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Lead exposure induced microgliosis and astrogliosis in hippocampus of young mice potentially by triggering TLR4–MyD88–NFκB signaling cascades

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HIGHLIGHTS

- In this study, we established a mouse model with lead (Pb) exposure.
- Lead exposure induced obvious microglial response, TLR4–MyD88–NFkB signaling activation, inflammatory cytokine generation and MAPK singling activation in the hippocampus.
- Increase of BrdU-incorporated cells and new-born astroglial cells, but no increase of DCX-labeled differentiated neuronal cells occurred in the dentate gyrus of hippocampus.
- Administration of MyD88 inhibitory peptide could relieve above Pb-induced effects.
- Data of this study suggested that lead exposure could induce microgliosis and astrogliogenesis in the hippocampal of young mice possibly by triggering TLR4–MyD88–NFkB signaling cascades.

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ABSTRACT

Proper proliferation and differentiation of neural stem cells or progenitors in hippocampus is critical to learn and memory functions, which might be disturbed by lead toxicity particularly in young individuals. While astroglial and microglial cells are known to play an important role in regulating neurogenesis of hippocampus, their abnormal response and influence on hippocampal neurogenesis remains unclear. In this study, therefore, glial response including microgliosis, astrogliogenesis and mediating involvement of TLR4–MyD88–NFκB signaling cascades were observed in hippocampus of young mice by animal model with lead (plumbum, Pb) exposure. It revealed that (1) significant microglial activation occurred in hippocampus soon following Pb exposure; (2) increased levels of TLR4, MyD88, NFκB expression were concomitantly detected; (3) BrdU-incorporated progenitor cells were observed in dentate gyrus with significantly-increased numbers at d28 in Pb insult group; (4) obvious astrogliogenesis was observed while these doublecortin-labeled differentiated neurons were not significantly changed in hippocampus; (5) administration of MyD88 inhibitory peptide attenuated or relieved above effects; (6) enhanced expression levels of IL-1β, TNFα, p38MAPK and ERK1/2 were also detected in hippocampus, indicating potential implication of inflammatory response and MAPK signaling activation in lead-induced microgliosis and astrogliosis. Data of this study overall have indicated that lead exposure could trigger or induce abnormal microgliosis and astrogliogenesis in the hippocampus of young mice through triggering TLR4–MyD88–NFκB signaling cascades, which might possibly thereafter disturb hippocampal neurogenesis and functional plasticity.

Abbreviations: BrdU, bromodeoxyurinine; DCX, doublecortin; DG, dentate gyrus; GFAP, glial fibrillary acidic protein; IL-1β, interleukin-1β; LPS, lipopolysaccharide; LTP, long-term potentiation; TLR4, toll-like receptor-4; TNFα, tumor necrosis factor-alpha; Pb, plumbum; PBS, phosphate buffered saline.

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1. Introduction

The dentate gyrus (DG) of hippocampus is one distinctive area in the mammalian brains that maintains active the neurogenesis for whole-life, which plays an important role in learn and memory function such as establishment of spatial representations (Cameron and Mckay, 2001; Zhao et al., 2008). Previous studies have indicated a tight correlation between neurogenesis and learning performance (Lemaire et al., 2000; Mohapel et al., 2005), and fewer new-born neurons result in poorer navigation learning and memory (Drapeau et al., 2003; Kempermann, 2002; Kempermann and Gage, 2002). Growing evidence has shown that various factors can influence neurogenesis and plasticity of hippocampus. For instance, astroglial and microglial cells play an important role in regulating neurogenesis of hippocampus. Apparently, astrocytes are fundamental for homoeostasis, development and plasticity of central neurons. Being active partners with neurons at synapses, astrocytes involved in hippocampal metaplasticity. Loss of astroglial function contributed to aging and neurodegenerative diseases. For instance, reactive astrocytes were associated with developing of neurite plaques in Alzheimer's disease (AD), particularly in hippocampal and cortical regions (Jones, 2015; Rodríguez-Arellano et al., 2015). Moreover, microglial cells were also involved in regulation of hippocampal neurogenesis in a temporally and spatially specific manner. Microglia might sense signals from inner and outer environments of brains, become activated and influence neurogenesis (Sato, 2015). Besides, these activated microglia cells present major source of the inflammatory cytokines. Activation of astrocytes and microglial cells exaggerated inflammatory cytokine production and hippocampal deterioration, and inflammatory response might greatly contribute to the impairment of memory function (Barrientos et al., 2015; Ojo et al., 2015). In addition, the hippocampal neurogenesis is also related to cognitive demand in adults (Dupret et al., 2008; Leuner et al., 2004). Accumulating evidences support a view that cognitive function depends on precise neurogenesis in hippocampus, and deficiency of newborn neuronal cells may impair learning and memory functions (Drapeau et al., 2003; Kempermann and Gage, 2002; Lemaire et al., 2000; Mohapel et al., 2005).

Lead (plumbum, Pb) is a heavy metal that widely exists in living surroundings, and lead exposure in high level can cause acute or chronic poisoning such as neuronal injury of the central nervous system (CNS), especially to children whose blood–brain barrier is fragile (Luo et al., 2012). Data showed that lead exposure could suppress generation or differentiation of CNS neurons, inhibit long-term potentiation (LTP), destruct secretion of neurotransmitters and interfere with calcium signaling (Gilbert et al., 2005; Goldstein 1990; Lasley and Gilbert, 2000). Besides, Pb exposure might also cause response of glial cells, which were critical in regulation of neurogenesis and cognitive function in hippocampus (Gilbert et al., 2005; Verina et al., 2007). While they actively functioned in modulation of neurogenesis, neuronal activity and LTP generation (Alvarez-Buylla and Garcı'a-Verdugo, 2002; Bélanger et al., 2011; Sierra et al., 2010), microglial cells and astrocytes appeared susceptive to lead exposure, and cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) were released as a pacemaker of inflammatory response or neuronal injury (Sansar et al., 2011; Strużyńska et al., 2007).

Until now, however, it still remains unclear on related mechanism for Pb-induced microglial response and neurogenesis dysfunction. It is well known that microglial cells can initiate innate immune reaction by toll-like receptors (TLRs) (Takeuchi and Akira, 2010), and TLRs modulate adult hippocampal neurogenesis and TLR deficiency impair neurogenesis in hippocampus (Rolls et al., 2007). But, there was also a report on microglial disruption in the developing hippocampus of young mice with chronic Pb exposure (Sobin et al., 2013). Besides, the adapter protein of most TLRs, myeloid differentiation primary response gene 88 (MyD88) transfers TLR signal to intracellular pathway (Watters et al., 2007; Vogel et al., 2003), and result in NFκB activation and inflammatory responses (Chen and Jiang, 2013). In this study, thus, we focused on mediating role of TLRs–MyD88–NFκB signaling activation in above glial responses of the hippocampus by using young mouse model with Pb exposure. Data of this study has provided new evidence that lead could induce abnormal microgliosis, astrogliosis and even abnormality of neurogenesis in the hippocampus, possibly by triggering TLR4–MyD88–NFκB signaling cascades.

