

Research Article

Arsenic Induces Continuous Inflammation and Regulates Th1/Th2/Th17/Treg Balance in Liver and Kidney In Vivo

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Numerous studies on arsenic-induced hepatonephritic toxicity including cancer have been reported. Given that chronic inflammatory response and immune imbalance are associated with oncogenesis, we investigated whether arsenic could influence the hepatic and nephritic expression of inflammatory factors and the differentiation of T cells. Mice were exposed to NaAsO₂ (0, 25, and 50 mg/L) for 1 and 3 months. Our data showed the destruction of the structure and inflammatory infiltration in the liver. The arsenic markedly increased the activity of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The myeloperoxidase (MPO) activities increased in the liver at 25 and 50 mg/L arsenic for 3 months as well as in the kidney at both 1 and 3 months. An increased expression of inflammatory indicators (IL-1 β , IL-12, and TNF- α) at 25 and 50 mg/L arsenic for 1 and 3 months in the liver and kidney, as well as IL-1 β in the liver for 3 months and in the kidney at 50 mg/L for 1 and 3 months were demonstrated in our experiments. Besides, a definite tendency toward Th1/Th17 cytokines in the liver while Th2/Th17 cytokines in kidney was also observed by arsenic. Moreover, arsenic enhanced the expression of MAPK/Nrf2/NF- κ B signaling molecules. In conclusion, the results of the study suggested that arsenic induces continuous immune-inflammatory responses in the liver and kidney.

1. Introduction

Arsenic is the most hazardous compound in the environment, which continues to be a major global health problem worldwide [1]. Chronic arsenic exposure could induce many cancers including skin, bladder, and lung, as well as noncancer diseases, such as diabetes, cardiovascular diseases, and anemia [2, 3]. The liver and kidney are the major target organs for arsenic poisoning since they play the important roles in arsenic metabolism and excretion, respectively [4]. Increasing studies have found that chronic arsenic exposure could cause liver injury, hepatoportal sclerosis, and liver cancer [5, 6]. Toxicological studies also have documented that arsenic exposure could induce renal edema and inflammatory infiltration and result in acute renal failure including

nephritis, nephritic syndrome, and nephrosis [7]. Despite the knowledge advancements from studies of arsenic exposure on the hepatotoxicity and nephrotoxicity underlying in vivo, a detailed molecular mechanism has not yet been well understood.

Except for its carcinogenicity, arsenic also has immunotoxicity. CD4⁺ T cells play a crucial role in regulating the immunity, inflammation, and cancer [8]. Animal studies have revealed that arsenic exposure affected CD4⁺ T cell numbers and CD4/CD8 ratios in the spleen and thymus [9, 10]. An epidemiologic study found that the maternal urinary arsenic concentrations were negatively associated with CD45RA⁺ CD4⁺ cells in cord blood [11]. CD4⁺ T cells could differentiate into Th1, Th2, Th17, and Treg by a series of corresponding transcription factors and cytokines [12]. It has

been reported that arsenic has immunosuppressive effect destroying Th1/Th2 imbalance [13], inhibiting Th17 cell differentiation, and promoting regulatory T (Treg) cell generation [14]. On the other hand, the liver and kidney were considered to possess macrophages, DCs, and other immune cells, which may also be involved in balancing immunity and tolerance [15, 16]. Growing evidences have indicated that chronic inflammation could continuously produce reactive oxygen species (ROS) and NO, which may cause cell damage and in turn induce cell proliferation, leading to DNA damage and gene mutation, and finally to the occurrence and development of tumor [17]. In addition, researchers also have proposed that the reduced immune surveillance could trigger different types of diseases including malignancies [18]. However, the study on the comprehensive immune-inflammatory response and potential mechanism by arsenic has not been reported so much.

The main mechanism for arsenic toxicity was the ROS generation and therefore the induction of oxidative stress [19]. In addition, excess ROS could trigger the activation of mitogen-activated protein kinase (MAPK) and nuclear factor E2-related factor 2 (Nrf2) signal pathways, which were involved in promoting and suppressing carcinogenesis [20]. It has been also reported that arsenic could activate the MAPK and Nrf2 pathway, then regulate the expression of inflammatory mediators including IL-1 β , TNF- α , IL-6, and IL-12, and result in neural inflammation and autophagy [21]. However, the knowledge on the effects of subchronic arsenic exposure on the hepatic and renal MAPK and Nrf2 pathways is still in initial stage.

Therefore, the study intended to investigate the immune dysfunction and inflammatory response in the liver and kidney by examining subchronic arsenic exposure model, hepatic and renal pathological and biochemical index alteration, the expression of inflammation (IL-1 β , IL-6, IL-12, and TNF- α), and the markers representing the T cell differentiation in the liver and kidney. Moreover, we also surveyed the relevant mechanism by observing the related immune-inflammatory modulatory pathway MAPK, nuclear factor kappa B (NF- κ B), and Nrf2. We are trying to provide a novel insight for understanding the carcinogenic mechanism of arsenic in the liver and kidney.

2. Materials and Methods

2.1. Reagents and Chemicals. Sodium arsenite ($\geq 99.0\%$) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Myeloperoxidase (MPO), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) detection kits were purchased from Jiancheng Biological Institute (Nanjing, China). Real-time polymerase chain reaction (real-time PCR) kits were from Takara Co. (Otsu Japan). Primary antibodies of ERK1/2, P-ERK1/2, JNK, P-JNK, P38, and P-P38 were purchased from Cell Signaling Technology (Cell Signaling, Danvers, USA), and NF- κ B, Nrf2, GSTO1/2, heme oxygenase 1, β -actin, and corresponding secondary antibodies were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals used were of analytical grade.

2.2. Animals and Experimental Procedures. Six-week-old female Kunming mice of 18–22 g were obtained from the Center for Experimental Animals at China Medical University (Shenyang, China) with a National Animals Use License number of SCXK-LN2013-0007. Mice were group-housed in stainless steel cages (10 mice per cage) in an air-conditioned room with temperature maintained at $22 \pm 2^\circ\text{C}$ and 12 h light/dark cycle for 1 week before the experiment. The mice were allowed standard mice chow diet and drinking water ad libitum throughout the study. All experiments and surgical procedures were approved by the Animal Care and Use Committee of China Medical University.

The dose for NaAsO₂ was selected on the basis of previously published studies [22], as well as our preliminary experiments. Mice were exposed to NaAsO₂ in drinking water at concentrations of 0, 25, and 50 mg/L for 1 and 3 months. The food and water consumption were measured every three days, and the mice were weighed every week during the experimental period. At the end day of the experiment, all mice were weighed and deeply anesthetized. Blood was collected through eyeball, extirpating into heparinized vials and centrifuged (3000x g, 4°C) for 10 min; the serum obtained was kept frozen at -80°C for measure. The entire liver and kidney were promptly removed and weighed, and small liver and kidney fractions were fixed with 4% paraformaldehyde for histopathological studies, and the remaining tissues were stored at -80°C for biochemical use.

