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Arsenic Activates the NLRP3 Inflammasome and Disturbs the Th1/Th2/Th17/Treg Balance in the Hippocampus in Mice

Hui Jing[1] · Nan Yan[2] · Ronghua Fan[3] · Zhou Li[1] · Qian Wang[1] · Kangjie Xu[1] · Xinkang Hu[4] · Lifeng Zhang[5] · Xiaoxu Duan[1]

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Abstract

Arsenic exerts neurotoxicity and immunomodulatory effects. Studies have shown that the nervous system is not considered to be an immune-privileged site. However, the effect of arsenic-induced neuroimmune toxicity has rarely been reported. We aimed to investigate the toxic effects of arsenic on the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome and the Th1/Th2/Th17/Treg balance in the brain tissue of mice. Mice were exposed to $NaAsO_2$ (0, 2.5, 5, and 10 mg/kg) for 24 h. Our results showed that 10 mg/kg arsenic exposure significantly decreased brain and hippocampal indices ($p < 0.05$). The mRNA and protein levels of the blood−brain barrier (BBB) tight junction protein occludin were decreased in the 5 and 10 mg/kg arsenic-treated groups. Compared with those in the control group, NLRP3 protein levels in 10 mg/kg arsenic-treated mice, caspase-1 protein levels in 2.5, 5, and 10 mg/kg arsenic-treated mice, and IL-1β protein levels in 5 and 10 mg/kg arsenic-treated mice were increased in the hippocampus ($p < 0.05$). In addition, arsenic induced a hippocampal inflammatory response by upregulating the mRNA levels of the proinflammatory factors IL-6 and TNF-α and downregulating the mRNA level of the anti-inflammatory factor IL-10. Moreover, arsenic decreased the mRNA levels of the Th1 and Th2 transcription factors T-bet and GATA3 and the cytokines IFN-γ and IL-4 and increased the mRNA levels of the Th17 transcription factor RORγt and the cytokine IL-22 ($p < 0.05$). Collectively, our study demonstrated that arsenic could induce immune-inflammatory responses by regulating the NLRP3 inflammasome and $CD4^+$ T lymphocyte differentiation. These results provide a novel strategy to block the arsenic-induced impairment of neuroimmune responses.

Keywords Arsenic · T lymphocyte · NLRP3 inflammasome · Hippocampus · Neuroimmune

Introduction

Arsenic is classified as a group I human carcinogen by the International Agency for Research on Cancer (IARC) [1]. The brain is one of the major target organs for arsenic poisoning because arsenic can cross the blood−brain barrier (BBB) and enter the brain [2]. Epidemiological reports have demonstrated that arsenic exposure could affect intellectual and cognitive function in children [3, 4]. Zhou et al. showed that postnatal days 4–10 (P4–P10) rats exposed to arsenic showed deficits in learning and social skills, as well as abnormal morphologic changes in the external granular layer and external pyramidal layer [5]. In addition to its neurotoxicity, arsenic is toxic to immune organs, immune cells, and immune molecules [6, 7]. 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 [8]. Neuroimmunological studies showed that

Hui Jing and Nan Yan contributed equally.

✉ Lifeng Zhang
zgykdxzlf@163.com

✉ Xiaoxu Duan
duanxiaoxu@symc.edu.cn

1 Department of Toxicology, School of Public Health, Shenyang Medical College, Shenyang 110034, China

2 Department of Medical Applied Technology, Shenyang Medical College, Shenyang 110034, China

3 Department of Health Inspection, College of Public Health, Shenyang Medical College, Shenyang 110034, China

4 Clinical Medicine ("5+3" integrated Training), The First Affiliated Hospital of China Medical University, Shenyang 110001, China

5 Department of Maternal, Child and Adolescent Health, School of Public Health, Shenyang Medical College, Shenyang 110034, China

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the nervous system is not an immune-privileged site. In contrast, nervous system contains many immune cells (microglia, astroglia, and lymphocytes), associated with the innate immune system and adaptive immune system, which play important roles in physiological and pathological processes. However, some studies have focused on the effects of inorganic arsenic on neuroimmunotoxicity.

