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RESEARCH

Arsenic Exposure Induces Neuro-immune Toxicity in the Cerebral Cortex and the Hippocampus via Neuroglia and NLRP3 Inflammasome Activation in C57BL/6 Mice

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Abstract

This study aimed to examine the immuntoxic effects of arsenic in the nervous system. Our results showed that arsenic increased corticocerebral and hippocampal weights ($p < 0.05$). Morris water maze tests revealed that arsenic significantly increased the time spent in latency to platform on the fourth day in 50 mg/L arsenic exposure and the fifth day in 25 and 50 mg/L arsenic exposure, as well as reduced the path length in target quadrant, time spent in target quadrant, and crossing times of the platform ($p < 0.05$). Hematoxylin–eosin staining showed that the vacuolated degeneration and pyknosis was found in the cerebral cortex and hippocampus of arsenic-treated mice. The mRNA levels of corticocerebral and hippocampal brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) were decreased in the 50 mg/L arsenic-treated group ($p < 0.05$). In addition, immunofluorescence staining showed that 25 and 50 mg/L arsenic all increased the expression of CD11b and glial fibrillary acidic protein (GFAP) in the cerebral cortex and hippocampus ($p < 0.05$). Arsenic markedly raised antigen-presenting molecule MHCII and CD40 mRNA levels in the cerebral cortex and hippocampus and upregulated the cell chemokine receptor 5 (CCR5) and CCR7 mRNA levels in the cerebral cortex at the 50 mg/L arsenic group, and increased the CCR7 mRNA levels in the hippocampus at the 25 and 50 mg/L arsenic groups ($p < 0.05$). Arsenic activated the nucleotide-binding domain-like receptor protein-3 (NLRP3) inflammasome, and enhanced its upstream promoter NF-κB protein level and downstream regulators IL-18 mRNA levels. Collectively, these results provide new evidences for the neuro-immune toxicity of arsenic.

Keywords Arsenic · Neuro-immune · Neuroglial Cell · NLRP3 Inflammasome

Introduction

Arsenic is recognized as the group 1 human carcinogen and widely exists in nature. Chronic arsenic exposure could exert numerous harmful effects including cancers of several systems such as respiratory, hepatobiliary, and urinary system [1] as well as non-cancer diseases, such as diabetes, cardiovascular diseases, and anemia [2, 3]. The central nervous system (CNS) is also the major target organ for arsenic poisoning because arsenic can cross the blood–brain barrier (BBB) and enter the brain [4]. Toxicological and epidemiological studies have shown that arsenic exposure could exert

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neurotoxicity including the decline of learning and memory abilities [5], cognitive impairment [6], and peripheral neuropathy [7]. The main mechanisms of arsenic neurotoxicity include oxidative stress [8], mitochondrial dysfunction [9], and apoptosis [10], which ultimately lead to the impairment of the structure and function of the nervous system. Inflammation and immunity are also one of the mechanisms of its neurotoxicity [11].

Arsenic also has certain immunotoxicity, which plays a role in arsenic-induced carcinogenesis and tissue injuries [12]. The traditional theory is that the nervous system is an immune-privileged compartment due to the presence of the BBB and the blood-cerebrospinal fluid barrier [13]. Increasing evidences showed that the nervous system contains many immune cells and immune molecules, consisting of innate and adaptive immune system. Innate immune system is the first line of defense in which innate immune cells activate against invasion of exogenous chemicals, which are also involved in brain development, brain maturation, and neuroinflammation and neurodegenerative diseases [14]. Microlia and astrocyte (two main kinds of cells in nervous system) are also the resident innate immune cells indicating that they could regulate neuro-immune response [15]. Antigen-presenting cells (APCs) are essential regulators of the innate immune response. Recent studies have reported that microglia and astrocyte are considered professional APCs as they express major histocompatibility complex class II (MHCII) and co-stimulatory molecules (CD40, CD80, and CD86), then participate in the brain impairment as well as regulate innate and adaptive immune response [16, 17]. Ansari et al. found that perinatal arsenic exposure increased the expression of M1 phenotypic marker CD86, TNF-α, and IL-6 and decreased the level of M2 phenotypic marker Arg1 and CD206 in the developing brain [18]. Evidence from in vitro experiments suggested that 0.6 μM iAs and 600 μM di-methylarsenic acid (DMAV) increased the expression of CD86 and CD206 of BV-2 microglia by immunofluorescence analysis, which suggested that arsenic induced microglia activation [19]. Astrocyte also express toll-like receptors (TLR), MHCI, and MHCII in multiple sclerosis (MS) [20]. However, studies in experimental animals on arsenic-induced neuroimmune toxicity are limited.

It is well known that the innate immune system can recognize damage-associated molecular patterns (DAMPs) by pattern recognition receptors (PRRs) including the NOD-like receptor (NLR) [21]. The NLRP3 inflammasome, as a major component of innate immunity, plays a critical role in innate immune homeostasis [22]. Once activated by exogenous and endogenous compounds, the NLRP3 recruit adaptor protein ASC activates caspase-1, secretion interleukin-1β (IL-1β), and IL-18, then enhances the innate immunity or inflammation [23]. Moreover, the activation of NLRP3 inflammasome could participate in a number of neurological injuries and diseases. In middle cerebral artery occlusion rats, the NLRP3 inflammasome was markedly activated, and NLRP3 and caspase-1 expression as well as the secretion of the inflammatory cytokines IL-1β and IL-18 increased in the peri-infarct cortex [24]. In the case of amyotrophic lateral sclerosis (ALS), not only microglia but also astrocyte expressed increased the NLRP3 levels and contributes to activation of the NLRP3 pathway [25]. Neuro-immune dysregulations especially innate immune imbalance is a common feature of many types of neurological diseases and injuries, but its role in arsenic-induced neurotoxicity has rarely been reported.

Therefore, the study is intended to investigate the effects of arsenic on innate immune modulation in the cerebral cortex and the hippocampus. We observed the ability of learning and memory, pathological changes, and neurotrophic factors including brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) levels. Moreover, we also investigated the alterations in microglia and astrocyte functionality and the NLRP3 inflammasome activation. We are trying to provide a new target for understanding the neurotoxicity mechanism of arsenic.

Materials and Methods

Reagents and Chemicals

Sodium arsenite (≥ 99.0%) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Real-time polymerase chain reaction (real-time PCR) kits were obtained from Takara Co. (Otsu Japan). RIPA lysis buffer and BCA protein assay kits were supplied by Beyotime Biotechnology (Beyotime Shanghai, China). Primary antibodies against Cd11b, GFAP, ASC, and caspase-1 and β-actin were purchased from Cell Signaling Technology (Cell Signaling, Danvers, USA); NF-κB, p-NF-κB, and NLRP3 were purchased from Wanleibio (Wanleibio, Shenyang, China); and the corresponding secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals used were of analytical grade.