2.3. Determination of Total Arsenic Concentration in Liver and Kidney. Measurement of arsenic species was performed as described by Li et al. [23]. Briefly, the liver and kidney was homogenized on ice with 10 ml deionized water per gram of tissue weight. iAs, monomethylarsonic acid (MMA), and dimethylarsinic acid (DMA) were determined by a high-performance liquid chromatography-hydride generation-atomic fluorescence spectrometer (HPLC-HG-AFS, SA-10 Atomic Fluorescence Species Analyzer, Titan Co., Beijing). Total arsenic (T-As) levels in the liver and kidney were then calculated by summing up the levels of iAs, MMA, and DMA totally.

2.4. Histopathological Analysis. Histopathological evaluations of the liver and kidney were performed according to the standard laboratory procedures. Briefly, the liver and kidney from three mice were removed and fixed with 4% paraformaldehyde for 48 h and embedded in paraffin blocks. 5 μm sections were prepared by microtome (EM UC7, Leica, Germany) and then stained for 15 min with hematoxylin-eosin (H&E) (Solarbio, Beijing, China), mounted, and analyzed using optical microscopy with a digital imaging system (Biodirect-Inc., Nikon, Japan). The injury score of the liver was evaluated as described by Kleiner et al. [24].

2.5. Determination of Serum Aminotransferase and Hepatonephric MPO Activities. ALT and AST in the serum, as well as MPO in the liver and kidney, were measured using commercially available kits according to the manufacturer's instructions.

2.6. Total RNA Isolation and Real-Time PCR Analysis. Total RNAs of the liver and kidney were isolated using a TRIzol reagent (Invitrogen, USA). 500 ng RNA was reversely transcribed into cDNA and amplified by using Takara reagent (Takara, Japan) according to the manufacturer's protocol; then, PCR amplification was performed by SYBR Premix ExTaqII kits (Takara, Japan). PCR was performed using the following thermal cycling conditions: 95°C 30 s; 40 cycles of denaturing at 95°C for 5 s; and annealing at 60°C for 30 s. PCR was performed using the following primers: IL-1 β (F): TGACCTGGGCTGTCCTGATG, (R): GGTGCTCATGT CCTCATCCTG, product length: 220 bp; IL-6 (F): CTGC AAGAGACTTCCATCCAG, (R): AGTGGT ATAGAC AGGTCTGTTG, product length: 131 bp; IL-12 (F): TGGT TTGCCATCGTTTTGCTG, (R): ACAGGTGAGGTTCA CTGTTTCT, product length: 123 bp; Tnf- α (F): CCCCCA AGGGATGAGAAGTTC, (R): GGCTTGTCACCTCGAAT TTTGAGA, product length: 148 bp; Ifn- γ (F): AAGCGT CATTGAATCACACCTG, (R): TGAC CTCAAACCTGG CAATACTC, product length: 92 bp; IL-13 (F): CACACA AGACCAGACTCCCCTG, (R): GGTTACAGAGGCCATG CAATATCC, product length: 155 bp; IL-23 (F): CCCGTA TCCAGTGTGAAGATG, (R): CCCTTTGAAGATGTCA GAGTC, product length: 128 bp; IL-10 (F): GGGGCCAGT ACAGCCGGGAAA, (R): CTGGCT GAAGGCAGTCC GCA, product length: 92 bp; GAPDH (F): TGTGTCCGT CGTGGATCTGA, (R): TTGCTGTTGAAGTCGCAGGAG, product length: 150 bp. $2^{-\Delta\Delta Ct}$ values were calculated to represent the amounts of different target genes.

2.7. Western Blot Analysis. The total proteins of the liver and kidney were extracted by commercial kits, and protein concentrations were quantified by bicinchoninic acid (BCA) protein kit (Beyotime, Shanghai, China). 45 μ g total protein was boiled for 5 min at 100°C before 7.5–10% SDS-PAGE and then transferred to 0.45 μ M polyvinylidene fluoride (PVDF) membrane (Amersham, Buckinghamshire, UK). After blocking for 2 h at room temperature, membranes were then probed with the primary antibodies of ERK1/2, P-ERK1/2, JNK, P-JNK, P38, P-P38, NF- κ B, Nrf2, GSTO1/2, and HO-1 (1 : 1000) at 4°C overnight, respectively. Finally, membranes were incubated with corresponding secondary antibodies (1–5000) for 2 h at room temperature. 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). β -Actin (1 : 5000) was used as the internal control.

2.8. Statistical Analysis. Data were expressed as mean \pm 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.

3. Results

3.1. General Status of Study Mice. In our study, mice were treated with 25 and 50 mg/L NaAsO₂ for 1 and 3 months,

respectively, by drinking water. All animals survived to the end of the experiment. The calculated average daily arsenic intake of different treatment groups was listed in Table 1. T-As levels in the liver and kidney were increased dramatically by arsenic exposure. We found that the weight of the liver significantly decreased in NaAsO₂-treated mice compared with corresponding control mice ($P < 0.05$), while no changes as to the kidney among different treatments. No statistically significant differences of body weight, as well as general status, have been observed during the whole study.

3.2. Subchronic Arsenic Exposure Induces Tissue Histopathology and Dysfunction of Liver and Kidney. We performed HE stain to investigate the histopathologic changes in the liver and kidney. As shown in Figure 1, the histological profile of the liver showed a normal hepatic architecture with hepatic lobules and hepatocytes in the control group (Figure 1(a)). Compared with the control group, the animals administered with 25 mg/L NaAsO₂ showed obvious inflammatory cell infiltration at 1 month (Figure 1(b)). With the increase of dose and time, we observed subsequently extensive disruption of the liver architecture including hepatocellular necrosis (Figures 1(c)–1(e)). However, we failed to find evident changes in the kidney of arsenic-treated groups (data not shown).

ALT and AST are the most common biochemical indexes of hepatic injuries. In our results, serum ALT enzyme activity upregulated dramatically with a dose-effect relationship by 25 and 50 mg/L NaAsO₂ exposure at 1 and 3 months (Figure 2(a)). Likewise, our results also verified the clear increase of AST enzyme activity in serum by arsenic exposure comparing with the control group (Figure 2(b), $P < 0.05$).

Our pathological results showed that arsenic induced marked inflammatory cell infiltration; we further evaluated inflammation-associated infiltration of neutrophils and monocytes within the tissue by measuring hepatic and renal MPO activity. The MPO activity in liver was increased by 65% and 105% at 25 and 50 mg/L NaAsO₂ in 3 months (Figure 2(c), $P < 0.05$). By contrast, we also found a notable enhancement of renal MPO activity at 25 and 50 mg/L NaAsO₂ in both 1 and 3 months (Figure 2(d), $P < 0.05$). These changes indicated that subchronic arsenic exposure could induce tissue damage and inflammation in mice.