Inflammation participates in the occurrence and development of many diseases, including neurological diseases and injuries. Wistar rats exposed to 10 mg/kg sodium arsenate for 8 days showed significant increases in the mRNA levels of the inflammatory markers IL-1β, TNF-α, and IFN-γ in the hippocampus [9]. In addition, some neurodegenerative processes, such as Alzheimer's disease (AD), vascular dementia (VAD), Parkinson's disease (PD), and multiple sclerosis (MS), were shown to be associated with neuroinflammation and immune dysregulation [10]. The NLRP3 inflammasome, which is a major component of innate immunity that consists of NLRP3, the adaptor protein ASC, and the protease caspase-1, plays a critical role in immune homeostasis and inflammation [11]. Moreover, the activation of the NLRP3 inflammasome could play an important role in a number of neurological injuries and diseases. In the Gulf War Illness rat model, 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, was increased in the hippocampus [12]. $CD4^+$ T cells are essential for mediating adaptive immunity in response to a variety of xenobiotics [13]. $CD4^+$ T cells can differentiate into T helper cells, including Th1, Th2, Th17, and regulatory T cells (Tregs) [14]. In Alzheimer's disease, an imbalance in Th17 and Tregs occurs, which is characterized by high levels of the cytokines IL-17 and IL-23 and decreased levels of TGF-β and IL-35 in the cerebral cortex and hippocampus, which may be associated with cognitive impairment [15].

To date, the immunomodulatory and inflammatory potential of arsenic in the nervous system has not been extensively investigated in vivo. In the present study, we first examined the inflammatory response and NLRP3 inflammasome activation in the hippocampus. Moreover, BBB alterations and $CD4^+$ T lymphocyte differentiation were examined. The resultant findings provide experimental evidence of inorganic arsenic-induced neuroimmune toxicity and have implications for the development of preventive and corrective targets to treat arsenic-induced neurotoxicity.

Methods and Materials

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 NLRP3 and caspase-1 were purchased from Cell Signaling Technology (Cell Signaling, Danvers, USA); CD4, occludin, and IL-1β 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: SYYXY2020031201). 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 for 1 week before the experiment. All laboratory mice had free access to a maintenance diet (Beijing Vital River Laboratory Animal Technology, Beijing, China) and drinking water ad libitum before and throughout the procedure.

The dose of $NaAsO_2$ was selected based on previously published studies [16], as well as our preliminary experiments. The mice were intragastrically administered 0, 2.5, 5, and 10 mg/kg $NaAsO_2$ for 24 h. At the end of the experiment, all mice were weighed and deeply anesthetized. Orbital blood was collected in heparinized vials and centrifuged (3000 × g, 4 ℃) for 10 min. The obtained serum was frozen at − 80 ℃ for analysis. The entire brain was promptly removed and weighed, and the hippocampal region was isolated and stored at − 80 ℃ for biochemical analysis.

Calculation of Brain and Hippocampus Indices

Brain and hippocampus indices were calculated according to the following formula: $(tissue\ weight/body\ weight) \times 100$.

Total RNA Isolation and Real-Time PCR Analysis

Total RNA was isolated from the hippocampus with TRIzol reagent (Invitrogen, USA). RNA (500 ng) was reverse transcribed into cDNA and amplified using Takara reagents (Takara, Japan) according to the manufacturer's protocol. Then, PCR amplification was performed by SYBR Premix ExTaq II kits (Takara, Japan). PCR was performed using the following thermal cycling conditions: 95 ℃ 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 listed in Table 1. The $2^{-\Delta\Delta Ct}$ values were calculated to represent the expression levels of different target genes.

Western Blot Analysis

Total proteins were extracted from 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 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 room temperature, the membranes were probed with primary antibodies against occludin, NLRP3, caspase-1, IL-1β, CD4, and β-actin (1:1000) at 4 ℃ overnight. Finally, the membranes were incubated with the corresponding secondary antibodies (1:5000) for 2 h at room temperature. The blots were detected with chemiluminescence reagents (PicoWest Super Signal, Pierce Biotechnology, IL, USA) and visualized using an electrophoresis gel imaging analysis system (MF-ChemiBIS 3.2, DNR Bio-Imaging Systems, Israel). β-actin (1:5000) was used as the internal control.