2. Materials and methods

2.1. Animals and animal model preparation

The C57/BL neonatal young mice were used in this study, and provided by the animal center of the Fourth Military Medical University (FMMU), China. These mice with their parents were housed in temperature-controlled, 12 h/12 h light/dark room, and allowed to access freely to pelleted semi-purified mouse chow (Solid, Vital Keao Feed Co., Beijing, China). When they grew to age of 11–12 days and weighing 5–8 g, mice ($n = 64$, totally) were taken for experiment. All animal experiments were carried out in according with the National Institute of Health guide for the care and use of Laboratory animals (NIH Publications No. 80-23) revised 1996. This study was approved by IACUC and the Committee of Animal Use for Research and Education of the Fourth Military Medical University, and all efforts were made to minimize animal suffering and reduce animals used.

The young mice were assigned into following groups. i.e., control, MyD88 inhibitory peptide, Pb and Pb + MyD88 inhibitory peptide. For preparation of Pb model, intraperitoneal injection with lead acetate (15 mg/kg, i.p.) was given to mouse group daily for 3 days, while MyD88 inhibitory peptide was administrated to single MyD88 inhibition or Pb + MyD88 inhibition group by intracerebroventricular injection, and saline injection was used as control. All mice of three groups were processed in two following batches: first animal batch ($n = 48$, d17) was used for serum Pb detection, immunohistochemical and western blot analysis of TLR4, MyD88, NFκB, inflammatory cytokine and MAPK signaling, and second animal batch ($n = 16$, d28) was mainly used for BrdU incorporation assay to progenitor cell proliferation, neuronal and astroglial differentiation in the hippocampus afterwards (Fig. 1a,c).

2.2. Serum Pb detection

For detection of serum Pb concentrations, the mice ($n = 16$) in above four groups were sacrificed in the day after the last injection of lead acetate, blood samples were collected into heparinized syringes and aliquots, which were taken immediately for determination of serum Pb concentration by atomic absorption spectrophotometry with a graphite furnace (AA Scan 1 Thermo Jarrell Ash).

2.3. Immunohistochemistry

The hippocampal samples and sections were prepared for immunohistochemical study. Briefly, mice ($n = 16$) were deeply anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde in 0.1 M

phosphate buffer (PB) for 30 min. Forebrains containing hippocampus were collected and equilibrated in the phosphate-buffered 20% sucrose with 2.5% paraformaldehyde overnight. The hippocampus were processed for frozen sections with 25 μm thickness on a cryostat, rinsed with PBS and collected for immunohistochemical staining.

Immunofluorescence and laser scanning confocal microscopy were performed to show cellular localization and changes of iba1, TLR4, NFκB, nestin, doublecortin (DCX), glial fibrillary acidic protein (GFAP) in hippocampal sections. In brief, sections were first incubated with primary antibody solution containing 10% donkey serum, 0.3% triton X-100 in 0.01 M PBS at 4 °C for 24 h, i.e., rabbit anti-iba1 (Wako, 019-19741, 1:1000), mouse anti-TLR4 (Abcam, ab30667, 1:200), rabbit anti-NFκB (p50, CS8242, 1:800), rabbit anti-nestin (Sigma, N5413, 1:400), rabbit anti-DCX (CS4604, 1:1000), rabbit anti-GFAP (DAKO, Z0334, 1:1000), mouse anti-GFAP (Millipore, MAB360, 1:800), respectively. After PBS wash, sections were incubated with Alexa Fluor-488 conjugated secondary antibody, or/and Alexa Fluor-559 conjugated secondary antibody (1:500, Molecular Probes) for 4 h at room temperature. After that, sections were counterstained with DAPI (Sigma, 1:10000) for 10 min, mounted with Fluorescence-preserving VECTASHIELD Mounting medium (Vector, H-1000), and examined under laser scanning confocal microscope (LSCM; FV-1000; Olympus, Japan). For control experiment, primary antibody was substituted with normal rabbit or mouse serum for immunochemistry, and immunoreactive cells were not detected in these control sections.

2.4. Western blot

For western blot, mice ($n = 16$) were flushed with saline and hippocampus were quickly separated. The fresh tissues were homogenized in RIPA buffer (1 g:10 mL) consisting of 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% NP-40, 1% deoxycholate, 1% Triton X-100, 10 mM PMSF, and 0.1% protease inhibitors cocktail (Roche, Switzerland). After standing in ice for 20 min, tissues were extracted holoprotein with centrifuge at 12,000 × g for 15 min in at 4 °C, and were defined for quantitative determination of proteins. After that, equivalent amounts of total protein containing 30 μg of protein were loaded on a single track of a SDS-polyacrylamide gel and transferred onto PVDF membrane. The PVDF membranes were blocked using 4% BSA in a solution of Tris-buffered saline with Tween-20 (TBS-T) (20 mM Tris–Cl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) at room temperature for 1 h, incubated with primary antibody, i.e., rabbit anti-TLR4 (AbGENT, AP1504a), goat anti-MyD88 (Sigma, SAB2500664), rabbit anti-p-IκB (CS2859), rabbit anti-NFκB (p50, CS8242), mouse anti-IL-1β (CS12242), rabbit anti-TNFα (CS11948), rabbit anti-ERK1/2 (CS4695), Rabbit anti-p-ERK1/2 (CS4370), Rabbit anti-p38MAPK (CS9212), rabbit anti-p-p38MAPK (CS4631), rabbit anti-β-actin (Sigma, internal control) overnight at 4 °C, respectively. After TBS wash, they were incubated in horseradish peroxidase conjugated secondary antibody (1:2000) at 37 °C for 4 h. After TBS wash, blots were visualized using West Pico Chemiluminescent kit (Pierce, Rockford, IL, USA), and immunoblot bands was measured by densitometry using LumiAnalyst Image Analysis software.

2.5. BrdU incorporation assay

For bromodeoxyurinine (BrdU) incorporation assay, BrdU administration was performed in the second animal batch ($n = 16$), and the earlier lead acetate injection and MyD88 inhibitory peptide treatment were the same as the first batch. On d21, they were caged separately and intraperitoneal injected with BrdU (Sigma–Aldrich, 50 mg/kg/day i.p.) for continuing 5 days. On the last day (d28), mice were transcardially perfused with 4% paraformaldehyde, brain samples containing hippocampus were collected and sectioned for immunohistocehmical visualization of BrdU-incorporated cells in hippocampus.