3.3. Subchronic Arsenic Exposure Increases the Expression of Inflammatory Cytokines in Liver and Kidney. Inflammatory cytokines IL-1 β , IL-6, IL-12, and TNF- α in the liver and kidney were determined by real-time PCR. The mRNA levels of hepatic IL-12 and TNF- α were all elevated markedly by 25 and 50 mg/L NaAsO₂ for 1 month (Figures 3(c) and 3(d), $P < 0.05$), 50 mg/L arsenic also enhanced the IL-6 mRNA levels in the liver (Figure 3(b), $P < 0.05$). In addition, the mRNA levels of hepatic IL-1 β , IL-6, IL-12, and TNF- α were all elevated markedly in the arsenic-treated group at 3 months (Figures 3(a)–3(d), $P < 0.05$). By contrast, we also found a notable enhancement of IL-6, IL-12, and TNF- α in different arsenic-treated groups, as well as a little increase of IL-1 β mRNA levels in the kidney by 50 mg/L NaAsO₂ exposure for 1 and 3 months (Figures 3(e)–3(h), $P < 0.05$).

TABLE 1: Average daily arsenic intake in drinking water, the concentrations of total arsenic (T-As, ng As/g tissue) of the liver and kidney, the body weight and liver and kidney weights in control and different experimental mice.

Experimental groups	Duration (months)	Dose (mg/L)	Calculated average daily arsenic intake (mg NaAsO ₂ /kg body weight/day)	T-As in liver (ng/g)	T-As in kidney (ng/g)	Body weight (g)	Liver weight (g)	Kidney weight (g)
Group 1	1	0	0	<LD	<LD	31.85 ± 1.08	1.44 ± 0.20	0.28 ± 0.02
Group 2	1	25	4.06 ± 0.90	93.97 ± 36.64	44.2 ± 3.18	31.64 ± 2.63	1.37 ± 0.16	0.29 ± 0.03
Group 3	1	50	6.47 ± 2.31	123.58 ± 22.65	64.28 ± 8.26	32.04 ± 1.56	1.35 ± 0.20 *	0.30 ± 0.03
Group 4	3	0	0	<LD	<LD	38.32 ± 1.86	1.36 ± 0.17	0.29 ± 0.03
Group 5	3	25	3.41 ± 0.23	213.29 ± 86.28	37.92 ± 6.16	36.05 ± 2.00	1.16 ± 0.18 *	0.29 ± 0.04
Group 6	3	50	6.47 ± 0.33	229.95 ± 21.04	66.36 ± 20.22	38.52 ± 0.89	1.13 ± 0.11 *	0.31 ± 0.03

Mice were treated with 25 and 50 mg/L NaAsO₂ by drinking water for 1 and 3 months, and total arsenic (T-As) levels of the liver and kidney were determined by the high-performance liquid chromatography-hydride generation-atomic fluorescence spectrometry (HPLC-HG-AFS) method, as described in Materials and Methods. Results were expressed as mean ± SD ($n = 3$). The limit of detection (LD) for T-As was 1 µg/L. * Denoted $P < 0.05$ compared with control mice.

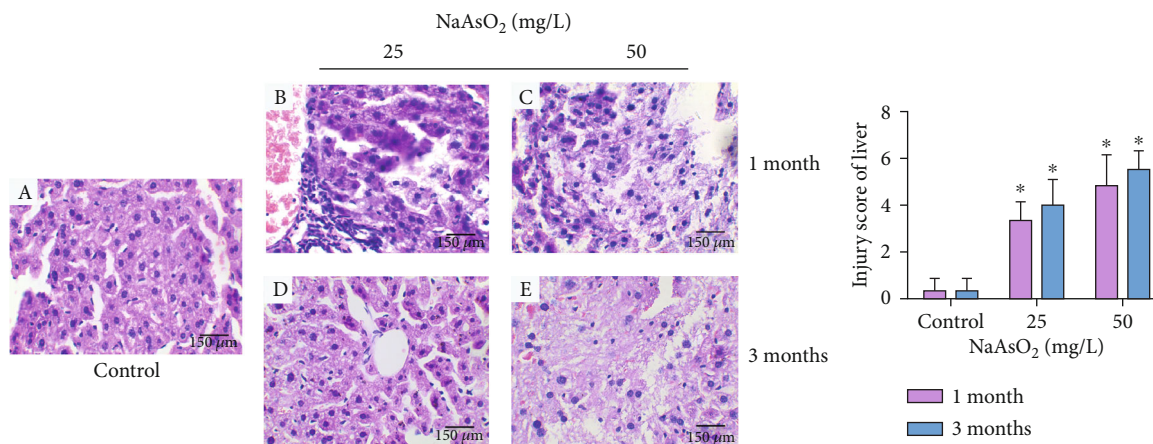


FIGURE 1: Histopathological changes in hepatic tissues of subchronic arsenic-treated mice by hematoxylin-eosin (H&E) assay (original magnification: $\times 400$). Mice were treated with 25 and 50 mg/L NaAsO₂ for 1 and 3 months: (a) Control; (b) 25 mg/L NaAsO₂-treated mice for 1 month; (c) 50 mg/L NaAsO₂-treated mice for 1 month; (d) 25 mg/L NaAsO₂-treated mice for 3 months; and (e) 50 mg/L NaAsO₂-treated mice for 3 months. (f) Analytical result of the liver injury score. Data were expressed as mean ± SD. * $P < 0.05$ compared with control mice.

These results indicated that subchronic arsenic exposure could affect the expression of inflammatory cytokines and induce the overall inflammatory response in both the liver and kidney.

3.4. Subchronic Arsenic Exposure Affects the Differentiation of CD4⁺ T Cell of Liver and Kidney. It was reported that the liver and kidney also include CD4⁺ T cell, macrophagocyte, and dendritic cells, which play important roles in maintaining immune homeostasis. Next, we measured the levels of mRNA encoding T helper 1 (Th1), Th2, Th17, and regulatory T cells- (Treg cell-) specific cytokines. Th1 cytokine IFN- γ , Th2 cytokine IL-13, Th17 cytokine IL-23, and Treg cytokine IL-10 mRNA levels all increased dramatically in the liver at arsenic-treated groups (Figures 4(a)–4(d), $P < 0.05$). By contrast, the levels of renal Th1 cytokine IFN- γ mRNA markedly increased by 100% and 282% at 25 mg/L NaAsO₂ with 1 and 3 months, 101% at 50 mg/L NaAsO₂ with 3 months (Figure 4(e), $P < 0.05$), while Th2 cytokine

IL-13 mRNA levels were upregulated by 85% at 25 mg/L NaAsO₂ with 3 months, 334% and 315% at 50 mg/L NaAsO₂ with 1 and 3 months in the kidney (Figure 4(f), $P < 0.05$). We also found a notable enhancement of Th17 cytokine IL-23 and Treg cytokine IL-10 in the kidney of different arsenic groups (Figures 4(g) and 4(h), $P < 0.05$).