Table 1 Primer sequences used in the real-time PCR

Gene	Primer sequences	Product length
Occludin	(F):TTGAAAGTCCACCTCCTTACAGA (R):CCGGATAAAAAGAGTACGCTGG	129 bp
IL-6	(F):CTGCAAGAGACTTCCATCCAG (R):AGTGGTATAGACAGGTCTGTTGG	131 bp
TNF-α	(F):CCCCAAAGGGATGAGAAGTTC (R):GGCTTGTCACTCGAATTTTGAGA	101 bp
IL-10	(F):GGGGCCAGTACAGCCGGGAAA (R):CTGGCTGAAGGCAGTCCGCA	92 bp
T-bet	(F):TCAACCAGCACCAGACAGAGA (R):TCCACCAAGACCACATCCAC	185 bp
GATA3	(F):CTAGGCCATTCGTACATGGAA (R):GGATACCTCTGCACCGTAGC	134 bp
RORγt	(F):ACGGCCCTGGTTCTCATCA (R):CCAAATTGTATTGCAGATGTT CCAC	80 bp
Foxp3	(F):CAGCTCTGCTGGCGAAAGTG (R):TCGTCTGAAGGCAGAGTCAGGA	190 bp
IFN-γ	(F):AAGCGTCATTGAATCACACCTG (R):TGACCTCAAACTTGGCAATACTC	201 bp
IL-4	(F): AAAATCACTTGAGAGAGATCA TCGG (R):GTTGCTGTGAGGACGTTTGG	102 bp
IL-22	(F):CGACCAGAACATCCAGAAGAA (R):GAGACATAAACAGCAGGTCCA	293 bp
TGF-β	(F):TGTGGAACTCTACCAGAAATA TAGC (R)GAAAGCCCTGTATTCCGTCTC	133 bp
GAPDH	(F):TGTGTCCGTCGTGGATCTGA (R):TTGCTGTTGAAGTCGCAGGAG	150 bp

Statistical Analysis

The data are expressed as the mean ± SD and were evaluated by one-way analysis of variance (ANOVA) using 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 intragastrically administered 2.5, 5, and 10 mg/kg $NaAsO_2$ for 24 h. All animals survived to the end of the experiment. As shown in Table 2, we found no changes in body weight or brain and hippocampal weights among the different groups. Compared with those in the control group, the brain and hippocampus indices were decreased in the arsenic-treated groups, and there was a significant difference only in the 10 mg/kg arsenic-treated group ($p < 0.05$).

Acute Arsenic Exposure Induces an Inflammatory Response in the Hippocampus

As shown in Fig. 1, the expression of the proinflammatory factors IL-6 and TNF-α was increased in the hippocampi of

Table 2 The body weights, as well as the weights and indices of the brain and hippocampus in mice

Experimental group	Body weight (g)	Brain weight (g)	Brain index (%)	Hippocampus weight (g)	Hippocampus index (%)
Control	18.86 ± 0.86	0.49 ± 0.05	2.61 ± 0.29	0.03 ± 0.01	0.18 ± 0.03
2.5 mg/kg $NaAsO_2$	18.69 ± 1.11	0.45 ± 0.05	2.40 ± 0.27	0.03 ± 0.00	0.15 ± 0.03
5 mg/kg $NaAsO_2$	18.78 ± 1.00	0.45 ± 0.04	2.41 ± 0.18	0.03 ± 0.01	0.16 ± 0.03
10 mg/kg $NaAsO_2$	18.52 ± 0.61	0.45 ± 0.02	2.45 ± 0.11*	0.03 ± 0.01	0.16 ± 0.03*

C57BL/6 mice were intragastrically administered 0, 2.5, 5, and 10 mg/kg $NaAsO_2$ for 24 h, and the brain and hippocampus were removed and weighed. The brain and hippocampus indices are expressed as the brain or hippocampus weight/body weight. The results are expressed as the mean ± SD ($n = 10$). *$p < 0.05$ compared with control mice

