The protein levels of TLR4, MyD88, TRAF6, TRIF, and β-actin in the control, PF and PAE pups' hippocampi 12 h after icv. injection of LPS or without any treatment (blank). *T*-test and One-way ANOVA analysis were used. The results are shown as the mean ± SD, n = 8. Significant differences: * P < 0.05, ** P < 0.01, *** P < 0.001, compared to the blank group.

Cell Signalling Technology. Inc, USA), respectively. The secondary antibodies were mentioned above. Hoechst 33342 was used for nuclei counterstaining. The staining was examined on a FV 1000 confocal microscope (Olympus, Japan).

2.5. Statistical analysis

All data were presented as mean ± standard deviation. The statistical significance of differences between groups was determined by one-way ANOVA analysis of variance. For the comparison only between two groups, *t*-test was applied. The statistical program SPSS 19.0 for windows (IBM SPSS, USA) was used for statistical analysis and the significance was considered at $P < 0.05$.

3. Results

3.1. Dam body weight during gestation and offspring body weight

The body weight of the dams and offspring in the control, PF and PAE groups was measured. No significant difference was found in the

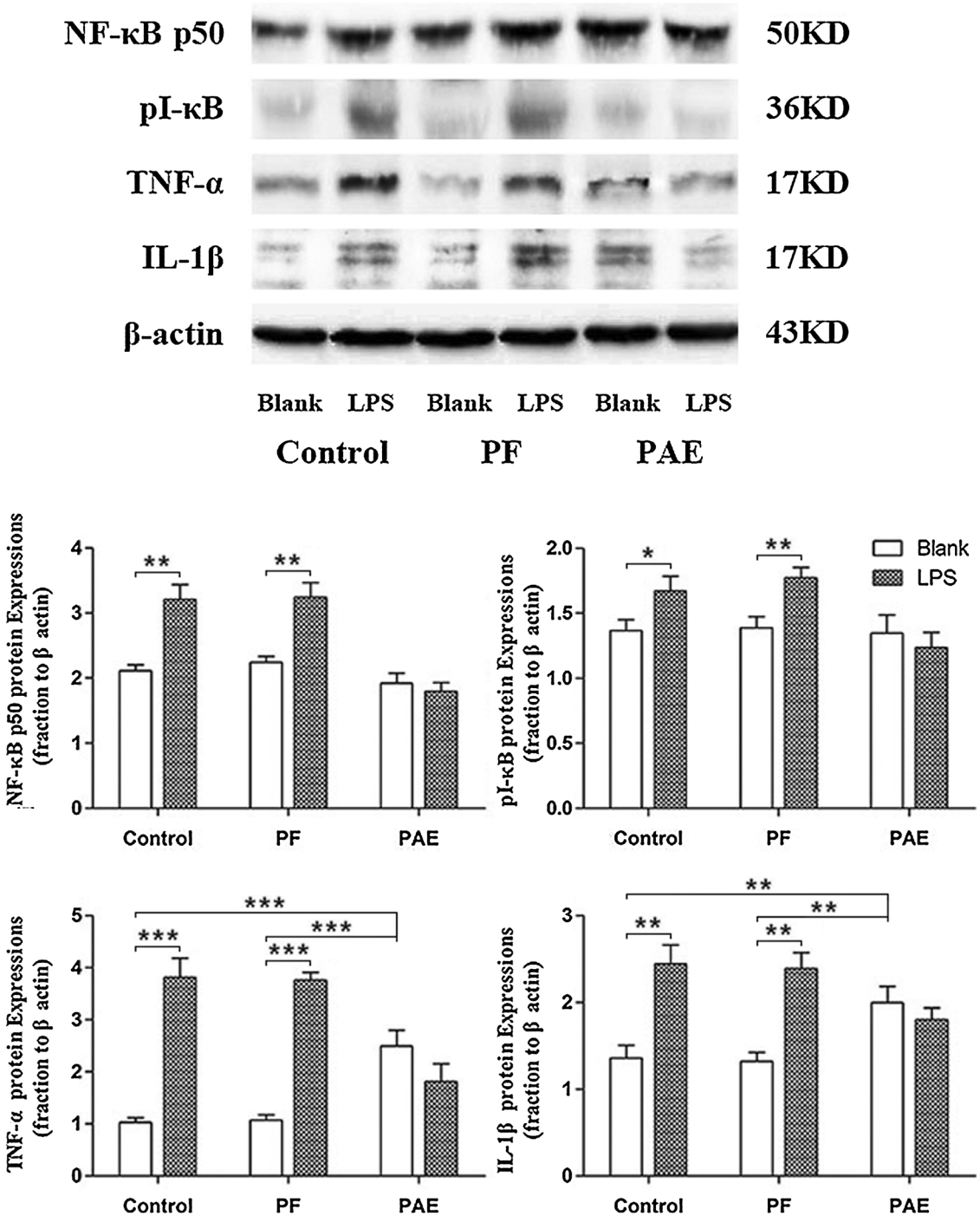

Fig. 3. The protein levels of NF-κB p50, pI-κB, TNF-α, IL-1β and β-actin in the control, PF and PAE pup brains 12 h after icv. injection of LPS or without any treatment (blank). *T*-test and One-way ANOVA analysis were used. The results are shown as the mean ± SD, n = 8. Significant differences: * P < 0.05, ** P < 0.01, *** P < 0.001, compared to the blank group.