Animals and Experimental Procedures

Six-week-old female C57BL/6 mice weighing 18–22 g were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China) (National Animals Use License Number: SYXK2019-0005). Animal use was approved by the Animal Use and Care Committee of Shenyang Medical College (protocol number: SYYXY2021031201). The mice were group-housed in stainless steel cages (10 mice per cage) in an air-conditioned room with a temperature of 22 ± 2 ℃ and a 12-h light/dark cycle starting 1 week before

the exposure. The mice were allowed standard mice chow diet and drinking water ad libitum throughout the procedure. As far as the experimental indexes determined in mice, no obvious gender differences were found in liver and kidney by arsenic exposure. In addition, male mice are aggressive and are more easily to hurt each other in the experimental periods. Consequently, female mice were selected to observe the effect of arsenic exposure in our study.

The dose of $NaAsO_2$ was selected based on previously published studies [26], as well as our preliminary experiments. The mice were exposed to arsenic in drinking water at concentrations of 0, 25, and 50 mg/L arsenic for 1 month. The mice were weighed every week during the experimental period. At the end of the experiment, all mice were weighed and deeply anesthetized with intraperitoneal injection of 3.3% chloral hydrate (dissolved in normal saline). The entire brain was promptly removed and weighed, brain samples of three mice were fixed with 4% paraformaldehyde for pathological examination and immunostaining, and the cerebral cortex and hippocampus regions of other brains were isolated and stored at − 80 °C for biochemical analysis.

Morris Water Maze

Morris water maze can assess the long-term memory. After 1-month arsenic exposure, six mice (selected randomly) from each group were tested in the Morris water maze (MWM). The apparatus was a circular white tank with 120 cm in diameter, 45 cm in high, filled with 22–26 ℃ water to a depth of 30 cm. Platform was placed at 2 cm below the surface of the water. A ceiling camera directly over the water maze was connected to computer with Any-Maze tracking software (Stoelting, Wood Dale, IL). On the first day, mice were trained for knowing the position of the platform, on the second to the sixth day, mice were released into the water from different points across trails and were allowed 90 s to locate the platform and remained on the platform for 2 s. The time of reaching the platform, swimming speed, and the time and distance spent were recorded by a video tracking system. On the seventh day, we removed the platform, the mice were allowed to swim for 90 s, then we counted the number of times they crossed the previous platform location.

Histopathological Examination

The cerebral cortex and the hippocampus were fixed in 4% paraformaldehyde, then tissues were rinsed with running tap water and placed in an automatic tissue dehydrator and dehydrated with gradient of high-percentage ethanol, cleared in xylene, dipped in wax, and embedded in paraffin. Tissues were sliced into 5-μm thickness with a microtome. The sections were deparaffinized in xylene, then gradient alcohol for rehydration, and soaked in distilled water for a few minutes. Finally, tissue sections were stained with hematoxylin and alcoholic eosin and examined under an optical microscope (Biodirect-Inc, Nikon, Japan), and the histopathological changes were analyzed.

Total RNA Isolation and Real-Time PCR Analysis

Total RNA was isolated from the cerebral cortex and the hippocampus with TRIzol reagent (Invitrogen, USA), and first-strand cDNA was synthesized from a 1-μg aliquot of the total RNA using the oligo-dT primer and reverse transcriptase. Then PCR amplification was performed by SYBR Premix ExTaqII kits (Takara, Japan). PCR was performed using the following thermal cycling conditions: 95 °C for 30 s, followed by 40 cycles of denaturing at 95 ℃ for 5 s, and annealing at 60 ℃ for 30 s. PCR was performed using the primers as follows:

Bdnf(F):CATAGACAAAAGGCACTGGAACTC, (R):TAAGGGCCCGAACATACGAT, product length 62 bp, *Vegf*(F):AGTGGTCCCAGGCTGCAC, (R):TCCATGAAC TTGATCACTTCAT, product length 67 bp, *MhcII*(F):AGA GACCA.

TCTGGAGACTTG, (R):CATCTGGAGTGTTGTTGGA, product length 131 bp, *Cd40*(F):CCTGGACAAGCTGTG AGGAT, (R):ACACCCCGAAAATGGTGATGA, product length 147 bp, *Il-18*(F): GGCTGCCATGTCAGAAGACT, (R):GTCTGGTC.

TGGGGTTCACTG, product length 121 bp, *Ccr5*(F): CAAGACAATCCTGATCG.

TGCAA, (R): TCCTACTCCCAAGCTGCATAGAA, product length 128 bp, *Ccr7*(F): GGGAAACCCAGGAAA AACGTG, (R)CTTGCTGATGAGAAGCACGC, product length 519 bp, *Gapdh*(F): TGTGTCCGTCGTGGATCTGA, (R): TTGCTGTTGAAG.

TCGCAGGAG, product length 150 bp. $2^{-\Delta\Delta Ct}$ values were calculated to represent the expression levels of different target genes.

Immunofluorescence Staining

Immunofluorescence staining was performed in tissue sections. First, the tissue was embedded in paraffin and cut to 4-μm paraffin sections. Next, tissue sections were gradient deparaffinized, rehydrated, and then pre-blocked with 10% goat serum for 30 min at room temperature. The expression of CD11b and GFAP was measured by adding primary antibodies, followed by the secondary antibody of goat CY3 anti-rabbit IgG (SA00009-2, Proteintech group, Wuhan, China). Nuclei were stained with DAPI (D10647, Aladdin Bio-Chem Technology Co., LTD, Shanghai, China) for 10 min at room temperature afterwards. After

three rinses and sealing sheet, fluorescence images were acquired by using a confocal microscope (DS-U3, Nikon, Japan).

Western Blot Analysis

Total proteins were extracted from the cerebral cortex and the hippocampus by commercial kits, and protein concentrations were quantified by a bicinchoninic acid (BCA) protein kit (Beyotime, Shanghai, China). Forty-five micrograms of total protein was boiled with the sample loading buffer for 3 min at 100 ℃ before being separated by 7.5–10% SDS-PAGE and then transferred to a 0.22 μM polyvinylidene fluoride (PVDF) membrane (Amersham, Buckinghamshire, UK). After being blocked for 2 h at 4 ℃, the membranes were probed with primary antibodies against NF-κB, p-NF-κB, NLRP3, ASC, caspase-1, and β-actin (1:1000) at 4 ℃ overnight, then rinsed with TBST five times for 7 min each time. Finally, the membranes were incubated with the corresponding secondary antibodies (1:5000) for 2 h at room temperature, and the cleaning method is the same as the primary antibody. Blots were detected with chemiluminescence reagents (PicoWest Super Signal, Pierce Biotechnology, IL, USA) and visualized using electrophoresis gel imaging analysis system (MF-ChemiBIS 3.2, DNR Bio-Imaging Systems, Israel), and analyzing the optical densities of target protein bands with the Image J software; β-actin (1:5000) was used as the internal control.