We also calculated the ratios of Th1/Th2 and Th17/Treg cytokines in the liver and kidney to more accurately reflect the effect of subchronic arsenic exposure on CD4⁺ T cell differentiation. The results showed that compared with the control group, hepatic IFN- γ /IL-13 ratio and IL-23/IL-10 ratio were increased at 25 mg/L NaAsO₂-treated group with 1 and 3 months and at 50 mg/L NaAsO₂-treated groups with 3 months (Figures 4(i) and 4(j), $P < 0.05$), while IL-23/IL-10 ratio decreased a little at the 25 mg/L NaAsO₂-treated group with 3 months (Figure 4(j), $P < 0.05$). In the kidney, IFN- γ /IL-13 ratio was upregulated at the 25 mg/L NaAsO₂-treated group with 1 month and decreased at the 25 mg/L NaAsO₂-treated group with 3 months as well as 50 mg/L

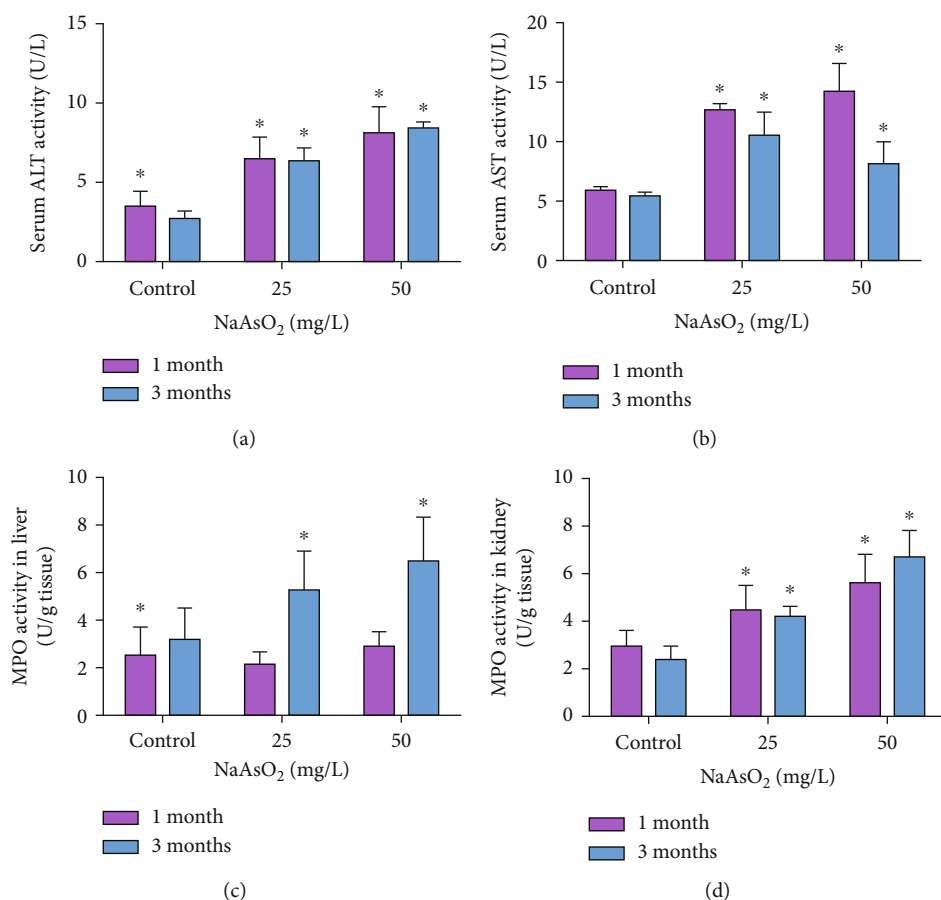


FIGURE 2: Subchronic arsenic exposure increased the activities of serum alanine amino transferase (ALT) and aspartate aminotransferase (AST), as well as hepatorenal myeloperoxidase (MPO) in mice. Mice were treated with 25 and 50 mg/L NaAsO₂ for 1 and 3 months. The activities of serum ALT (a) and AST (b) and MPO in the liver (c) and kidney (d) were determined. Data were expressed as mean \pm SD. * $P < 0.05$ compared with control mice.

NaAsO₂-treated groups with 1 and 3 months (Figure 4(k), $P < 0.05$). The ratio of IL-23/IL-10 in the kidney was increased in all arsenic-treated groups (Figure 4(l), $P < 0.05$). These results indicated that subchronic arsenic exposure could affect the expression of CD4⁺ T cell subpopulation-related cytokines and induce a prominent advantage of Th1 and Th17 in the liver but Th2 and Th17 in the kidney.

3.5. Subchronic Arsenic Exposure Activates MAPK/NF- κ B/Nrf2 Pathway of Liver and Kidney. We observed that phosphorylations of ERK1/2, JNK, and P38 were significantly induced by arsenic in the liver and kidney (Figure 5). Moreover, the expression of NF- κ B protein was markedly upregulated 1.5–2 times of control in the liver by arsenic (Figure 5(a)). The upregulation of renal NF- κ B protein was consistent with the clear increase of the liver (Figure 5(c), $P < 0.05$). As shown in Figures 6(a) and 6(c), arsenic treatment showed a clear increase of Nrf2 and GST protein in the liver and kidney ($P < 0.05$). The antioxidant enzyme HO-1 was elevated markedly in the 25 and 50 mg/L NaAsO₂ groups with 3 months, and the upregulation of renal HO-1 protein was consistent with the clear increase of liver. Totally, the subchronic arsenic exposure induced the contin-

uous activation of the MAPK/NF- κ B/Nrf2 pathway, which might also be associated with the arsenic-induced immune-inflammatory imbalance response.

4. Discussion

Arsenic is well known to cause numerous cancers including the liver and kidney; mechanistic studies have also reported that it may be associated with genotoxicity-related DNA methylation, oxidative stress, and altered cell proliferation. Recent years, the critical roles of decreased immune surveillance and chronic inflammatory response in the occurrence and development of cancer have attracted great interest. So, we investigated the effect of subchronic arsenic exposure on hepatic and renal immune-inflammatory responses as well as the underlying mechanism in a mouse model.

Histopathological examination is viewed as the “gold standard” to determine organ damage. One year of 6 μ g/gm arsenic body weight/day feed induced the pathologic changes including mild hepatic steatosis, inflammation, necrosis, and significant fibrosis in mice [25]. Our results were corroborated by the similar findings of the liver. In another study, Xu et al. observed early glomerular sclerosis,