For visualization of BrdU incorporation, frozen selections were pretreated with HCl solution for 40 min and balanced out with

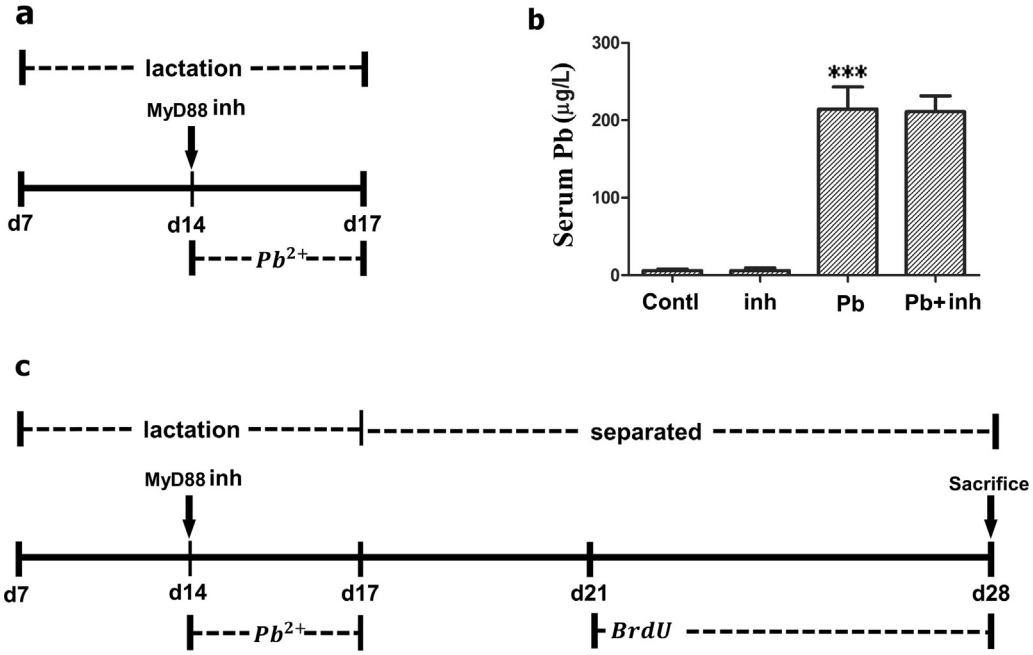

Fig. 1. Animal group design and blood serum Pb detection in mouse model with lead exposure. (a) young mice at d17 for detection of serum Pb and microglial response; (b) blood serum Pb concentrations increase significantly in Pb group compared with control; (c) mice at d28 for detection of gliosis and neurogenesis in hippocampus. ANOVA: $^{**}P < 0.01$ vs control ($n = 4$).

0.1 M sodium tetraborate solution (pH 8.5) for 30 min. After PBS washing, selections were incubated in the primary antibody solution containing mouse anti-BrdU (Millipore, MAB3262, 1:300) and 1% Triton X-100 at 25 °C for 24 h, and then incubated in secondary antibody of Alexa Fluor-488 conjugated donkey anti-mouse IgG, or Alexa Fluor-559 conjugated donkey anti-mouse IgG for 4 h at room temperature. Finally, brain sections were mounted, examined under a LSCM, and interested images were captured for demonstration.

2.6. Statistical analysis

For data analysis, the interested images were captured and analyzed by software ImageJ and Image Tool. The numbers of iba1, TLR4, NFκB, BrdU, nestin, DCX, GFAP-positive cells in unit field (0.25 mm^2) of hippocampus were counted and presented in mean ± SEM. Western blot bands of TLR4, MyD88, p-IκB, NFκB, IL-1β, TNFα, p-p38, p38, p-ERK1/2 and ERK1/2 were analyzed by comparison with internal control β-actin level and data were expressed as mean ± SEM. The differences between means were analyzed by one-way ANOVA (SPSS 18.0). When ANOVA showed significant difference among means, the pair-wise comparisons between means were performed by post hoc testing and significance level was set at a P value of less than 0.05, and significance was shown verse control or Pb group, respectively.

3. Results

3.1. Significant increase of serum Pb concentrations in animals with lead injection

The serum Pb concentrations were first detected in mice at d17 after intraperitoneal injection of lead acetate solution. A dramatic increase of Pb^{2+} concentration was observed in Pb group compared to that of control or MyD88 inhibitory peptide alone. The serum Pb^{2+} concentration in Pb + MyD88 inhibitory peptide group also remained in high level, and it did not show significant difference compared with that of Pb group (Fig. 1b).

3.2. Obvious activation of microglial cells with increased TLR4 level in hippocampus

By iba1/TLR4 double immunofluorescence and laser scanning confocal microscopy, distribution of reactive microglial cells were examined in the hippocampus of mice of control, MyD88 inhibitory peptide, Pb, and Pb + MyD88 inhibitory peptide (Fig. 2a). Obvious microglial activation with strong iba1-positivity was observed in the hippocampus after Pb exposure, and MyD88 inhibitory peptide showed relieving effect on the microglial response induced by Pb exposure. Total numbers of iba1 + microglial cells increased significantly in Pb group compared