Statistical Analysis

The data were expressed as mean ± standard deviation (SD). Comparisons among groups were made using one-way analysis of variance (ANOVA) with LSD post hoc test using the SPSS 25.0 statistical analysis software. $p < 0.05$ was considered to be statistically significant.

Results

General Status of the Study Mice

In our study, the mice were treated with 0, 25, and 50 mg/L arsenic for 1 month by drinking water. All animals survived to the end of the experiment. As shown in Fig. 1A, the body weight of control or arsenic-treated mice all increased with time of exposure. No statistically significant differences of body weight and brain weight have been observed at the end of the experiment. The corticocerebral and the hippocampal weights in the arsenic-treated groups were consistently higher than those in the control group (Fig. 1C, D, $p < 0.05$).

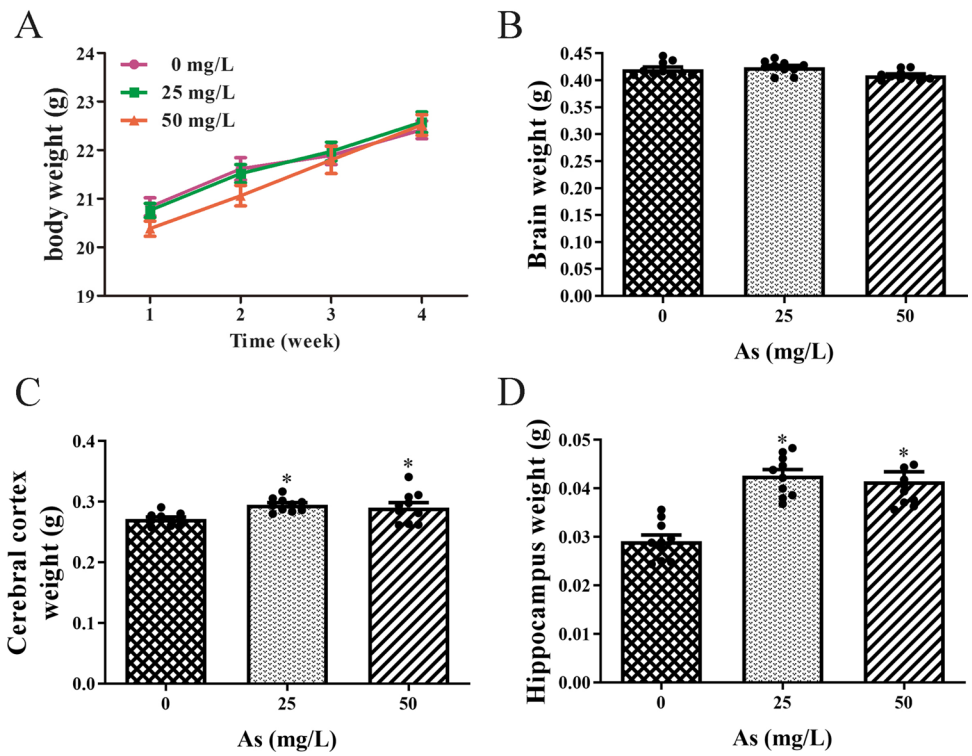

Fig. 1 Effects of arsenic exposure on body weight and brain, corticocerebral, and hippocampal weights in mice. Mice were treated with 0, 25, and 50 mg/L arsenic by drinking water for 1 month; the mice were weighed every week during the experimental period. The cerebral cortex and hippocampus were removed and weighed. **A** Body weight; **B** brain weight; **C** cerebral cortex weight; **D** hippocampus weight. The results were expressed as the mean ± SD ($n = 10$). *$p < 0.05$ compared with the control mice

Arsenic Injuries Learning and Memory Impairment in Mice

The effects of arsenic on learning and memory ability were detected with Morris water maze. As the training days increased, the latency to platform showed decreasing trend in every group. Compared with the control group, arsenic markedly increased the latency to platform on the fourth training day in 50 mg/L and the fifth training day in 25 and 50 mg/L (Fig. 2A, $p < 0.05$). On the sixth day, the stage was removed from the maze; we found the time of the arsenic-treated group in path length in target quadrant, the time spent in target quadrant, and crossing times of the platform were decreased; there also existed significant difference in path length in target and time spent in target quadrant between 25 and 50 mg/L arsenic-treated groups (Fig. 2B, C, D, $p < 0.05$).

Arsenic Induces Pathological Changes in the Cerebral Cortex and the Hippocampus

Histopathological changes in the cerebral cortex and the hippocampus were examined by HE staining. As shown in Fig. 3, the structure of the cerebral cortex and the hippocampus in the control group was normal and arranged orderly, with even distribution of neuron. In contrast, the tissue structure of the cerebral cortex and the hippocampus in arsenic-exposed mice was sparsely arranged, and the neurons were arranged in an irregularity way with more vacuolated degeneration and pyknosis, especially in 50 mg/L arsenic-treated mice. Otherwise, the number of pycnotic cells of mice in the 50 mg/L arsenic-treated group is more than lower than the 25 mg/L arsenic-treated group in cerebral cortex and those of mice at CA3 and DG region had significant difference in the number of pycnotic cells between 25 and 50 mg/L arsenic-treated groups.

Arsenic Decreases the Expression of Angiotrophic and Neurotrophic Factors

It has been reported that neurotrophic factors (NTFs) such as BDNF and VEGF can affect the process of learning and memory. We observed the mRNA levels of BDNF and VEGF in the brain. As shown in Fig. 4A, arsenic down-regulated the mRNA levels of BDNF and VEGF in the cerebral cortex, and there was a significant difference only in the 50 mg/L arsenic-treated group ($p < 0.05$). Consistent with these effects in the cerebral cortex, the mRNA levels of BDNF and VEGF were also markedly decreased in the hippocampus in the 50 mg/L arsenic-treated group when compared with the control group and the 25 mg/L arsenic-treated group (Fig. 4B, $p < 0.05$).