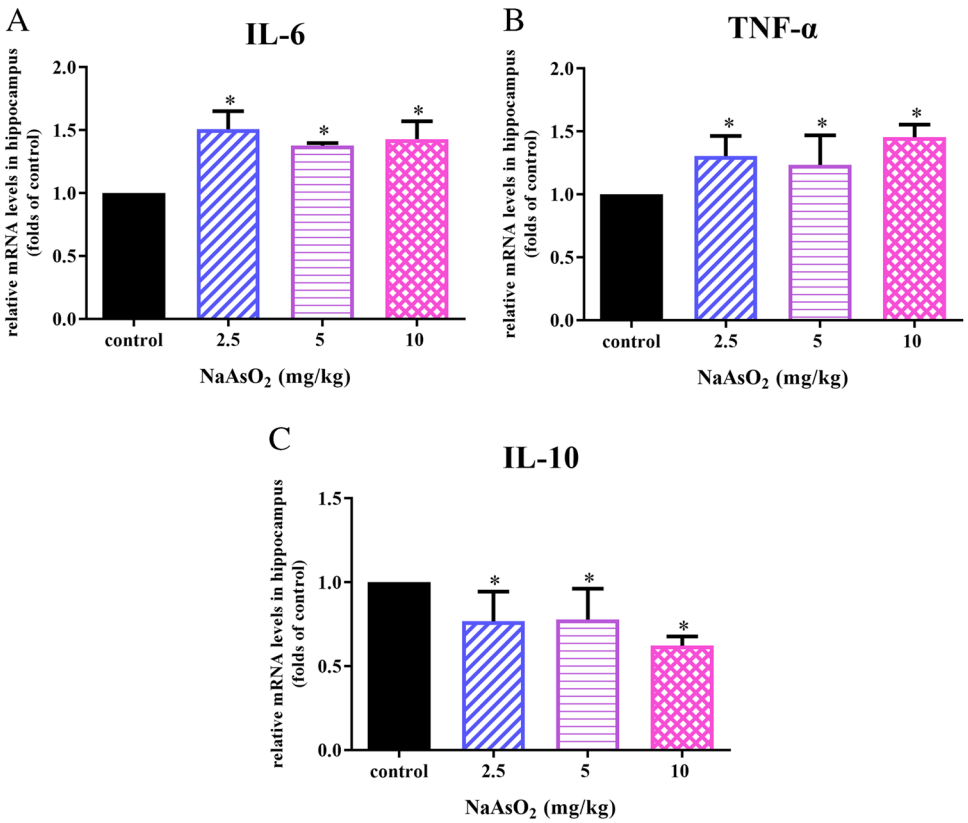

Fig. 1 Effect of acute arsenic exposure on the expression of inflammatory factors in the hippocampus. C57BL/6 mice were intragastrically administered 0, 2.5, 5, and 10 mg/kg $NaAsO_2$ for 24 h. The mRNA levels of IL-6 (**A**), TNF-α (**B**), and IL-10 (**C**) were assessed by real-time PCR. The results are expressed as the mean ± SD ($n = 4$). $^{*}p < 0.05$ compared with control mice, $^{\#}p < 0.05$ compared with 25 mg/L arsenic-treated mice

arsenic-treated mice compared with control mice ($p < 0.05$). Arsenic also downregulated the mRNA levels of the anti-inflammatory factor IL-10 in the hippocampus ($p < 0.05$).

Acute Arsenic Exposure Activates the NLRP3 Inflammasome in the Hippocampus

The NLRP3 inflammasome has been shown to play a vital role in the innate immune response. As shown in Fig. 2, the protein expression of NLRP3 was increased in the hippocampi of 10 mg/kg arsenic-treated mice ($p < 0.05$). Caspase-1 expression was increased by 64.85%, 89.55%, and 81.93% in the 2.5, 5, and 10 mg/L arsenic-treated groups, respectively ($p < 0.05$). In addition, arsenic increased the protein levels of the NLRP3-regulated cytokine IL-1β in the hippocampus in the 5 and 10 mg/L arsenic-treated groups ($p < 0.05$).

Acute Arsenic Exposure Induces Blood–Brain Barrier Disruption in Mice

Occludin, which is a tight junction protein, was used to assess BBB permeability. Occludin mRNA levels were decreased in the hippocampus after 5 and 10 mg/kg arsenic exposure (Fig. 3A). Treatment with 5 and 10 mg/kg arsenic also decreased the protein levels of occludin in the hippocampus ($p < 0.05$) (Fig. 3B).

Acute Arsenic Exposure Destroys the Th1/Th2/Th17/Treg Balance in the Hippocampus

Once the BBB is damaged, peripheral T cells can enter the brain. Therefore, we next analyzed the levels of $CD4^+$ T lymphocytes in the hippocampus in arsenic-exposed mice by western blotting. As shown in Fig. 4A, arsenic markedly increased the protein expression of CD4 in the hippocampus in the 2.5, 5, and 10 mg/kg arsenic-treated groups ($p < 0.05$). We next analyzed the differentiation of $CD4^+$ T lymphocytes in the hippocampus. Arsenic decreased the mRNA levels of the Th1 transcription factor T-bet and the cytokine IFN-γ in the 2.5, 5, and 10 mg/kg arsenic-treated groups ($p < 0.05$). The mRNA levels of the Th2 transcription factor GATA3 and the cytokine IL-4 were downregulated in the 2.5, 5, and 10 mg/L arsenic-treated groups compared with the control group ($p < 0.05$). In contrast, Th17 transcription factor ROR-γt was significantly increased by 4.65%, 60.04%, and 59.19%. The Th17 cytokine IL-22 was increased by 15.44%, 53.54%, and 178.56% in the 2.5, 5, and 10 mg/kg arsenic-treated groups, respectively. However, we found no change in the Treg transcription factor *Foxp3* or the cytokine TGF-β between the control group and arsenic-treated groups ($p > 0.05$).