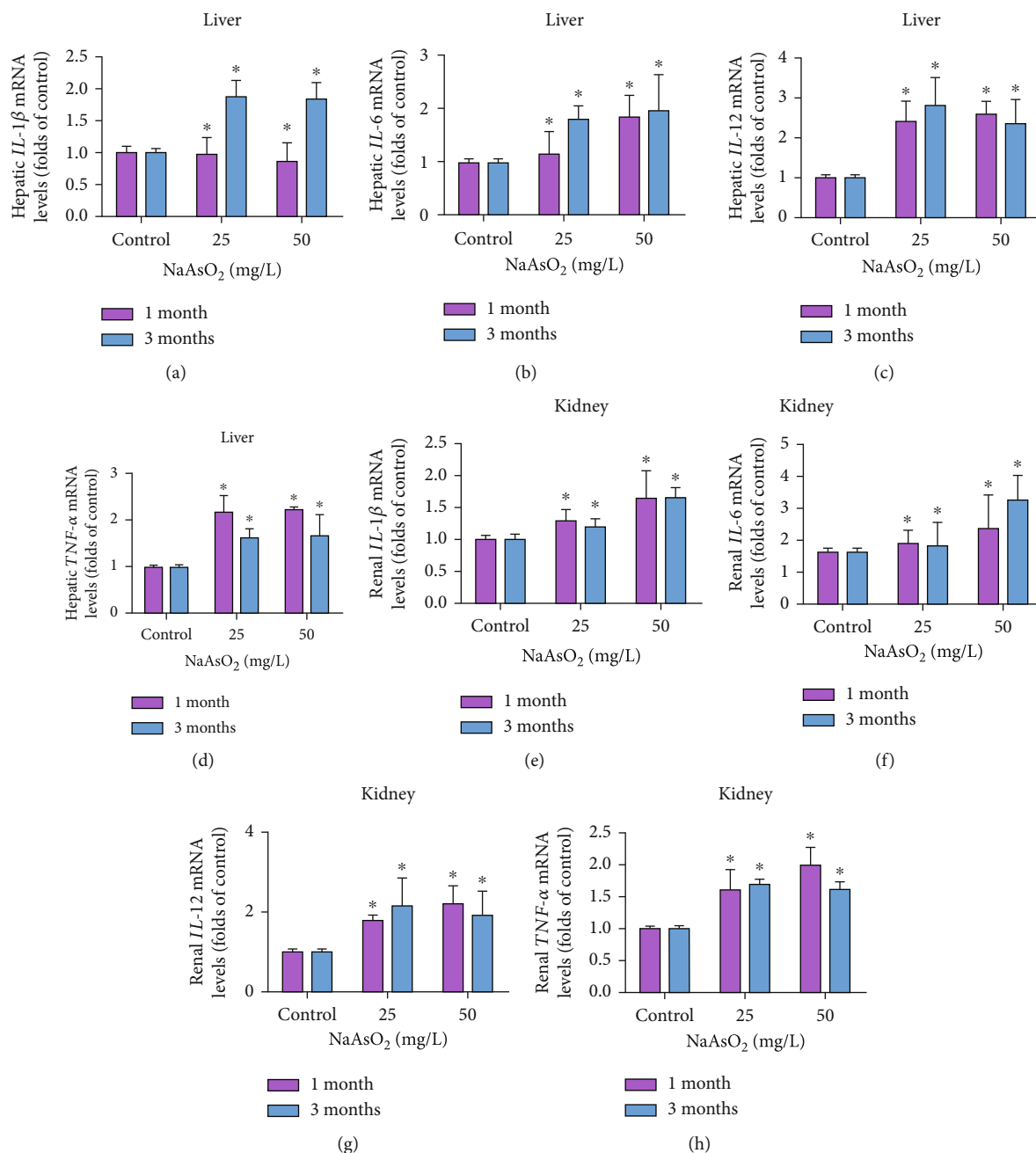


FIGURE 3: Subchronic arsenic exposure raised the levels of inflammatory cytokines in the liver and kidney. Mice were treated with 25 and 50 mg/L NaAsO₂ by drinking water for 1 and 3 months. The mRNA levels of IL-1β, IL-6, IL-12, and TNF-α in liver (a–d), as well as in kidney (e–h) were determined by real-time PCR. Data were expressed as mean ± SD. **P* < 0.05 compared with control mice.

tubular atrophy, and marked chronic inflammatory cell infiltration of the kidney interstitial space in the high-arsenic treatment group [26]. However, there were no obvious renal pathological changes in our results. Based on the important role of infiltration of neutrophils and monocytes in inflammatory response, we analyzed hepatic and renal MPO activities which have been used as a reliable marker of tissue inflammation. The significant increase in MPO activities of the liver and kidneys tissue after arsenic exposure in our study is consistent with other studies [27]. We also tested the ALT and AST activities in the serum, which are the

important indicators for liver damage and immune response. Many studies have reported that the activities of ALT and AST in the plasma were significantly higher in arsenic-treated rats than in normal control rats [28]. Consistent with these studies, we observed markedly raised levels of serum ALT and AST in arsenic-treated mice; it may be associated with arsenic-induced hepatocyte membrane damage.

Inflammatory cell infiltration could increase secretion of various inflammatory cytokines and thus impair immune function [29]. In addition, chronic inflammatory response has been found to be one of the most important factors that