Arsenic Induces Microglia and Astrocyte Activation in the Cerebral Cortex and the Hippocampus

Innate immune response can participate in brain injury and neuronal death. Microglia and astrocyte are the known resident innate immune cells of the central nervous system, so

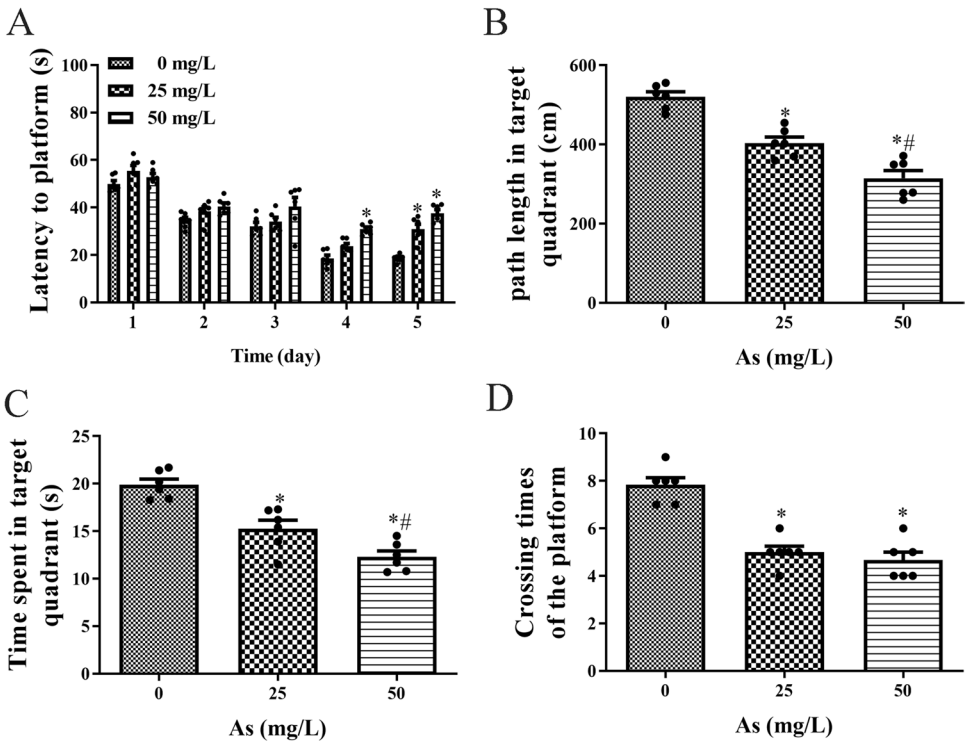

Fig. 2 Effect of arsenic exposure on learning and memory ability in the mice. Mice were treated with 0, 25, and 50 mg/L arsenic by drinking water for 1 month. After the treatment, Morris water maze test was performed to analyze the learning and memory ability of the mice. **A** Latency to platform; **B** path length in target quadrant; **C** time spent in target quadrant; **D** crossing times of the platform. The results were expressed as the mean ± SD ($n = 6$). *$p < 0.05$ compared with the control mice. #$p < 0.05$ compared with the 25 mg/L arsenic-treated mice

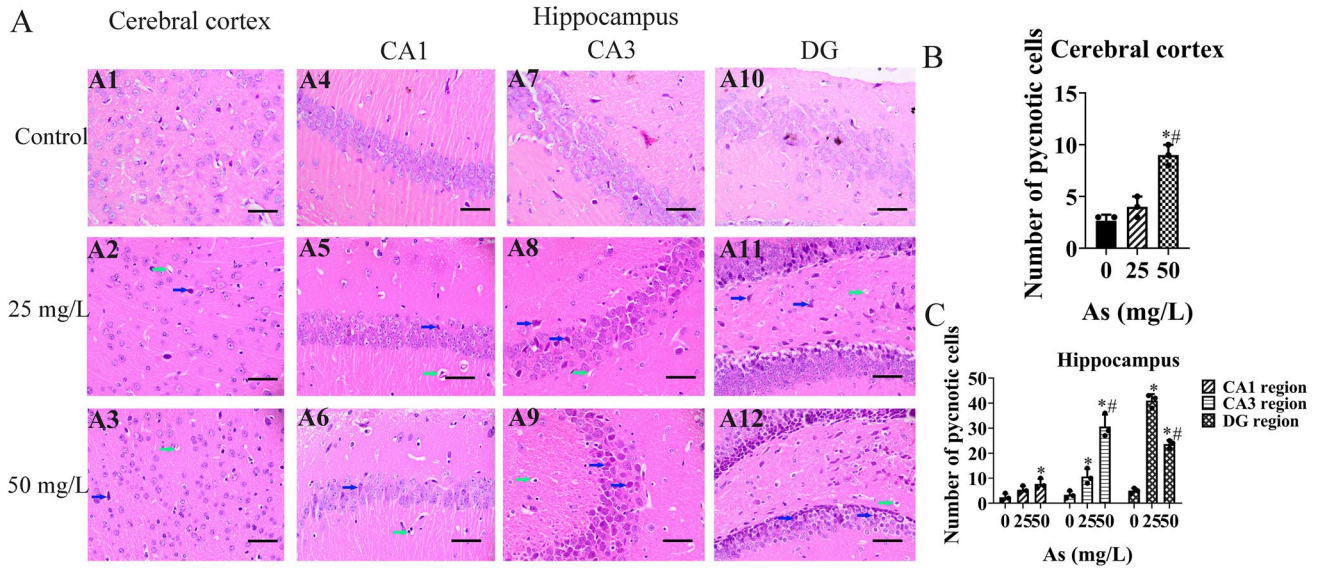

Fig. 3 Histopathological changes in corticocerebral and hippocampal tissues of arsenic-treated mice by hematoxylin–eosin (HE) assay (original magnification, ×400). The mice were treated with 0, 25, and 50 mg/L $NaAsO_2$ for 1 month. (**A1**) Cerebral cortex in the control group; (**A2**) cerebral cortex in the 25 mg/L arsenic-treated group; (**A3**) cerebral cortex in the 50 mg/L arsenic-treated group; (**A4**) hippocampal CA1 region in the control group; (**A5**) hippocampal CA1 region in the 25 mg/L arsenic-treated group; (**A6**) hippocampal CA1 region in the 50 mg/L arsenic-treated group; (**A7**) hippocampal CA3 region in the control group; (**A8**) hippocampal CA3 region in the 25 mg/L arsenic-treated group; (**A9**) hippocampal CA3 region in the 50 mg/L arsenic-treated group; (**A10**) hippocampal DG region in the control group; (**A11**) hippocampal DG region in the 25 mg/L arsenic-treated group; (**A12**) hippocampal DG region in the 50 mg/L arsenic-treated group. Green arrows point to the vacuolated degeneration of neurons. Blue arrows point to the pyknosis of neurons. The number of pyknotic cells in the picture using photoshop by manual count. Scale bars, 50 μm. Quantity analysis of pycnotic cells numbers in the cerebral cortex and hippocampus is shown in panels B and C, respectively. $^{*}p < 0.05$ compared with the control mice, $^{\#}p < 0.05$ compared with the 25 mg/L arsenic-treated mice