body weight among groups except that the weight of the control dams was significantly higher than that of the PF and PAE maternal rats at GD 21 (Fig. 1).

3.2. Basic and LPS-stimulated TLR4 signaling in the hippocampus of the control, PF and PAE rats

Western blot showed no significant difference in the protein levels of TLR4, MyD88 or TRAF6 among the three groups, without LPS treatment. Only TRIF was significantly increased in the PAE group, compared with those in the control and PF groups (Fig. 2). For downstream signaling of TLR4, there was no significant difference in phosphorylated I-κB (pI-κB) and NF-κB p50 levels among groups, but significant increase of TNF-α and IL-1β was detected in the hippocampus of PAE rats, compared with the other two groups (Fig. 3).

LPS stimulation significantly increased the expressions of TLR4, MyD88, TRAF6 and TRIF in the hippocampi in both the control and PF groups. In the PAE group, however, no significant difference was found between LPS treatment and non-LPS treatment in the protein levels of MyD88 or TRAF6. Moreover, the protein levels of TLR4 and TRIF were even significantly down-regulated by LPS (Fig. 2). In the same way, the levels of pI-κB, NF-κB p50, TNF-α and IL-1β were significantly up-

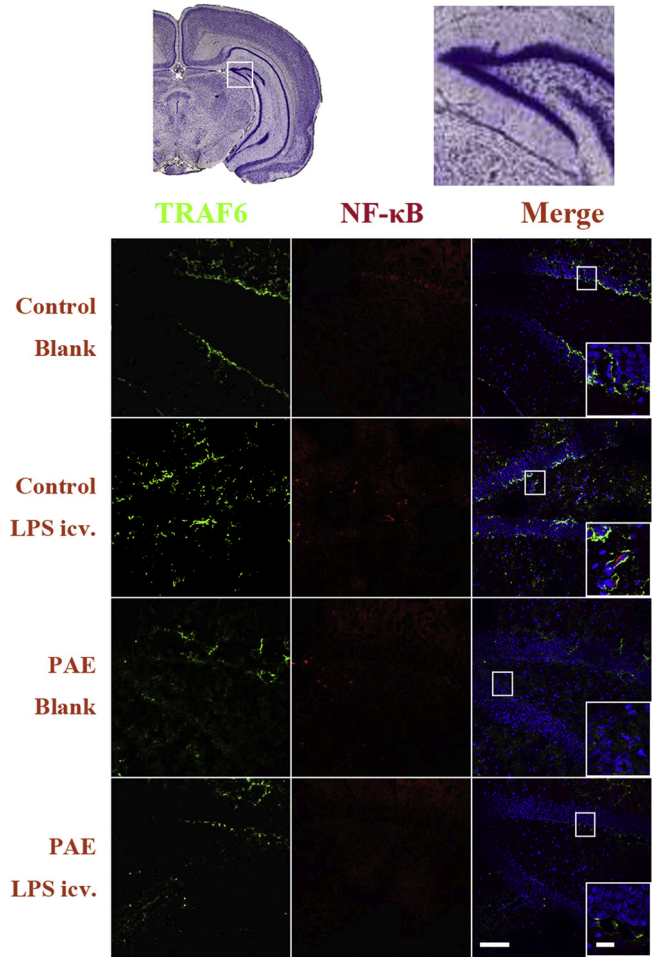

Fig. 4. Representative micrographs of immunofluorescent labeling for TRAF6 (green) and NF-κB p50 (red) in rat DGs in control blank, control LPS, PAE blank and PAE LPS groups. Bar outside the box for all the images, 50 μm; Bar inside the box for all the insertions, 10 μm.