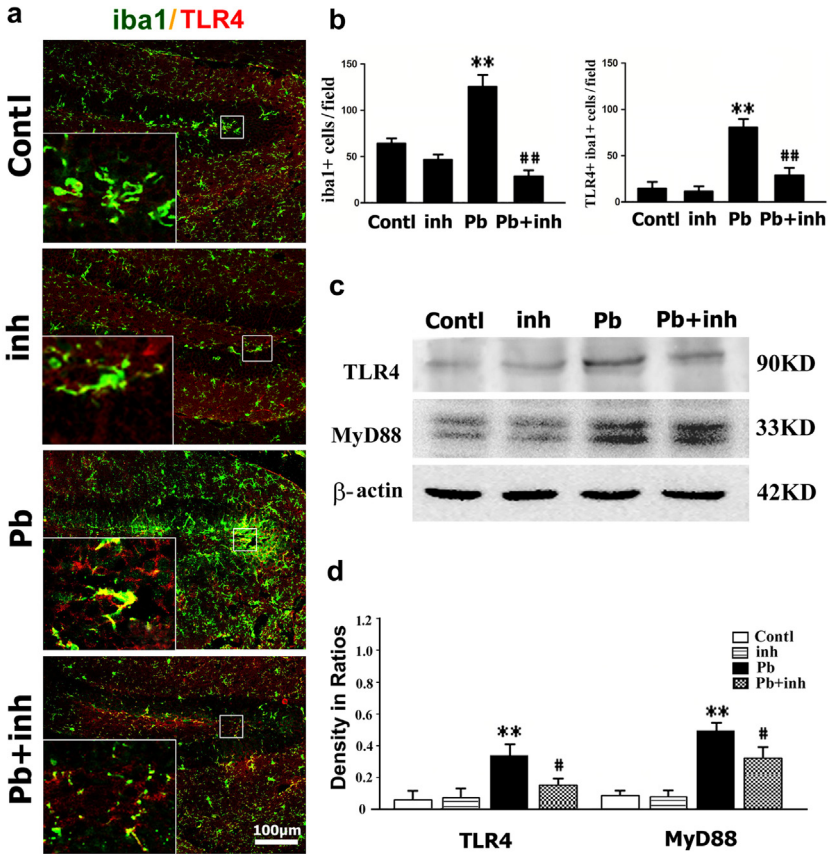

Fig. 2. Microglial response and accompanied TLR4 and MyD88 increasing expression in the hippocampus after Pb exposure. (a) The iba1/TLR4 immunoreactive microglial cells distributed in the dentate gyrus of control, MyD88 inhibitory peptide, Pb, and Pb + MyD88 inhibitory peptide group; (b) comparison of iba-1+ and iba1+/TLR4+ microglial cells among above groups; (c) immunoblot shows TLR4 and MyD88 expression; (d) comparison of TLR4 and MyD88 levels among above groups. ANOVA: $^{**}P < 0.01$ vs control, $\#P < 0.05$, $\#\#P < 0.01$ vs Pb group ($n = 4$).

with that of control, while it decreased in Pb + MyD88 inhibitory peptide group compared with Pb insult group (Fig. 2b). Besides, iba1+/TLR4+ doubly-immunostained microglial cells also increased significantly in Pb group compared with that of control, and decreased significantly in Pb + MyD88 inhibitory peptide group compared with Pb insult group (Fig. 2b).

3.3. Significant activation of TLR4–MyD88–NKkB signaling cascade in hippocampus

Expression of TLR4–MyD88–NFκB signal cascades were examined in the hippocampus of control, MyD88 inhibitory peptide, Pb, and Pb + MyD88 inhibitory peptide group by both western blot and immunohistochemistry (Figs. 2 and 3). Expression levels of TLR4 and MyD88 concomitantly increased significantly in Pb group compared with control or MyD88 inhibitory peptide alone group, and they were relieved in Pb + MyD88 inhibitor peptide group compared to that of Pb insult group (Fig. 2c,d).

By immunofluorescence, NFκB-staining intensity remarkably increased in the Pb group (Fig. 3a). Cell count showed that NFκB+ cells increased significantly in Pb group compared with control or MyD88 inhibitory peptide alone, and went down in Pb + MyD88 inhibitory peptide group compared with that of Pb insult group (Fig. 3b). Western blot further confirmed significant increases of both p-IκB and NF-κB expression in Pb group compared with control, while they were reduced in Pb + MyD88 inhibitory peptide group compared with that of Pb insult group (Fig. 3c,d), indicating that activation of NFκB signaling or release of active NFκB molecule occurred in the hippocampus of mice with Pb exposure.

3.4. Aberrant increase of BrdU+ progenitors instead of DCX+ neurons in hippocampus

To examine proliferation and neuronal differentiation of neural stem cells in the hippocampus of mice at d28, BrdU incorporation, BrdU/nestin, and BrdU/DCX double immunofluorescence were performed in control, MyD88 inhibitory peptide, Pb and Pb + MyD88 inhibitory peptide group. The BrdU+, Nestin+, and BrdU/Nestin+ cells were mainly distributed in the dentate gurus of hippocampus (Fig. 4a), Cell count showed that both BrdU+, Nestin+ and BrdU/Nestin+ cells in Pb group increased significantly compared with that of control and they were reduced in Pb + MyD88 inhibitory peptide group (Fig. 4b–d). Data indicated that increased proliferation of neural stem cells or progenitors occurred in the dentate gyrus of hippocampus after Pb exposure.

DCX immunostained cells were also numerously observed in the in the dentate gurus of hippocampus (Fig. 5a). DCX-staining intensity and DCX+ differentiated neuronal cells did not show significant changes among in Pb and Pb + MyD88 inhibitory peptide group. Cell count on BrdU/DCX double labeling showed slight increase of BrdU/DCX+ cells in both Pb and

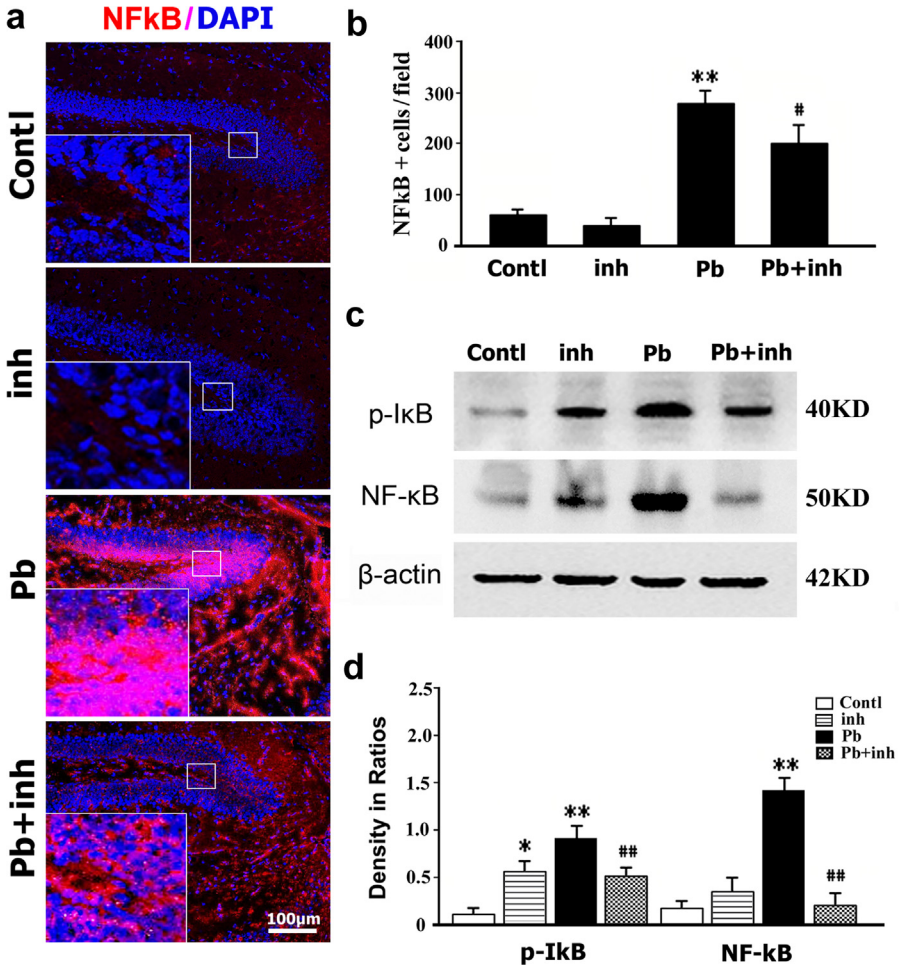

Fig. 3. Microglial activation and increase of NFκB expression in the hippocampus after Pb exposure. (a) NFκB+ microglial cells distributed in the dentate gyrus of control, MyD88 inhibitory peptide, Pb, and Pb + MyD88 inhibitory peptide group; (b) comparison of NFκB+ cells among above groups; (c) immunoblot shows p-IκB and NFκB expression; (d) comparison of p-IκB and NFκB levels among above groups. ANOVA: $^{*}P < 0.05$, $^{**}P < 0.01$ vs control, $\#P < 0.05$, $\#\#P < 0.01$ vs Pb group ($n = 4$).