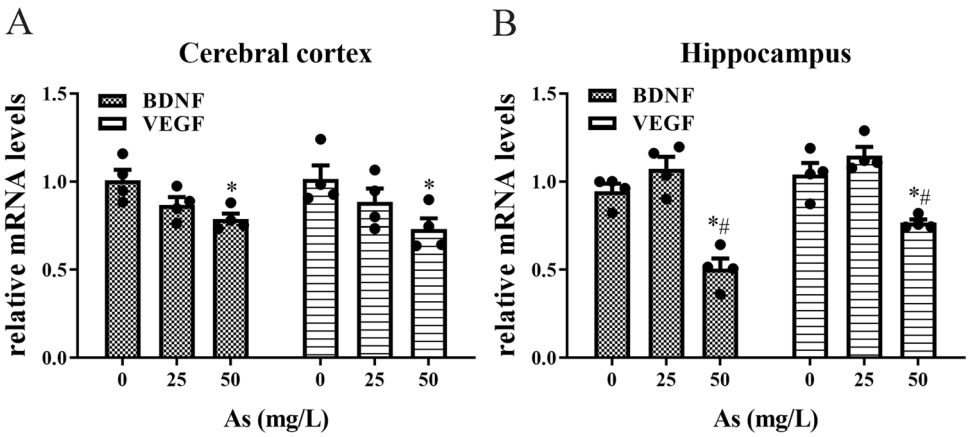

Fig. 4 Effect of arsenic exposure on the expression of neurotrophic factors in the cerebral cortex and the hippocampus. The mice were treated with 0, 25, and 50 mg/L arsenic by drinking water for 1 month. Total RNA of the cerebral cortex and the hippocampus was isolated and real-time PCR was conducted. The mRNA levels of neurotrophic factors BDNF and VEGF were shown in the cerebral cortex (**A**) and the hippocampus (**B**). The results were expressed as the mean ± SD ($n = 4$), $^{*}p < 0.05$ compared with the control mice, $^{\#}p < 0.05$ compared with the 25 mg/L arsenic-treated mice

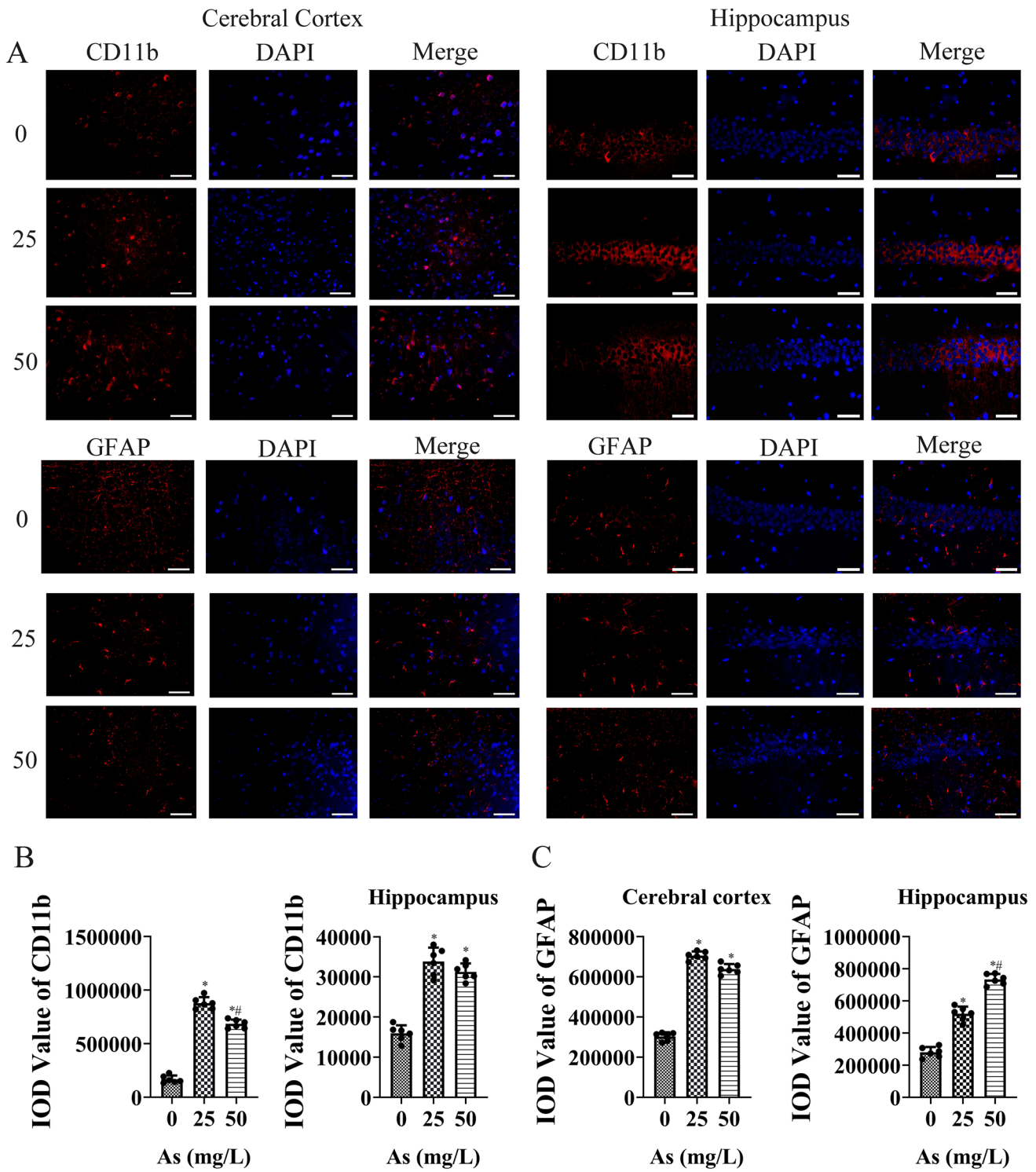

Fig. 5 Effect of arsenic exposure on microglia and astrocyte activation in the cerebral cortex and the hippocampus by immunofluorescence staining (400×). The mice were treated with 0, 25, and 50 mg/L arsenic by drinking water for 1 month. **A** The cerebral cortex and the hippocampus sections were stained with FITC and DAPI to visualize CD11b-positive and GFAP-positive cells (red) and cell nuclei (blue), respectively. Scale bars = 50 μm. **B** Analysis of the integral optical density (IOD) of CD11b-positive cells in cerebral cortex and hippocampus. **C** Analysis of the integral optical density (IOD) of GFAP-positive cells in cerebral cortex and hippocampus. The results were expressed as the mean ± SD. Each result was detected by randomly selected zone, and each experiment was carried out under the condition of selecting three samples within each group. $^{*}p < 0.05$ compared with the control mice, $^{\#}p < 0.05$ compared with 25 mg/L arsenic-treated mice

we detected the expression of CD11b and GFAP, which were used as microglia and astrocyte activation markers in the cerebral cortex and the hippocampus. As shown in Fig. 5, arsenic increased the IOD of CD11b-stained microglia cells and GFAP-stained astrocyte cells in the cerebral cortex and the hippocampus, in which the IOD of corticocerebral CD11b-stained microglia cells in the 50 mg/L arsenic-treated group were also significantly lower than that of the 25 mg/L arsenic-treated group; the hippocampal GFAP-stained astrocyte cells in the 50 mg/L arsenic-treated group also increased significantly compared with the 25 mg/L arsenic-treated group (Fig. 5B, C, $p < 0.05$).