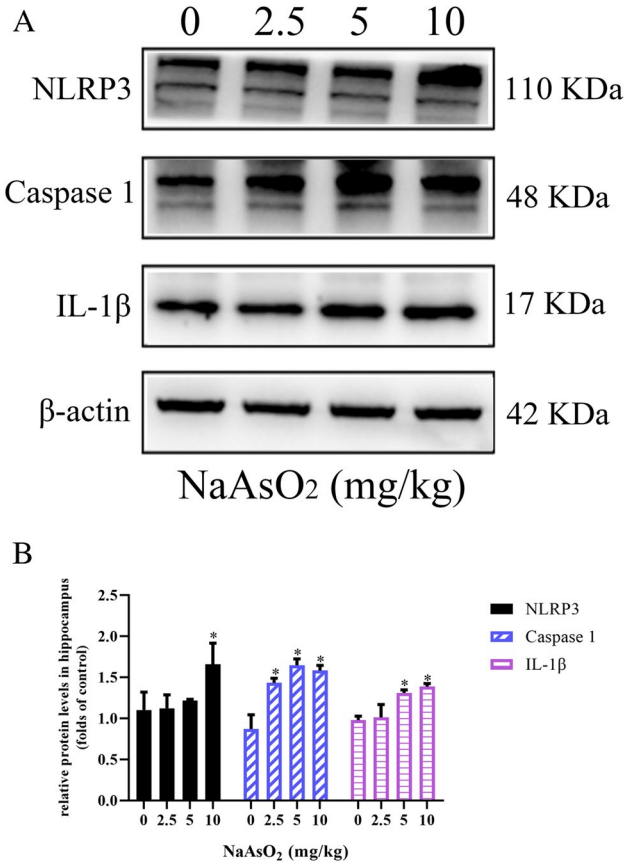

Fig. 2 Effect of acute arsenic exposure on the NLRP3 inflammasome in the hippocampus. C57BL/6 mice were intragastrically administered 0, 2.5, 5, and 10 mg/kg $NaAsO_2$ for 24 h. The expression of NLRP3, caspase-1, and IL-1β in hippocampus (**A**) and the corresponding quantitative analysis (**B**) were assessed by western blotting, and β-actin was used as the loading control. The results are expressed as the mean ± SD. $^*p < 0.05$ compared with control mice