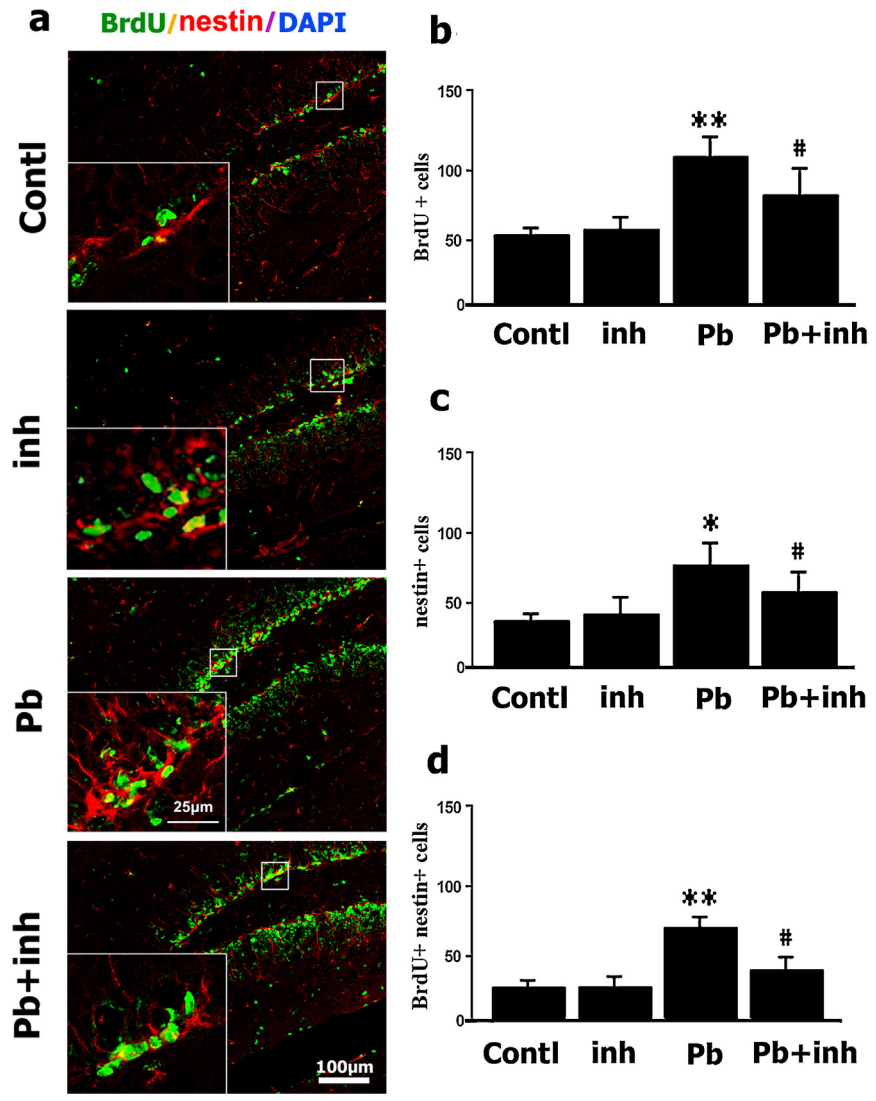

Fig. 4. BrdU incorporation and nestin-expressing cells in the dentate gyrus of hippocampus. (a) BrdU/nestin double-labeling in dentate gyrus of control, MyD88 inhibitory peptide, Pb, and Pb + MyD88 inhibitory peptide group; (b) comparison of BrdU+ cells among above groups; (c) comparison of Nestin+ cells among above groups; (d) comparison of BrdU+/Nestin+ cells among above groups. ANOVA: $^{*}P < 0.05$, $^{**}P < 0.01$ vs control, $\#P < 0.05$ vs Pb group ($n = 4$).

Pb + MyD88 inhibitory peptide group, but they did not have significant difference among above groups (Fig. 5b–d). Data thus suggested that no increase of neuronal differentiation from neural stem cells or progenitors occurred in the hippocampus after lead exposure, even though increasing BrdU+ proliferative cells distributed in hippocampus.

3.5. Abnormal increase of new-born astrocytes and migration in hippocampus

Furthermore, BrdU/GFAP and nestin/GFAP double-labeling were done to show astroglial differentiation of neural stem cells or astrogliogenesis in the hippocampus of control, MyD88 inhibitory peptide, Pb and MyD88 inhibitory peptide group. Abundant and increase of GFAP+ astrocytic cells were clearly observed in the hippocampus of mice with Pb exposure (Fig. 6a,b). Cell count data indicated that not only total GFAP+ astrocytes, but also BrdU/GFAP and nestin/GFAP double-labeled cells increased in Pb group compared with that of control, while MyD88 inhibitory peptide relieved the Pb-induced effect (Fig. 6c–e), indicating that obvious increase of new-born astrocytes or astroglial differentiation from neural progenitors occurred in the hippocampus following lead exposure.

3.6. Changes of inflammatory cytokines and MAPK signaling in the hippocampus

Western bot was also applied to show expression of IL-1β and TNFα in the hippocampus tissue. The immunoblots of IL-1β and TNFα were detected in the hippocampus of control, MyD88 inhibitory peptide, Pb and Pb + MyD88 inhibitory peptide group (Fig. 7a). Being inflammatory cytokine, IL-1β and TNFα significantly increased expression levels in response to Pb exposure, while administration of MyD88 inhibitory peptide reduced expression levels of IL-1β and TNFα ($p < 0.05$–0.01) (Fig. 7b). Data thus confirmed a significant increase of inflammatory cytokine generation in hippocampus induced by Pb exposure.