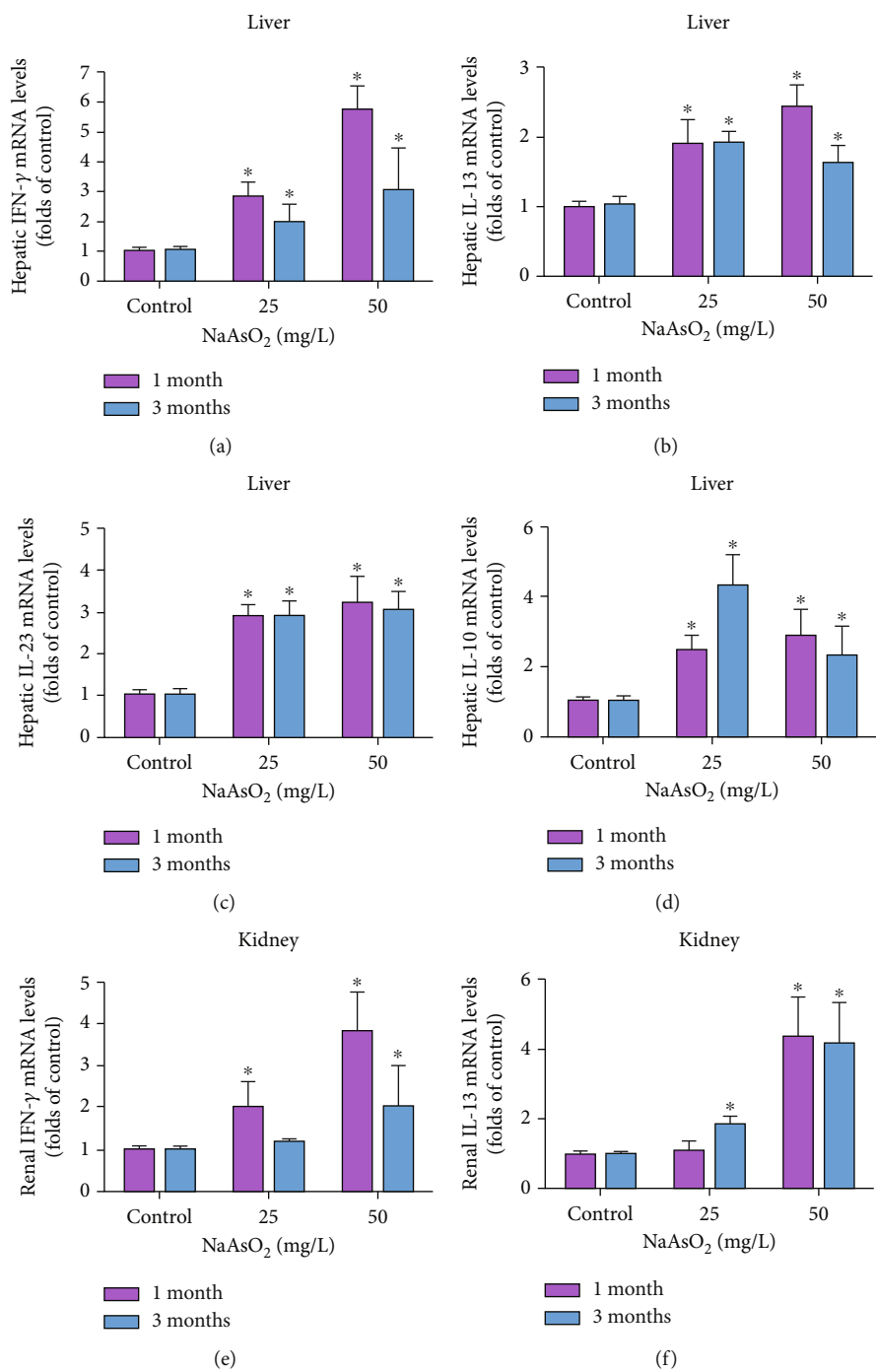


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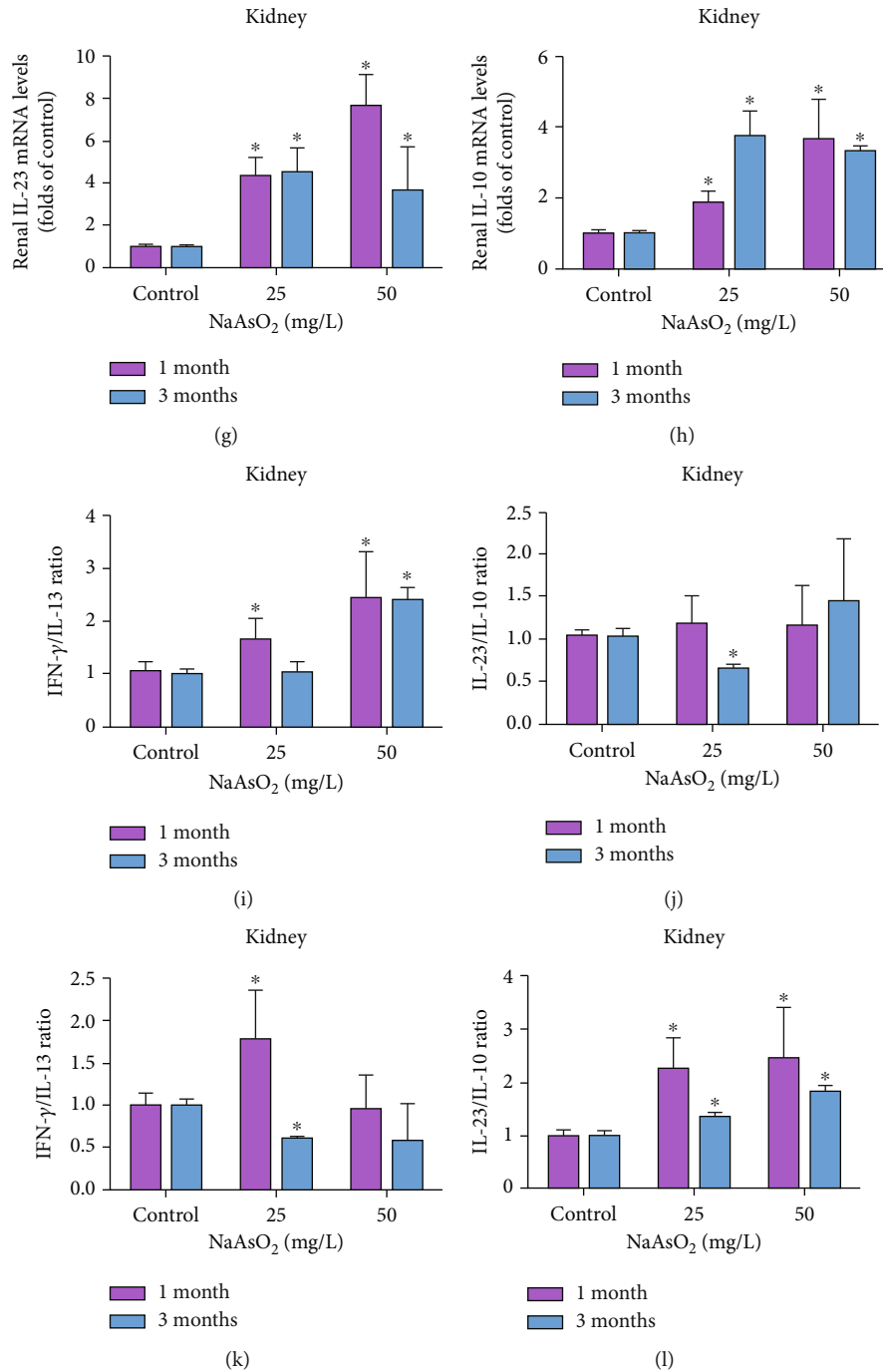


FIGURE 4: Suchronic arsenic exposure affected the expression of CD4⁺ T cell-specific signature cytokines in the liver and kidney. Mice were treated with 25 and 50 mg/L NaAsO₂ by drinking water for 1 and 3 months. The mRNA levels of T cell signature cytokines IFN- γ , IL-13, IL-23, and IL-10 in the liver (a-d) and kidney (e-h) were determined by real-time PCR; the ratios of IFN- γ /IL-13 and IL-23/IL-10 were calculated in liver (i-j) and kidney (k-l). Data were expressed as mean \pm SD. * P < 0.05 compared with control mice.

contribute to cancers [30]. It was reported that proinflammatory cytokines TNF- α , IL-6, and IL-1 β are crucial to immune response, inflammatory, and hematopoiesis, as well as development and progression of tumor [17]. Animal studies have determined that arsenic exposure stimulates proinflammatory cytokine TNF- α , IL-6, and IL-8 gene expressions in the liver of the cocks [31]. In another study, arsenic reportedly stimulated the expression of inflammatory

gene iNOS, cyclooxygenase2 (COX-2) and TNF- α protein, and mRNA in the kidney of chicken [32]. Our experimental results showed that arsenic significantly increased the levels of hepatonephric IL-1 β , IL-6, IL-12, and TNF- α in both 1 and 3 months, suggesting that subchronic arsenic exposure could lead to hepatorenal persistent inflammatory response, which may be further meaningful to raise the incidences of liver and kidney cancers.