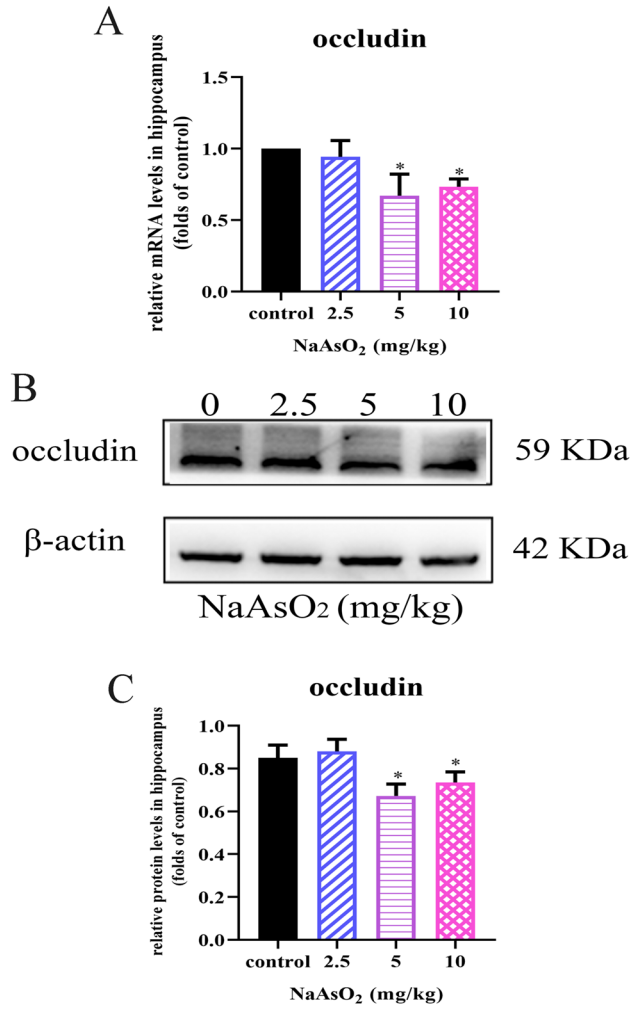

Fig. 3 Effect of acute arsenic exposure on blood−brain barrier permeability in mice. C57BL/6 mice were intragastrically administered 0, 2.5, 5, and 10 mg/kg $NaAsO_2$ for 24 h. The mRNA levels of occludin (**A**) were assessed by real-time PCR. The expression of occludin in the hippocampus (**B**) and the corresponding quantitative analysis (**C**) were assessed by western blotting, and β-actin was used as the loading control. The results are expressed as the mean ± SD ($n = 4$), and three independent experiments were performed. $^*p < 0.05$ compared with control mice

Discussion

The present study examined the effect of acute arsenic exposure on hippocampal inflammatory responses and $CD4^+$ T cell subpopulation differentiation in a C57BL/6 mouse model. The mice were intragastrically administered sodium arsenite for 24 h, and arsenic-exposed animals showed marked decreases in indices in the brain and hippocampus, which suggests neurotoxicity due to acute arsenic exposure. The mechanisms may be associated with arsenic-induced apoptosis [17] and altered DNA damage [18].

It has been reported that arsenic exposure can induce robust inflammation in the brain. Arsenic treatment increases the expression levels of the proinflammatory cytokines interleukin 1 beta (IL-1β), IL-6, interferon gamma (IFN-γ), and TNF-α in cultured microglia and astrocytes [19, 20]. In our study, mice that were treated with 2.5, 5, and 10 mg/kg arsenic for 24 h showed marked increases in the levels of the inflammatory factors IL-6 and TNF-α and decreased IL-10 mRNA levels. Wu et al. showed that 2–8 mg/kg arsenic trioxide exposure could decrease the anti-inflammatory markers IL-4 and IL-10 and increase the proinflammatory markers TNF-α, IL-1β, IL-18, IL-2, IL-6, INOS, and COX-2 in the brains of ducks [2]. These findings suggested that stimulation of inflammatory cytokines could be involved in arsenic-induced neurotoxicity.

The NLRP3 inflammasome is an important component of the innate immune system. In response to activation, Asc recruits procaspase-1 through interactions with the CARD domain of caspase-1. Caspase-1 then facilitates IL-1β and IL-18 maturation, which ultimately leads to the