Finnaly, western blot was performed to detect changes of MAPK signals after Pb insult. The immunoblots of p-p38MAPK, p38MAPK, p-ERK1/2 and ERK1/2 were observed in the hippocampus of control, MyD88 inhibitory peptide, Pb and Pb + MyD88 inhibitory peptide group (Fig. 8a). Expression levels of total p38MAPK and

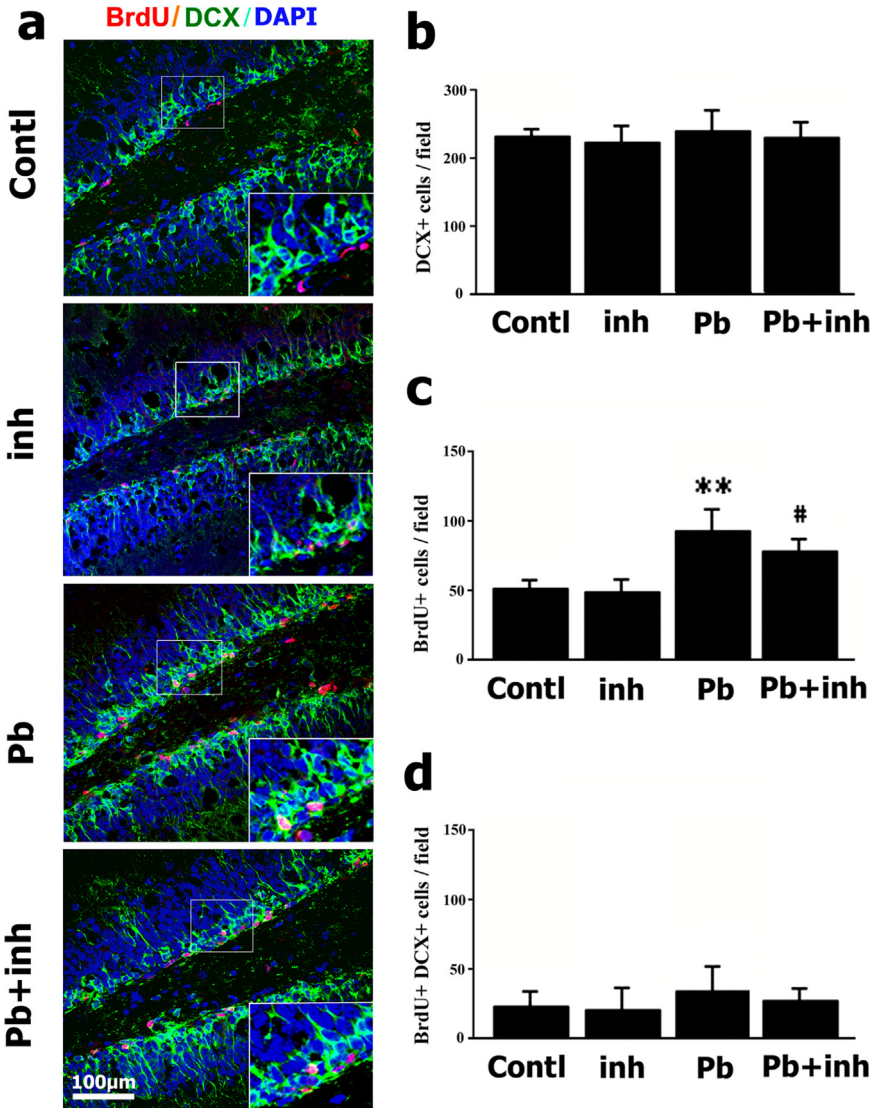

Fig. 5. BrdU/DCX double-labeled neuronal cells in the dentate gyrus of hippocampus. (a) BrdU/DCX double-labeled cells with DAPI counterstaining in dentate gyrus of control, MyD88 inhibitory peptide, Pb and Pb + MyD88 inhibitory peptide group; (b) comparison of DCX+ cells among above groups; (c) comparison of BrdU+ cells among above groups; (d) comparison of BrdU+/DCX+ cells among above groups. ANOVA: $^{**}P < 0.01$ vs control, $\#P < 0.05$ vs Pb group ($n = 4$).

ERK1/2 significantly increased and their phosphorylated levels increased concomitantly in Pb group, while administration of MyD88 inhibitory peptide showed reducing effect on their expression levels (Fig. 8b,c). Data thus indicated that activation of p38MAPK and ERK1/2 signaling pathways might also occur in the hippocampus of young mice after Pb exposure.

4. Discussion

This study demonstrated that lead exposure could induce significant microgliosis and astrogliosis in hippocampus of young mice, which is attained most possibly by triggering TLR4-MyD88-NFκB signaling cascades. While significantly-increased BrdU-incorporating progenitors occurred, no significant increase of BrdU/DCX labeled neuronal differentiation was observed in the dentate gyrus. Instead, abnormal increase new-born astrocytes or astrogliogenesis were detected in the hippocampus. More interestingly, administration of MyD88 inhibitory peptide could partially but significantly relive aforementioned Pb-induced effects. Based on previous literatures, this peptide showed inhibition on MyD88-dependent TLR/IL-1R signaling activity by interefering with MyD88 homodimer formation. The functional inhibition ability was confirmed by in vivo inhibition and in vitro blocking assay (Benedetti et al., 2014; Li et al., 2014). In addition, our study showed increased expression level of MyD88 after Pb insult while it was partially blocked in Pb + MyD88 inhibitory peptide. Data of our study suggests that MyD88 inhibitory peptide might inhibit both activity and expression of MyD88 in the hippocampus. This study suggested that lead might devastate neurogenesis by increasing microgliosis and astrogliosis in the hippocampus of young mice.

Many previous evidences have shown a tight relationship between new-born neurons in hippocampus and learning or cognation function (Abrous et al., 2002; Zhao et al., 2008). The new-born neurons influenced learning and acted in temporary storage of information (Gould et al., 1999; Gross, 2000; Kempermann, 2002). In humans, abnormal or decreased hippocampal neuronal genesis was found in various neurological disorders such as epilepsy, cerebral ischemia, AD and Parkinson's disease (Lemaire et al., 2000). For analysis of adult neurogenesis state, BrdU incorporation was utilized to mark the proliferation of progenitor cells and new-born neurons in hippocampus (Wei et al., 2012; Zhao et al., 2008). In the present study, BrdU-positive cells increased