Arsenic Affects the Expression of Antigen-Presenting Function-Related Molecules

Microglia and astrocyte are considered the CNS antigen-presenting cells. Antigen presentation requires the expression of MHC-II and co-stimulatory molecules CD40. Thus, the mRNA levels of MHC-II and CD40 in the cerebral cortex and the hippocampus were assessed by real-time PCR. In Fig. 6A, the mRNA levels of the corticocerebral MHCII and CD40 were elevated markedly by 25 and 50 mg/L arsenic exposure ($p < 0.05$). We also found notable increases in the mRNA levels of the MHCII in different arsenic-treated groups, as well as an increase of CD40 in the hippocampus by 50 mg/L arsenic exposure (Fig. 6B, $p < 0.05$). In addition, CD40 mRNA levels in the 50 mg/L arsenic-treated group was also higher than each 25 mg/L arsenic-treated group.

Given that microglia and astrocyte migration are crucial for antigen-specific activation of T cells, we further investigated the mRNA levels of the chemokine receptors CCR5 and CCR7. The mRNA levels of CCR5 and CCR7 were increased by 39.24% and 43.19% in the cerebral cortex with 50 mg/L arsenic treatment, respectively (Fig. 6C, $p < 0.05$). There also existed significant difference in CCR5 mRNA levels between 25 and 50 mg/L arsenic-treated groups ($p < 0.05$). Our results showed significant increases of CCR7 in the hippocampus by 25 and 50 mg/L arsenic treatment (Fig. 6D, $p < 0.05$).

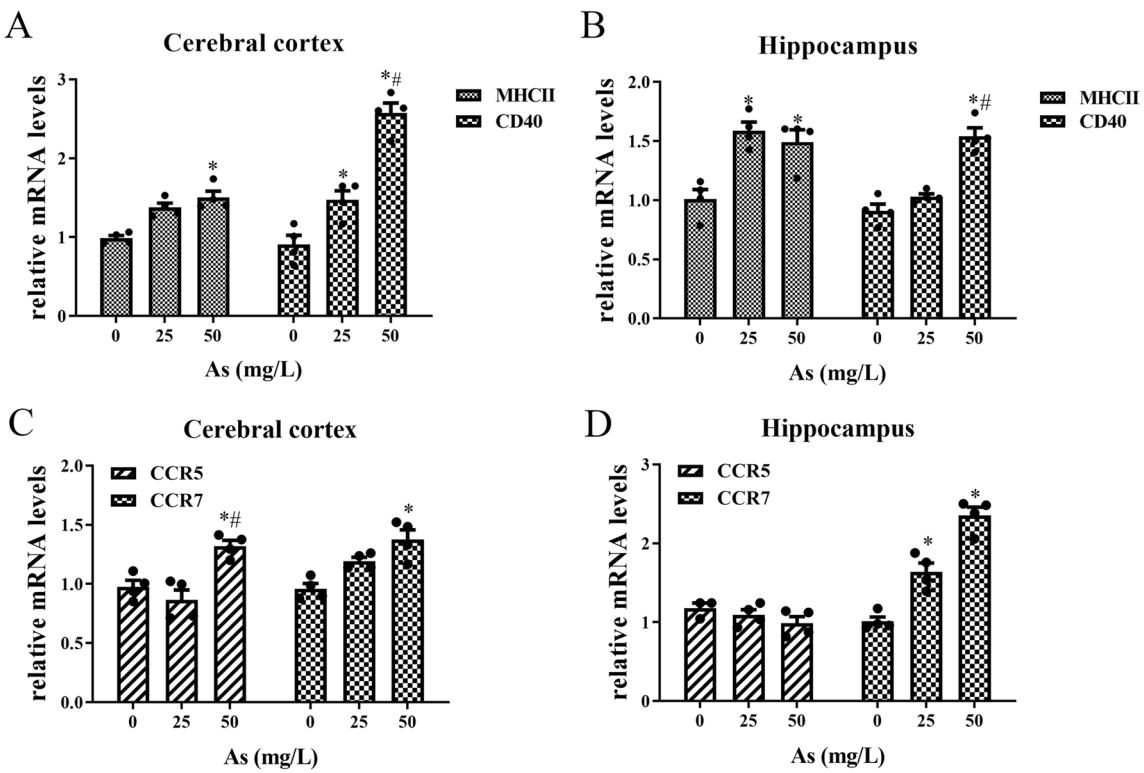

Fig. 6 Effect of arsenic exposure on the antigen-presenting function related molecules in the cerebral cortex and the hippocampus. The mice were treated with 0, 25, and 50 mg/L arsenic by drinking water for 1 month. Total RNA of the cerebral cortex and the hippocampus was isolated and real-time PCR was conducted. The mRNA levels of antigen-presenting molecule MHCII and costimulatory molecules CD40 were shown in the cerebral cortex (**A**) and the hippocampus (**B**), as well as the mRNA levels of chemokine receptor CCR5 and CCR7 were shown in the cerebral cortex (**C**) and the hippocampus (**D**). The results were expressed as the mean ± SD ($n = 4$), $^{*}p < 0.05$ compared with the control mice, $^{\#}p < 0.05$ compared with the 25 mg/L arsenic-treated mice