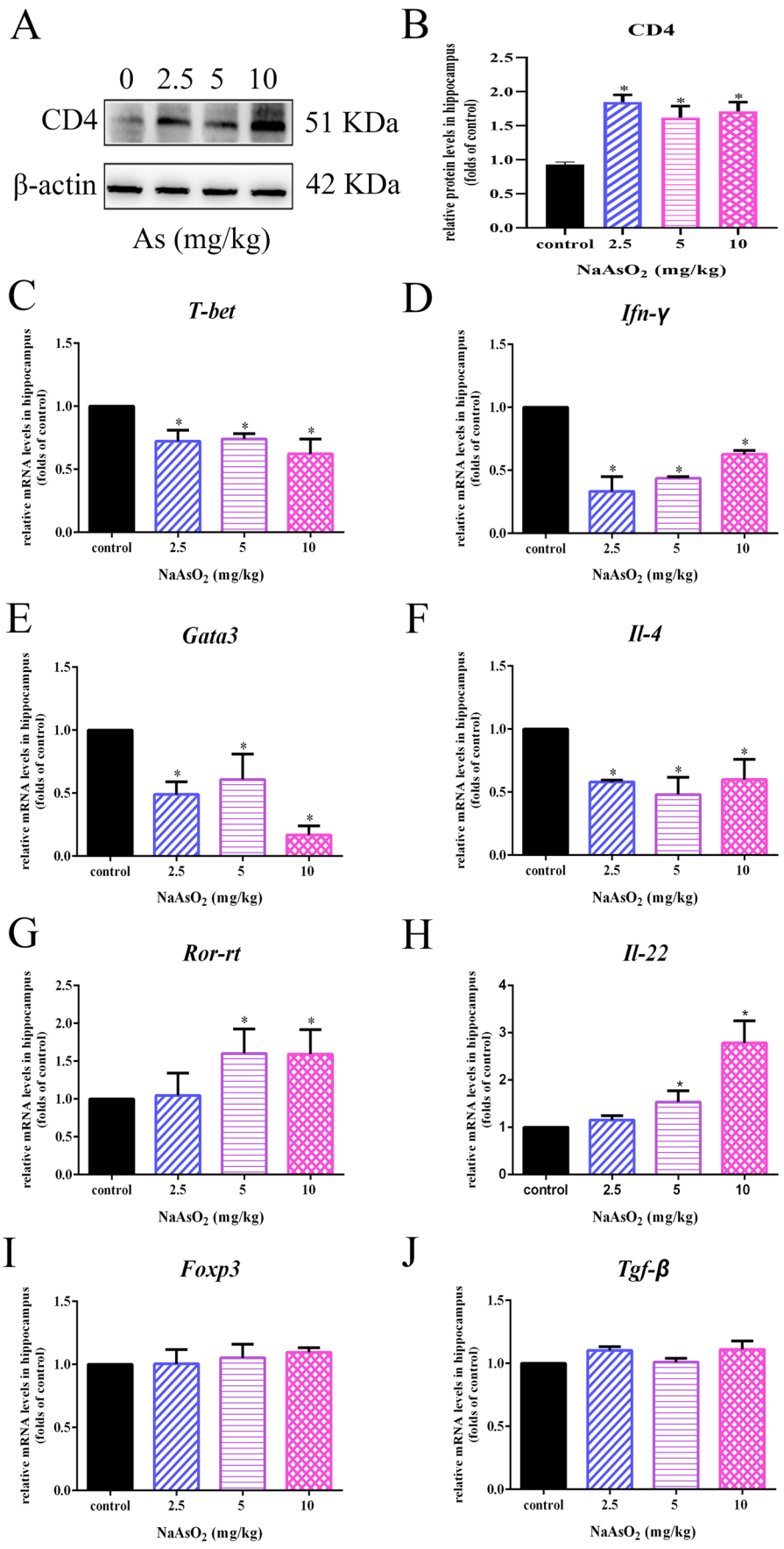

Fig. 4 Effect of acute arsenic exposure on the expression of inflammatory factors in the hippocampus. C57BL/6 mice were intragastrically administered 0, 2.5, 5, and 10 mg/kg $NaAsO_2$ for 24 h. The expression of CD4 in the hippocampus (**A**) and the corresponding quantitative analysis (**B**) were assessed by western blotting, and β-actin was used as the loading control. The mRNA levels of the T lymphocyte-specific transcription factors T-bet (**C**), Gata3 (**E**), RORγ-t (**G**), and Foxp3 (**I**), as well as the signature cytokines IFN-γ (**D**), IL-4 (**F**), IL-22 (**H**), and TGF-β (**J**), in the hippocampus were assessed by real-time PCR. The results were expressed as the mean ± SD ($n = 4$). *$p < 0.05$ compared with control mice

inflammatory response and tissue damage [21]. Our study showed that caspase-1 is more expressed in 5 mg/kg arsenic group compared to the 10 mg/kg arsenic group. We thought it may be caused by a phenomenon called hormesis, which is ubiquitously existed upon certain toxicants exposure characterized by a low-dose stimulation, while high-dose inhibition effects. Consequently, the increased effect of caspase-1 in high dose of arsenic exposure (10 mg/kg) would be lower than in low-dose group (5 mg/kg). Furthermore, the NLRP3 inflammasome is implicated in both neuroinflammation and cognitive impairment in neurodegenerative diseases [12]. Yang et al. found that 5 and 10 mg/kg sodium arsenite exposure for 7 days resulted in the activation of NF-κB p65 and the NLRP3 inflammasome, as well as the upregulation of IL-1β and IL-6 expression in the livers of mice [22]. Huang et al. found that following 8 weeks of exposure to 2,5-hexanedione (HD), the protein levels of NLRP3, ASC, caspase-1, and IL-1β in the brain and spinal cord homogenates of rats exposed to 400 mg/kg HD were significantly increased [23]. Our present findings showed activation of the NLRP3 inflammasome in the hippocampus. These results suggested that the NLRP3 inflammasome could be involved in the neuroimmune abnormalities by arsenic.