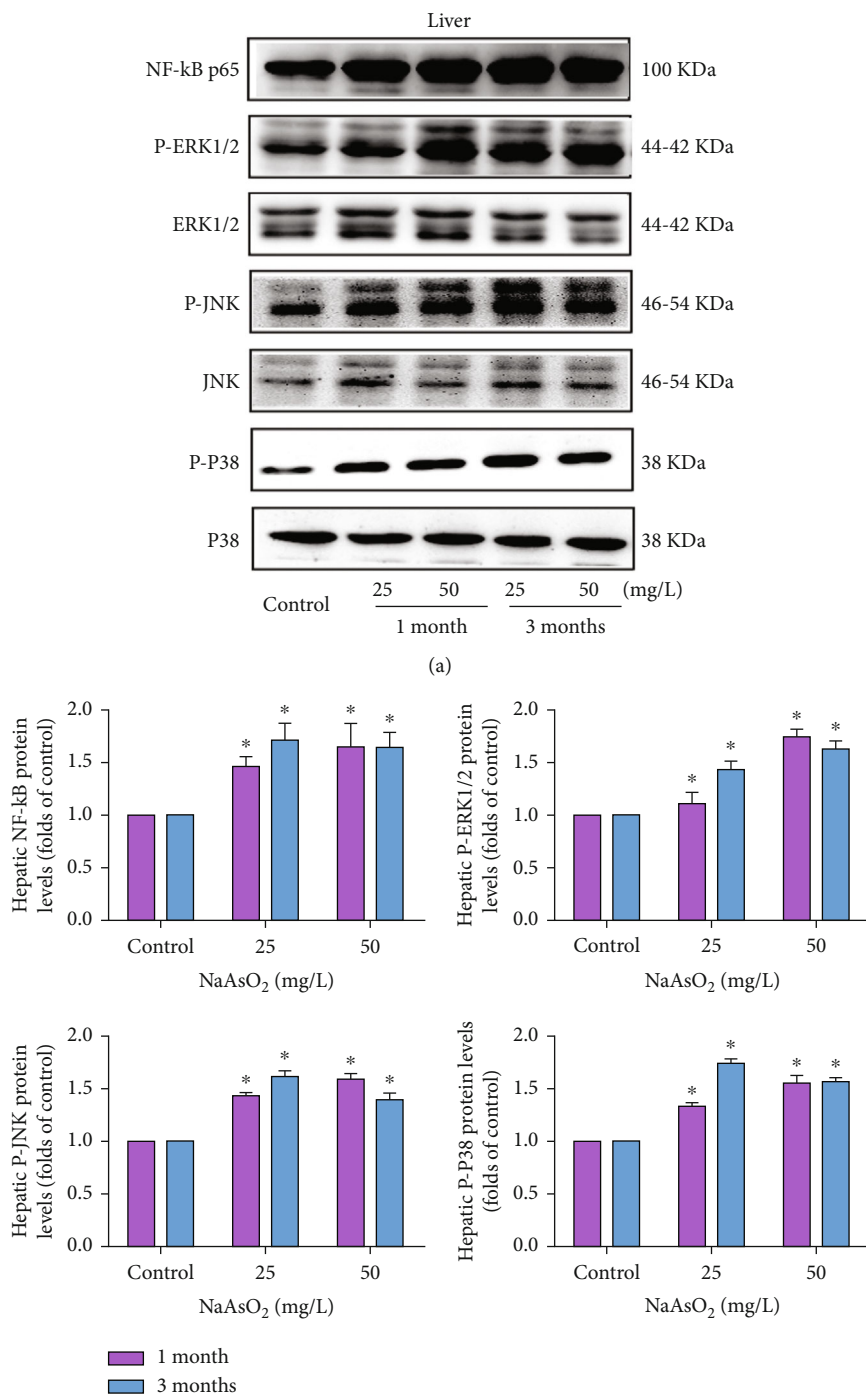


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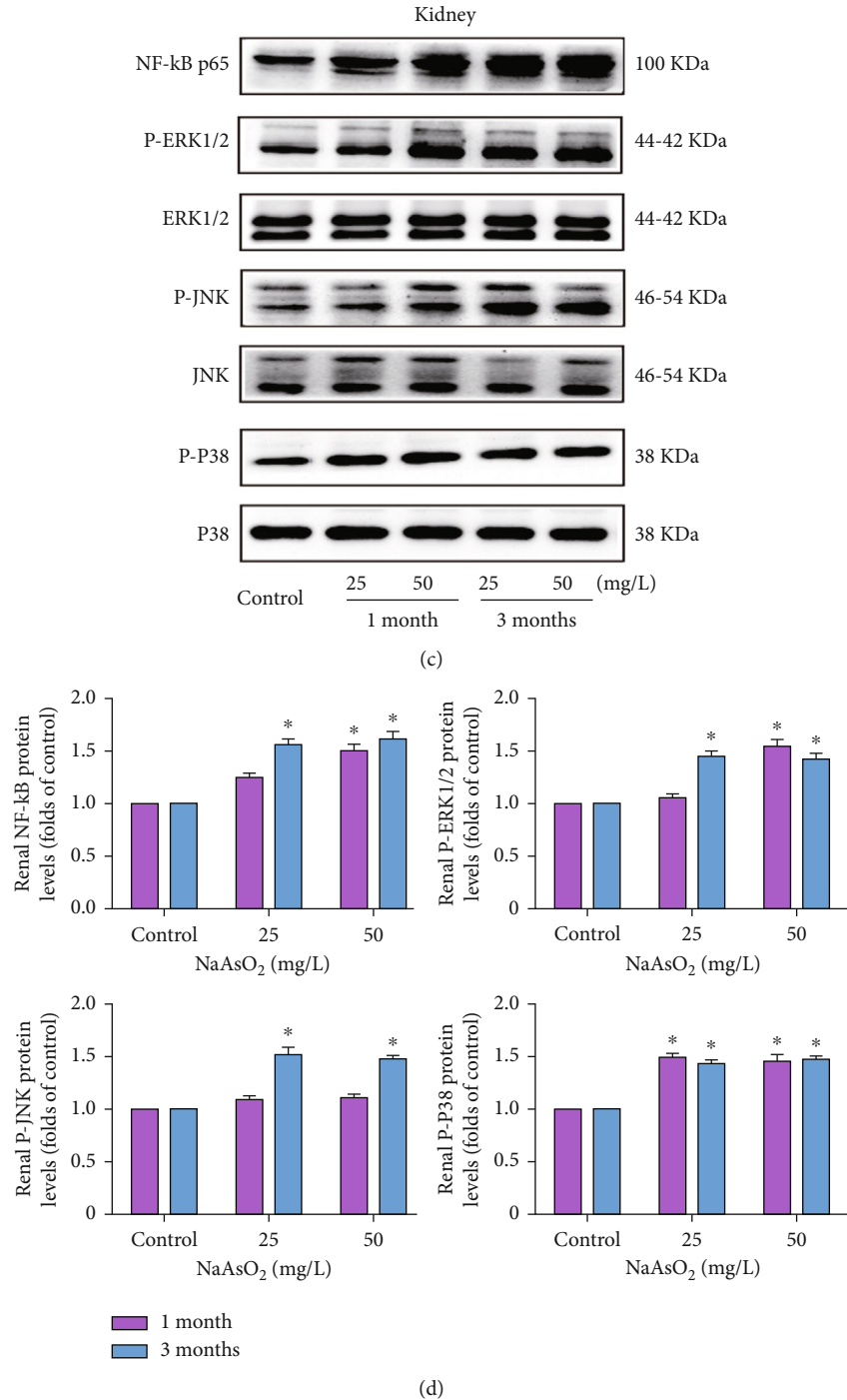


FIGURE 5: Subchronic arsenic exposure activated MAPKs/NF- κ B in the liver and kidney. Mice were treated with 25 and 50 mg/L NaAsO₂ by drinking water for 1 and 3 months. Expression of NF- κ B, P-ERK1/2, P-JNK, and P-P38 in the liver (a) and kidney (c), and the corresponding quantitative analysis (b, d) were assessed by western blotting; β -actin was blotted as the loading control. Data were expressed as mean \pm SD. * P < 0.05 compared with control mice.