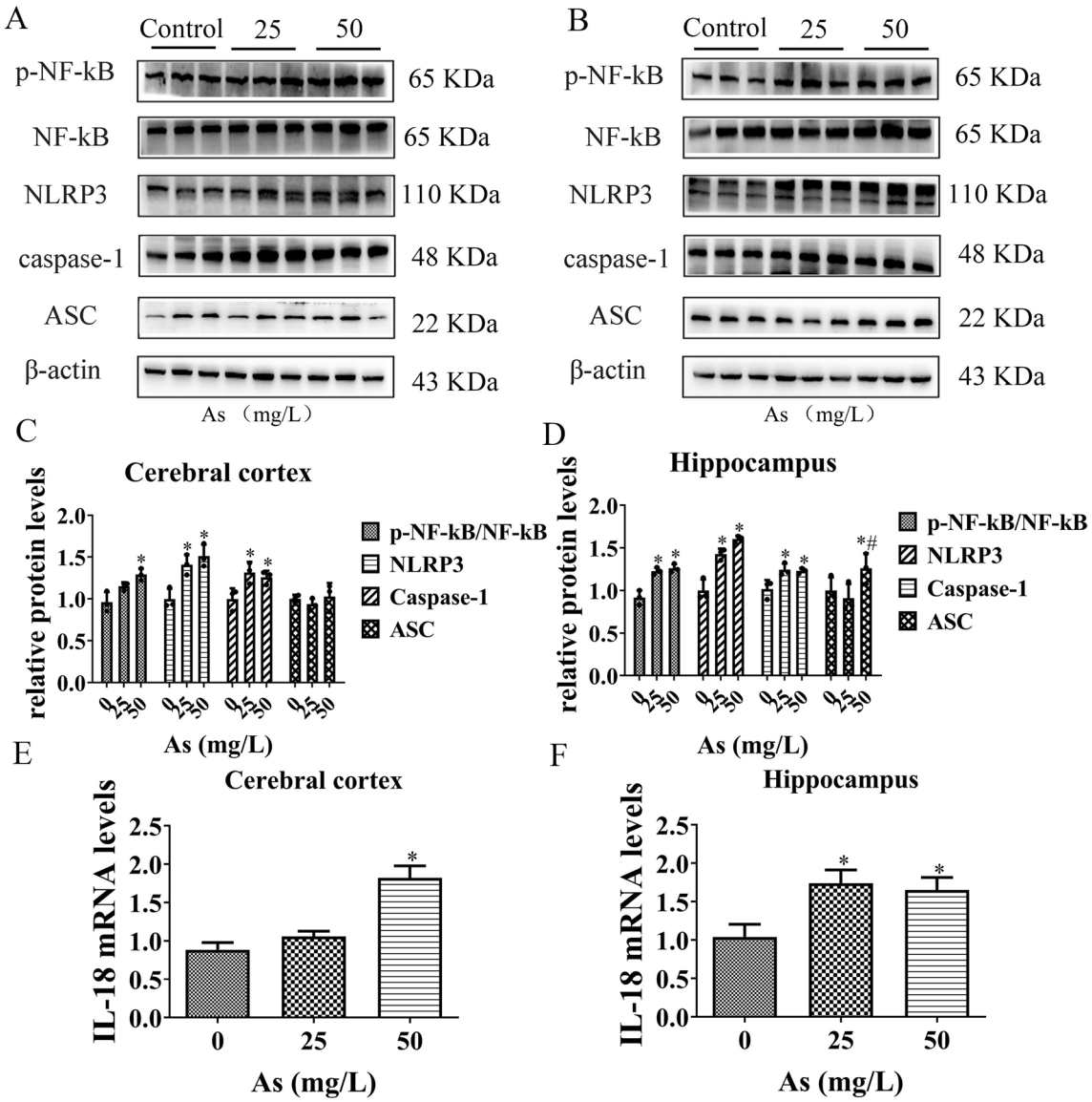

Fig. 7 Effect of arsenic exposure on the NF-κB/NLRP3 inflammasome in the cerebral cortex and the hippocampus. The mice were treated with 0, 25, and 50 mg/L arsenic by drinking water for 1 month. Expression of p-NF-κB, NF-κB, NLRP3, caspase-1, and ASC in the cerebral cortex (**A**) and the hippocampus (**B**) and the corresponding quantitative analysis (**C**, **D**) were assessed by western blotting, the expression of p-NF-κB, expressed as a ratio relative to that of NF-κB. β-Actin was blotted as the loading control. Total RNA of the cerebral cortex and the hippocampus was isolated and real-time PCR was conducted. The mRNA level of IL-18 was shown in the cerebral cortex (**E**) and the hippocampus (**F**). The results were expressed as the mean ± SD. $^{*}p < 0.05$ compared with the control mice, $^{\#}p < 0.05$ compared with the 25 mg/L arsenic-treated mice

Arsenic Activates NF-κB and the NLRP3 Inflammasome in the Cerebral Cortex and the Hippocampus

The NLRP3 inflammasome has been shown to play a vital role in the innate immune responses. As shown in Fig. 7, the protein expression of NLRP3 and caspase 1 were increased in the corticocerebral and the hippocampi of 25 and 50 mg/L arsenic-treated mice (Fig. 7C, D, $p < 0.05$). In addition, the 50 mg/L arsenic increased the protein levels of ASC in the hippocampus compared with the control group and 25 mg/L arsenic-treated mice (Fig. 7D, $p < 0.05$). NF-κB is an essential transcription factor which involved in inflammation and immunity. Activated NF-κB can activate the NLRP3 inflammasome; p-NF-κB expression was markedly and consistently increased in the cerebral cortex and the hippocampus (Fig. 7C, D). In addition, arsenic increased the mRNA levels of the NLRP3 regulated downstream cytokine IL-18 not only in the cerebral cortex in 50 mg/L arsenic-treated mice but also in the hippocampus in 25 and 50 mg/L arsenic-treated

mice (Fig. 7E, F, $p < 0.05$). However, the P-NF-kB, NLRP3, and caspase-1 mRNA levels showed no significant changes among arsenic groups in cerebral cortex and hippocampus.

Discussion

Epidemiological and animal model studies have demonstrated that arsenic can induce cognitive dysfunction, and linked to neurodegenerative disorders [27]. It has long been believed that brain was an immune privileged tissue. Recently, neuroscientists showed that the central nervous system does not display absolute immunological privilege, because it has some immune cells such as microglia and astrocyte, which could induce the innate immunity and the adaptive immune response [28]. The imbalance of immune homeostasis was reported to be involved in the occurrence and development of the nervous system diseases and nerve injury [29]. However, effects by which innate immune response contribute to the progression of arsenic neurotoxicity are poorly defined. The weights and indexes of organs are generally used to evaluate the tissue functions. Mehta et al. found that mice were administered with As_2O_3 (2.0 and 4.0 mg/kg bw) for 45 days, and the results showed a significant decrease in brain weight compared to the control group [30]. However, in our study, mice study treated with 25 and 50 mg/L arsenic for 1 month showed marked increases in the weight in the cerebral cortex and the hippocampus. These disparities in the results might be associated with different arsenic doses and durations, as well as diverse mice strains. The effects of arsenic on neurological behavior and learning and memory ability were detected with Morris water maze. Arsenic treatment markedly increased latency to platform on the fourth and fifth day as well as decreased path length in target quadrant, time spent in target quadrant, and crossing times of the platform, which is consistent with previous reports [31, 32]. Histopathologically, arsenic-induced structural changes of the cerebral cortex and the hippocampus accompanied by vacuolated degeneration and pyknosis was observed. Wu et al. found the changes in the brain structure include neuronal pyropysis and vacuolar degeneration by arsenic trioxide [33]. These results suggested that arsenic can induce neurotoxicity in vivo.