The blood−brain barrier is critical for maintaining nervous system homeostasis and protects the nervous system from toxins, inflammation, and injury [24]. Occludin is one of the major components of tight junctions (TJs), which can maintain paracellular permeability [25]. In our study, the mRNA and protein levels of occludin were markedly decreased after arsenic exposure in the hippocampus. Manthari et al. found that 15 mg/L As_2O_3 exposure in drinking water resulted in the significant downregulation of occludin mRNA and protein expression in the cerebral cortex and hippocampus in PND 42 mice [26]. Wang et al. found that chronic arsenic exposure decreased occludin mRNA and protein levels [27]. These studies suggested that arsenic could damage BBB integrity, which can ultimately damage the nervous system.

Our results showed that arsenic caused blood−brain barrier disruption in the hippocampus (Fig. 3), which could result in T cell and other immune cell infiltration into the nervous system and induce an immune-inflammatory response [28]; thus, we further focused on the levels of T cells in the hippocampus. It has been reported that the number of lymphocytes is increased in autism spectrum disorder (ASD) brains compared to control brains, and $CD3^+$ T lymphocytes predominate over $CD20^+$ B lymphocytes and $CD8^+$ T lymphocytes predominate over $CD4^+$ T lymphocytes [29]. In our study, CD4 protein levels were increased in the hippocampi of arsenic-treated mice. $CD4^+$ T cells can differentiate into Th1, Th2, Th17, and Treg cells, so we also examined $CD4^+$ T cell subpopulation differentiation in the hippocampus. It has been reported that Th1/Th2 Th17/Treg imbalance is associated with neurological diseases and injuries, including multiple sclerosis (MS), rheumatoid arthritis (RA), and necrotizing encephalitis [30, 31]. In ASD, dysregulation of Th1, Th2, Th17, and Treg cells is characterized by high levels of T-bet, GATA, and RORγt and decreased Foxp3 expression in the brain, which may be associated with cognitive impairment [32]. Our results showed that arsenic decreased the Th1 transcription factor T-bet and the cytokine IFN-γ and the Th2 transcription factor GATA3 and the cytokine IL-4 in the hippocampus; however, the change was much more pronounced for Th2 cytokines than Th1 cytokines, suggesting that acute arsenic treatment induced Th1-polarized immune responses in the hippocampus. Some studies have shown that arsenic induced the downregulation of Th1 cytokines and Th2 cytokines [33]. Another study reported that arsenic increased Th1 cytokines and upregulated Th2 cytokines [6]. These disparities in the literature might be associated with different arsenic doses and durations, as well as diverse effector organs. It has also been reported that Th17 cells contribute to autoimmunity and inflammation, while Treg cells maintain immune homeostasis [34]. Huang et al. observed that Th17 cell levels were increased, whereas Treg cell levels were decreased in the brains of MCAO rats compared with the ratio in sham-operated rats. Moreover, 2-(-2-benzofuranyl)-2-imidazoline reciprocally regulated the Th17/Treg balance induced by ischemic stroke in rats [35]. We found that the mRNA levels of the Th17 transcription factor RORγt and the cytokine IL-22 were dramatically increased in hippocampal homogenates. Taken together, these results suggest that arsenic induced abnormalities in $CD4^+$ T cell subpopulations, including the polarization of Th1 subpopulations, and increased Th17 cells, providing a deeper understanding of arsenic-induced neuroimmune imbalance.

Conclusions

In the current study, we conclusively showed that arsenic induced neuroimmune toxicity by inducing inflammation, activating NLRP3 inflammasomes, and modulating $CD4^+$ T lymphocyte subpopulation differentiation. Our findings provide a novel target for treating arsenic-induced neurotoxicity. Further studies are needed to identify the mechanisms of neuroimmune toxicity and develop novel therapeutic strategies to protect against the neurotoxicity of arsenic exposure.

Author Contribution Conceptualization, funding acquisition, data curation, formal analysis, visualization, and writing—original draft, H.J.; software, data curation, and validation, R.H.F.; formal analysis and software, N.Y.; investigation, Z.L.; methodology, Q.W., K.J.X., X.K.H.; resources, validation, writing—review, supervision and editing, L.F.Z.;

funding acquisition, writing—review and editing, supervision, project administration, X.X.D.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval Animal use has been approved by the Animal Use and Care Committee at Shenyang Medical College (protocol number: SYYXY2020031201).

Consent for Publication All authors have read and agreed to the published version of the manuscript.

Conflict of Interest The authors declare no competing interests.

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