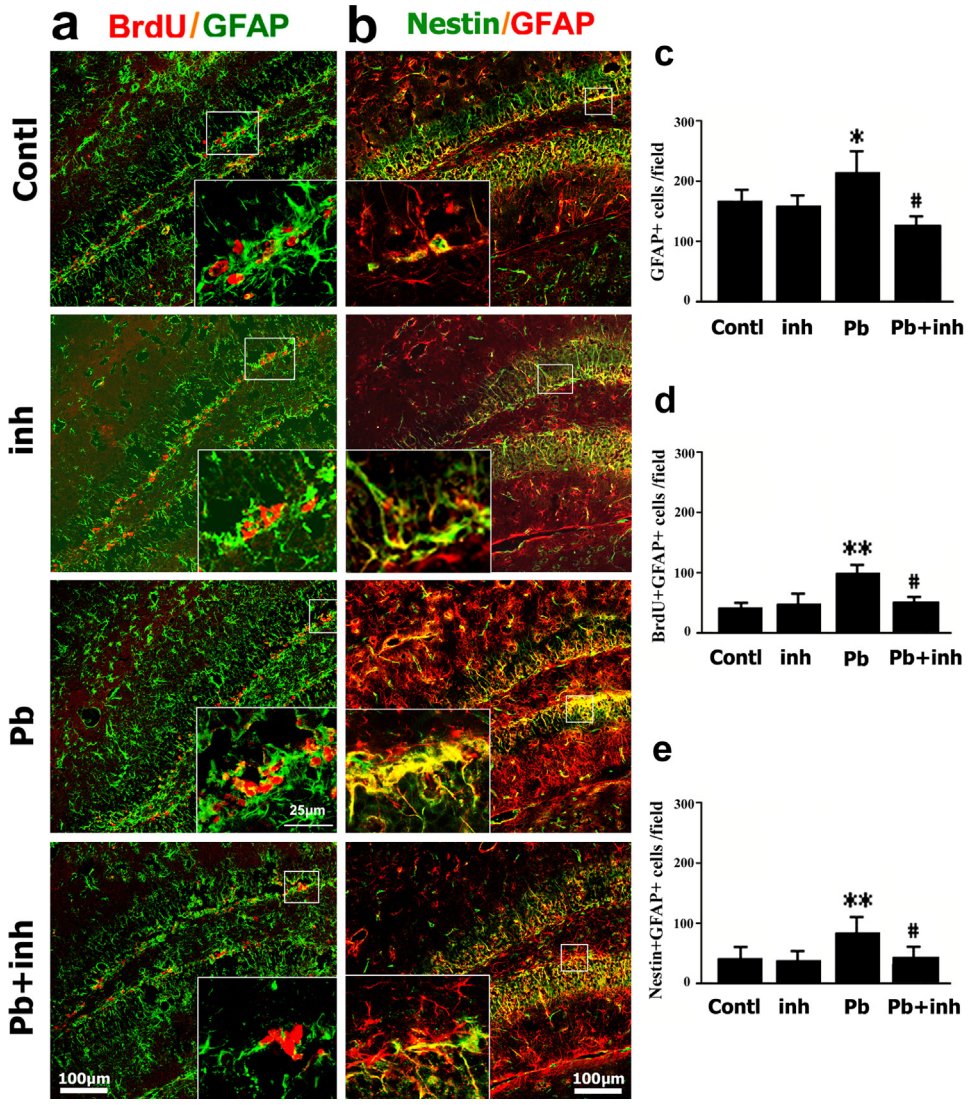

Fig. 6. Activation and proliferation of GFAP+ newborn astrocytes in the hippocampus after Pb exposure. (a) BrdU/GFAP immunostained cells of control, MyD88 inhibitory peptide, Pb and Pb + MyD88 inhibitory peptide group; (b) Nestin/GFAP immunostained cells of control, Pb and Pb + MyD88 inhibitory peptide group; (c) comparison of GFAP+ astrocytes among above groups; (d) comparison of BrdU+/GFAP+ astrocytes; (e) comparison of Netsin+/GFAP+ astrocytes. ANOVA: $^{*}P < 0.05$, $^{**}P < 0.01$ vs control, $\#P < 0.05$, $\#\#P < 0.01$ vs Pb group ($n = 4$).

significantly after lead exposure, but most of BrdU-labeled cells were differentiated into astrocytes instead of neuronal cells indicated by DCX labeling. Our data was basically consistent with some previous reports on lead toxicity on neurogenesis (Li et al., 2013; Liu et al., 2013; Mousa et al., 2015; Verina et al., 2007). It was found that lead exposure decreased generation of granular neurons in the hippocampus (Verina et al., 2007). Abnormality of hippocampal neurogenesis or cognitive dysfunction also occurred after lead exposure and this lead-induced neurotoxicity was also detected in elegans (Li et al., 2013; Liu et al., 2013; Mousa et al., 2015).

In this study, for the purpose to show microglial activation and inflammatory response, immunostaining and western blot were applied to detect iba1, TLR4, MyD88, NFκB, IL-1β, and TNFα expression in the hippocampus of young mice at d17. While iba1-positivity and TLR4-positivity marked microglial activation and TLR4 co-localization in microglial cells, expression patterns of MyD88 and NFκB indicated TLR4–MyD88–NFκB singling activation. Up-regulation of IL-1β, and TNFα level might further imply the inflammatory response in the hippocampus of mice with Pb insult (Pomilio et al., 2015). Furthermore, for the purpose to show proliferation and differentiation state of neural stem cells (or progenitors) in the dentate gyrus of hippocampus, immunostaining was applied to detect nestin, BrdU, GFAP and DCX in hippocampus of young mice at d28. Nestin, an intermediate filament protein, was used to label neural stem cells or progenitors, BrdU incorporation used to label proliferation state of these cells. GFAP was used to label astroglial differentiation and DCX used to label differentiation of immature neurons in the hippocampus (Ceizar et al., 2015; Latchney et al., 2015). Quantitative analysis on nestin, BrdU, GFAP and DCX-positive cell numbers among distinct groups indicated that lead exposure could trigger proliferation of the neural stem cells and increase astrogliogenesis instead of neuronal differentiation in the hippocampus.

Moreover, our study has provided new evidence on involvement of TLR4–MyD88–NFκB signaling in mediation of microgliosis, astrogliosis and abnormality of neuronal genesis in hippocampus caused by lead exposure. Other authors also found that neurogenesis and cognitive function was influenced by TLR signal and inflammatory cytokines in adult hippocampus (Das and Basu, 2008; Rolls et al., 2007). MyD88, being a critical molecule mediating TLR signal to inflammatory cytokines, regulates NFκB

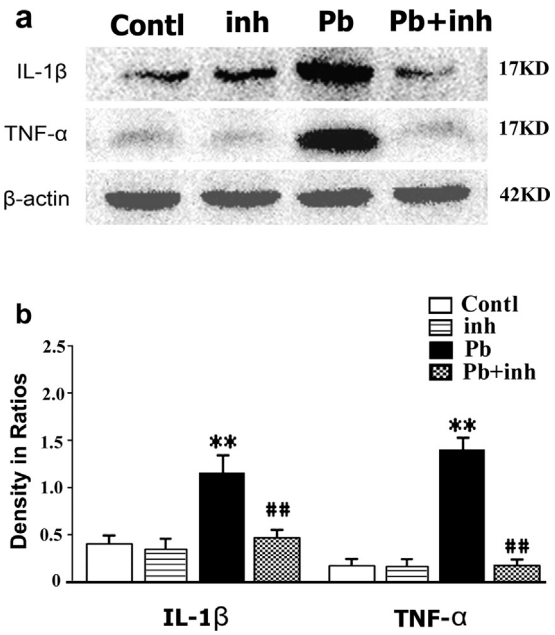

Fig. 7. Increased expression of inflammatory cytokine IL-1β and TNFα in the hippocampus after Pb exposure. (a) Immunoblot of IL-1β and TNFα in control, MyD88 inhibitory peptide, Pb and Pb + MyD88 inhibitory peptide group; (b) comparison of IL-1β and TNFα expression levels among above groups in densitometry in ratios to β-actin. ANOVA: $^{**}P < 0.01$ vs control, $\#\#P < 0.01$ vs Pb group ($n = 4$).