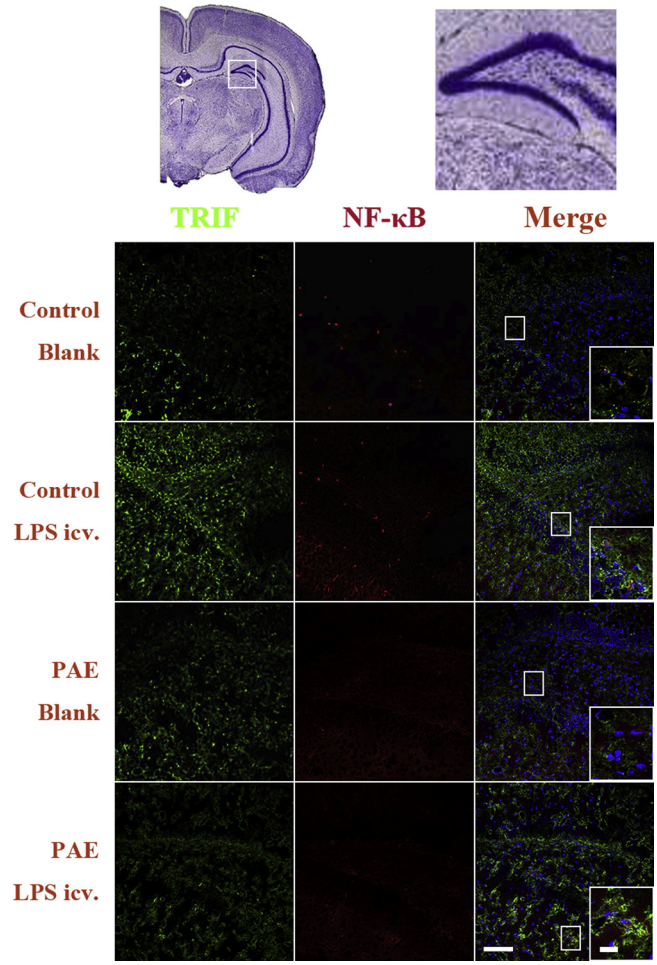

Fig. 5. Representative micrographs of immunofluorescent labeling for TRIF (green) and NF-κB p50 (red) in rat DGs in control blank, control LPS, PAE blank, and PAE LPS groups. Bar outside the box for all the images, 50 μm; Bar inside the box for all the insertions, 10 μm.

regulated by LPS stimulation in the control and PF groups. While in the PAE group, there was no significant difference between LPS treatment and non-LPS treatment in those protein levels (Fig. 3).

3.3. Co-localization of TRAF6 and TRIF with NF-κB p50

Immunohistochemistry showed that few cells were positively labeled by TRAF6 or NF-κB p50 antibody in the DG of the hippocampus of untreated control and PAE animals. While NF-κB p50 immunostaining was dramatically enhanced in the hippocampi of LPS-treated animals in the control group, some of the NF-κB p50 positive cells were overlaid by TRAF6 immunostaining as well. However, in the PAE group, animals receiving LPS treatment did not show obviously enhanced immunostaining of TRAF6 or NF-κB p50, compared to animals without any treatment (Fig. 4). The immunolabeling of TRIF presented the similar tendency (Fig. 5).

4. Discussion

Based on the previous studies on PAE [16], the rat models of classic liquid ethanol diet were established in our study. No significant difference was detected in the body weight of the offspring among groups, which implied that relative moderate level of PAE in this study did not affect their general physical development (Fig. 1.).

In the hippocampus of the PAE rats, we found that TNF-α, IL-1β and TRIF were significantly up-regulated without any postnatal stimulation, which is consistent with previous reports [8,15].

Notably, there was no significant change in the protein levels of TLR4, MyD88, TRAF6 or NF-κB p50 in the hippocampus of the PAE rats and this observation seems not parallel to the increased inflammatory cytokines. In contrast, TRIF, a key molecule of MyD88-independent pathway, was significantly elevated by PAE at the same time point. TRIF mediates the rather delayed activation of TLR-associated signaling cascades [11]. Whether the up-regulated inflammatory cytokines mainly depend on the TRIF cascade in the developing brain of PAE rats is not known. However, our findings suggest that TRIF may serve to PAE-induced neuroinflammation at least in the delayed phase. Furthermore, as PAE induces long-term elevation of inflammatory cytokines, the developing brain is likely exposed not only to ethanol but also to raised cytokine signals [21]. In addition to their function in the immune response, cytokines play important roles in multiple aspects in the CNS development.