NTFs, such as BDNF and VEGF, were the neurobiological markers of learning and memory disorders [34]. BDNF was neurotrophic factor involved in synaptic plasticity and neurogenesis [35]. VEGF is pleiotrophic growth factor expressed by the neurons and astrocyte, which could mediate vascular permeability, neurotrophic activity, and so on. Mehta et al. found that As_2O_3 (2 and 4 mg/kg bw) that induced the behavioral deficits were accompanied by reduction of protein levels of BDNF in the hippocampus of mice [30]. In pathological conditions, such as ALS, Alzheimer's disease (AD), and brain injury, VEGF was marked downregulated in the hippocampus, further destroyed the blood–brain barrier, and induced cognitive impairment [36]. Our results demonstrated that BDNF and VEGF were significantly decreased in the cortex and the hippocampus of arsenic-treated mice, suggesting the suppression of NTF expression might be one of the main reasons for arsenic-induced memory impairment.

Microglia and astrocyte are the main innate immune cells in the nervous system, which can release pro-inflammatory and chemokines cytokine, and initiate innate immune response [37, 38]. CD11b (also known as OX42, Mac-1) is a common surface marker of microglia; the increased expression of CD11b in the brain likely represents priming and activating of microglia; activated astrocyte is also represented by increasing levels of GFAP and functional impairment [27]. It has been reported that microglial activation can contribute to brain injury [39], neuroinflammation [40], and neurodegenerative diseases including AD and Parkinson's disease (PD) [12]. Shuhua et al. found that 5–20 mg/L NaF can stimulus BV-2 cells to change into activated microglia displaying upregulated OX-42 expression [41]. Zhang et al. found chronic arsenic exposure (50 mg/L $NaAsO_2$ for 24 weeks) can induce microglia activation in the cerebral cortex and the hippocampus of mice [19]. Upregulated expression of GFAP in the brain was observed by Kuzu et al. [42] in mice exposed to 10 mg/kg $NaAsO_2$ for 15 days. In our study, arsenic exposure showed trend to increase the expression of CD11b and GFAP in the cerebral cortex and the hippocampus, suggesting that arsenic induced the activation of microglial and astrocyte in the brain and affect the innate immune response.

It was reported that microglia and astrocyte are CNS APCs that mediate innate and adaptive immune response [43]. APCs could express high levels of antigen-presenting molecule MHCII and costimulatory molecules (CD40, ICAM-1, CD80, and CD86) to present antigens to T cells, then promote T cell activation and proliferation, and finally initiate adaptive immune responses [44]. The overexpression of MHCII, CD80, and CD86 was observed in the lung and spleen of the arsenic-treated mice [45]. In the aspect of neurological research, the expression of MHCII and CD40 was markedly increased by activated microglia in response to neuronal damage and neuroinflammation [46]. Similar to microglia, reactive astrocyte increased the expression of MHCII in the MS lesions, suggesting the role for microglia and astrocyte in the antigen presentation [47]. In our study, arsenic exposure induced tendency toward increasing the mRNA levels of MHCII and CD40 in the cerebral cortex and the hippocampus. Chemokines, a vital part of host immunity, play important roles in the regulating innate, adaptive immune response and immune surveillance by interaction chemokine receptor [48]. Beside dendritic cells (DC),

natural killer (NK), macrophages, and chemokine receptors CCR5 and CCR7 were present in microglia and astrocyte of the nervous system which can mediate the migration, pathological neuroinflammation, and neuronal development of immune cells [49, 50]. Kikumura et al. found the mRNA levels of CCR5 and CCR7 were upregulated in the brain of murine ocular toxoplasmosis [51]. Zhao et al. confirmed that arsenic can increase the levels of CCR5 and CCR7 in the spleen and the lung [45]. Our experimental results showed that arsenic significantly increased the levels of corticocerebral CCR5 and CCR7 and hippocampal CCR7. These results hypothesized that arsenic might affect the antigen presentation in the nervous system and lead to neuro-immune imbalance.

The NLRP3 inflammasome is a major component of the innate immunity. The activation of the NLRP3 inflammasome is widely reported in multiple endogenous and exogenous chemical-induced tissue damage, and it could also be activated by NF-κB pathway [52, 53]. Once NLRP3 activated, pro-inflammatory cytokines IL-1β and IL-18 initiate an inflammatory cascade that leads to the recruitment of innate immune cells and induces subsequent adaptive immune response [54]. In addition, NLRP3 was located in microglia, astrocyte, and neurons in the brain, which was central to the pathogenesis of many brain diseases including AD, PD, ALS, and traumatic brain injury (TBI) [55]. Jia et al. found that 2.5 and 5 mg/kg $NaAsO_2$ exposure via oral gavage for 3 months resulted in a significant upregulation in NLRP3, ASC, procaspase-1, caspase-1, IL-1β, and IL-18 proteins of the liver in SD rat [56]. Doxorubicin could enhance protein expression of p-NF-κB, NLRP3, procaspase-1, caspase-1, and IL-1β in the hippocampi and the prefrontal cortices of rats, then induced memory impairment [57]. In our study, the proteins levels of p-NF-κB, NLRP3, and caspase-1 as well as the mRNA levels of IL-18 were all elevated in the cerebral cortex and the hippocampus of the arsenic-treated mice. Moreover, the protein levels of ASC were markedly increased in the hippocampus in the 50 mg/L arsenic-treated groups. These results suggested that the NLRP3 inflammasome was also involved in the neural-immune abnormalities by arsenic.

Conclusions

In the current study, we conclusively showed that arsenic induced the neuro-immune toxicity by decreasing the expression of the neuroprotective factor, inducing the expression of immune cells such as microglia and astrocyte, modulating the function of antigen presenting and the expression of chemokine receptor–related molecular as well as activating the NLRP3 inflammasome. These results provide a novel target for treating arsenic-induced neurotoxicity.

Author Contributions Nan Yan: conceptualization, data curation, formal analysis, visualization and writing—original draft. Zhengdong Wang: revision, data curation. Zhou Li: resources, validation, supervision and editing. Yang Zheng: software, investigation, revision, data curation and validation. Nan Chang: formal analysis and software. Kangjie Xu: investigation. Qian Wang: methodology. Xiaoxu Duan: funding acquisition; writing—review and editing; supervision; project administration.

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Data availability The data supporting the findings of this study are presented within the manuscript.

Declarations

Competing Interests The authors declare no competing interests.

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