and AP-1 translocation to nucleus (Chen and Jiang, 2013; Muzio et al., 1997). NFκB action is regulated by phosphorylation of I-kappa-B kinase, with degradation of IκB-α and release of p-IκB. Consistently, increase of inflammatory cytokine such as IL-1β and TNFα were detected in hippocampus after lead exposure in this study, and release of inflammatory cytokines might be mainly from active microglial cells under lead exposure or inflammation (Khan et al., 2011; Strużyńska et al., 2007; Xu et al., 2006). Inflammatory cytokines could interact with nitric oxide synthase and contribute to neuronal injury and impairment of cognitive function (Liu et al., 2012; Nava-Ruíz et al., 2010), and it also depended on balance with anti-inflammatory molecules (Ekdahl et al., 2009).

In this study, there was a significant increase of p-IκB in MyD88 inhibitory peptide group compared with control, but, an increase of p-IκB level was more evidently observed in Pb group and partially decreased in Pb + MyD88 inhibitory peptide group. However, it is a question why treatment of MyD88 inhibitory peptide alone could also induce p-IκB increase in the hippocampus. As we know, there were MyD88-dependent and independent pathways for NFkB activation. One possibility is that a functional balance may exist between two pathways, and a response of p-IκB increase from MyD88-independent pathway might occur after blockage of MyD88-dependent pathway by its inhibitor, which was referred as delayed NFκB response (Brieger et al., 2013; Fitzgerald et al., 2003). Administration of inhibitory peptide in naïve mice might relieve predominance of MyD88-dependent pathway on one hand, and trigger MyD88-independent pathway and delayed NFκB activation on the other hand. This speculation was supported by a MyD88 knock-out study (O'Halloran et al., 2014).

Finally, the activation of p38MAPK and ERK1/2 signaling was also observed in the hippocampus of young mice with Pb insult. The p38MAPK and ERK1/2, being two major members of MAPK signaling pathway, actively function in microglia, astrocytes and neuronal cells. Dynamic of MAPK signaling was found in lead and lipopolysaccharide stimulation (Cheng et al., 2002, 2004), and enhanced phosphorylation was detected in hippocampus of immature rats after lead exposure (Cordova et al., 2004). In our study, the correlated trends of p38MAPK and ERK1/2 expression and phosphorylation levels suggested their possible involvement

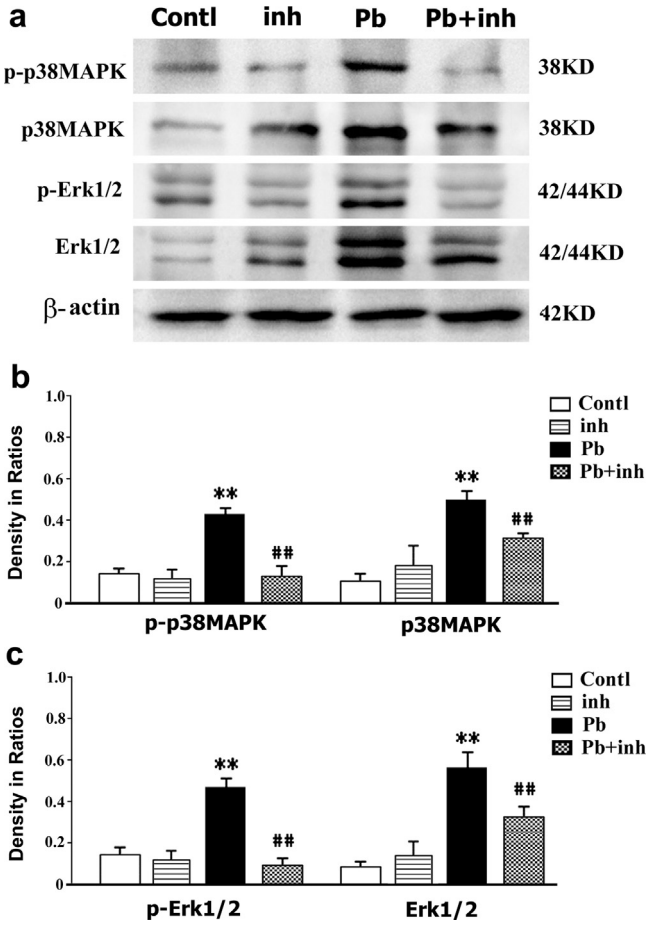

Fig. 8. Increased expression and phosphorylation levels of p38MAPK and ERK1/2 in the hippocampus after Pb exposure. (a) Immunoblot of phosphorylated and total p38MAPK and ERK1/2 in control, MyD88 inhibitory peptide, Pb and MyD88 inhibitory peptide group; (b) comparison of p-p38MAPK and p38MAPK expression levels among above groups in densitometry in ratios to β-actin. (c) comparison of p-ERK1/2 and ERK1/2 expression levels among above groups. ANOVA: $^{**}P < 0.01$ vs control, $\#\#P < 0.01$ vs Pb group ($n = 4$).

in down-stream effect of TLR–MyD88–NFκB signaling in the microgliosis or astrogliosis happened in the hippocampus after Pb exposure. Abnormality in astrocytic and microglial function or activation could hamper their promoting assistance in the neuronal differentiation and maturation in the hippocampus, a specific brain region that maintains active neurogenesis in whole life (Bélanger et al., 2011; Liu et al., 2012).

5. Conclusion

In summary, lead exposure could induce fast microglial response, TLR4–MyD88–NFκB signaling activation, inflammatory cytokine generation and followed MAPK singling pathway activation in hippocampus of young mice. Afterwards, BrdU-incorporated proliferating cells and new-born astrocytes increased remarkably, but no significant increase of DCX-positive differentiated neurons was observed in the dentate gyrus. Administration of MyD88 inhibitory peptide could significantly relieve above Pb-induced effects. Results of this study overall suggested that lead exposure could trigger abnormal microgliosis and astrogliogenesis in the developing hippocampus of young mice, which might possibly thereafter disturb hippocampal neurogenesis and functional plasticity.

Conflict of interest

The authors declare that there are no conflicts of interest.

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