Accumulating evidence shows that the alterations of CNS innate immunity are involved in the etiology of numerous neurodevelopmental disorders including schizophrenia, Alzheimer's disease and autism [22–24]. Therefore, PAE-induced innate immune response in the CNS may contribute to the etiology of neurodevelopmental disorders, likely including FAS.

We previously demonstrated that TLR4 response could be induced by LPS administration in the brain of neonatal rats even at PND 1 [25]. However, in this study, LPS stimulation did not activate the TLR4-mediated innate immune response in the PAE rat hippocampus at PND

30. Neither molecules of MyD88-dependent pathway (including TRAF6) nor NF-κB p50 (including pI-κB) was up-regulated. TLR4 and TRIF, another adaptor in MyD88-independent pathway, were even significantly down-regulated in the hippocampus of PAE rats following LPS. These findings indicate a dampening effect of moderate PAE on LPS induced innate immune response in the young rat CNS, and MyD88-independent pathway were probably more severely affected than the MyD88-dependent pathway. We did not include more time points to see this dampening effect by PAE. But we deduce that PAE suppresses TLR4 response should be constant in the hippocampus of young rats. Because it was still detectable at PND 30, one month after PAE.

The effects of PAE on the CNS innate immune response vary, depending on the experiment paradigms. For instance, intraperitoneal LPS administration after low level (70 mg/dL blood alcohol concentration) of PAE led to exaggerated IL-1β expression in the hippocampus of male rats at PND 60 [26]. While, alcohol exposure during the third-trimester-equivalent (the first 1–2 weeks of postnatal life in rodents) blunts the LPS-induced increase of IL-1β mRNA levels in the frontal cortex of female rats [27]. Moderate PAE in our study caused attenuated immune response to LPS, consistent with early reports that PAE attenuates LPS-induced fever in rats [28,29], which may attribute to TLR tolerance: exposure of innate immune cells to TLR ligands (ethanol) induces a state of temporary refractoriness to a subsequent exposure to a TLR ligand (LPS). And LPS hyporesponsiveness could possibly be a damaging response to the brain [30].

The hippocampus is highly vulnerable to the effects of alcohol during development [31]. DG has been shown to be an important target of PAE [32]. Mamalian DG area also possesses life-long potential of neurogenesis [33]. Though we did not find significant difference in the number of new born cells among groups of these rats, using BrdU labeling (data not shown here), it could be possible that glial proliferation is suppressed by PAE [7]. Glial loss or reduced genesis may lead to a dampening effect on TLR4 response.

The immunofluorescence staining of the coronal sections in the DGs showed results consistent with that from western blot. There were more NF-κB p50 positive cells, part of which express TRAF6 or TRIF after the direct stimulation of LPS in the control than in the PAE group. The results suggested that PAE may suppress both MyD88-dependent and MyD88-independent innate immune in the CNS.

In summary, the present study used a relatively moderate PAE model and showed that: (1) moderate PAE itself induced a long term up-regulation of inflammatory cytokines in the rat brain; (2) PAE led to resistance of the innate immune reaction to direct LPS challenge, particularly of the MyD88-independent pathway in the rat brain. Accumulating evidence from clinic and laboratories showed that both innate and adaptive immune systems are necessary for normal brain development [34]. Although the mechanism needs to be further studied, our data suggests that a moderate level of PAE probably challenges the development of CNS as a mild neuroinflammation and may result in attenuated innate immune response of the brain at young age. Since innate immunity affects neurodevelopment as well, our work might provide a deeper understanding of alcohol-associated neurodevelopmental disorders.

Author contributions

P.W. and B.-Y.L. prepared all the animal models and completed most of the experiments; M.-M.W. took care of the animals and carried out the Western Blot assay; X.-Y.W. performed the confocal microscopy assays and cut the brain sections; S.S. participated in data analysis; S.-W.Y. took part in the study design; L.-X.S. was involved in the study design, data analysis and manuscript writing; F.K. designed and directed the study.

Conflicts of interest

The authors declare no conflict of interests.

Acknowledgments

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