Many studies have reported that arsenic could regulate Th1 and Th2 response by affecting the section of their representative cytokines such as IFN- γ , IL-4, and IL-13 [32, 33]. Th17 has strong plasticity and can be transformed into Tregs and Tr1 (Type I regulatory T cells) under pathological conditions, therefore exerting immunosuppressive effects [25]. IL-13 and IL-23 are essential for the differentiation of Th2

as well as Th17 [34, 35]. In addition, IL-23 is found to be overexpressed in many human cancers as well as mouse tumors [34]. Treg cells, which are characterized by IL-10 production, can modulate Th1, Th2, and Th17 immune responses via multiple mechanisms [36]. Our results showed that subchronic arsenic exposure raised IFN- γ , IL-13, IL-23, and IL-10 in the liver and kidney, which suggests that

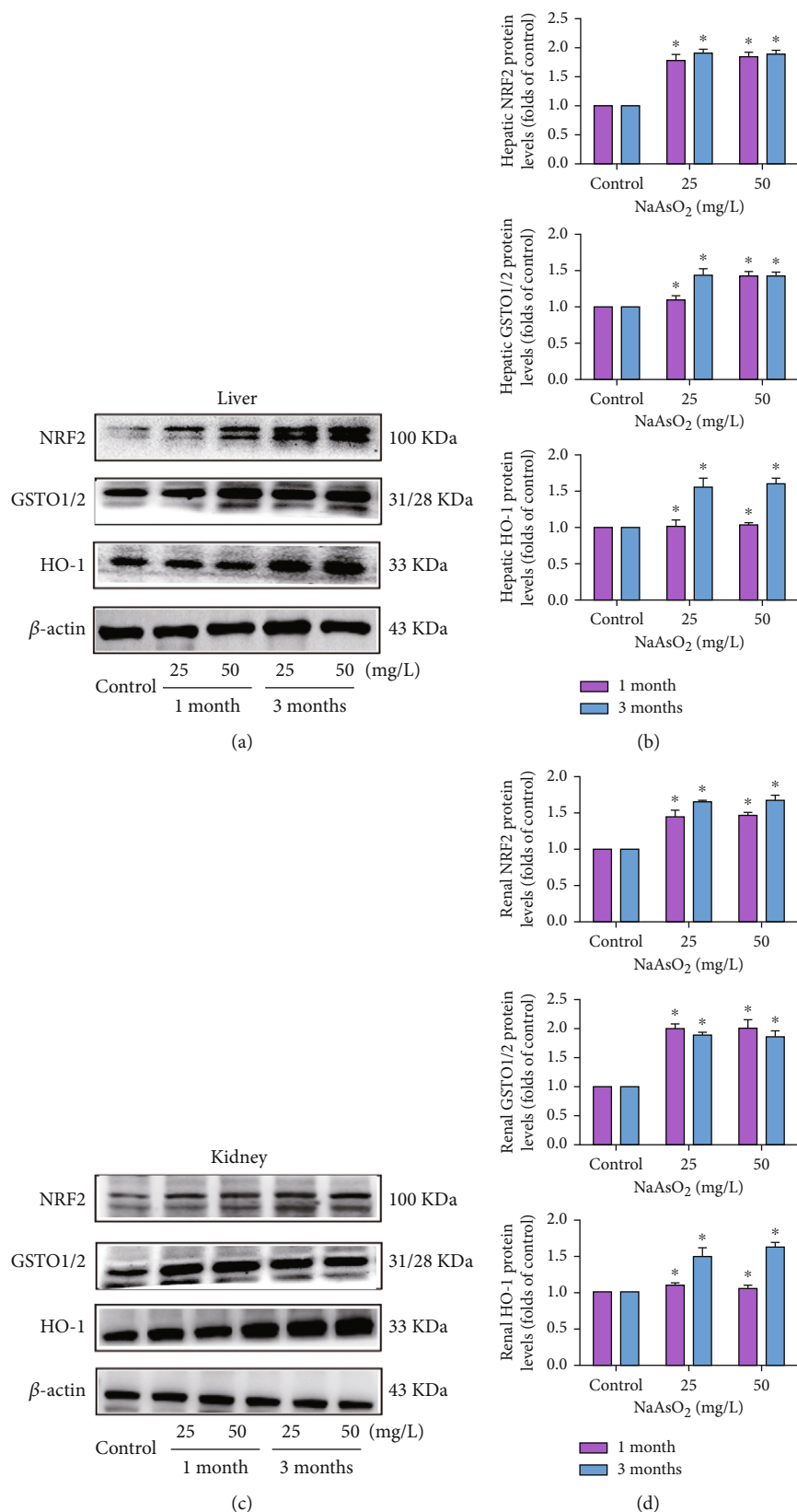


FIGURE 6: Subchronic arsenic exposure activated the Nrf2 pathway in the liver and kidney. Mice were treated with 25 and 50 mg/L NaAsO₂ by drinking water for 1 and 3 months. Expression of Nrf2, GSTO1/2, and HO-1 in the liver (a) and kidney (c) and the corresponding quantitative analysis (b, d) were assessed by western blotting; β -actin was blotted as the loading control. Data were expressed as mean \pm SD. * $P < 0.05$ compared with control mice.

arsenic exposure could disrupt the homeostasis of Th1/Th2/Th17/Treg to induce immune inflammatory responses. Those are consistent with partial conclusions that arsenic altered the level of immune cytokines in preschool children [37] as well as male workers exposed to arsenic in Bangladesh [38]. In addition, researchers also found that arsenic inhibited the cellular immunity by changing the expression of Th1-related cytokines but not affecting Th2 in preschool children [33]. Another in vitro study found that low-dose arsenic (0.25–2 μmol) exposure reduced the secretion of IFN- γ without influencing IL-4 and IL-13 [39]. It is reported that IL-23 could affect the numbers and their capacity to secrete IL-10 of Treg cells [40]. In our study, it was found that the ratio of IFN- γ to IL-13 increased in the liver exposed to arsenic as well as decreased in the arsenic-treated groups except the 25 mg/L NaAsO₂ group with 1 month in the kidney, which indicated that the effect of arsenic on Th1 is greater than Th2 in the liver and a dominate Th2 in the kidney. The difference in results between the liver and kidney may be due to tissue diversity. In patients with coal-burning arsenic poisoning, the imbalance of Th17 and Tregs in peripheral blood mononuclear cells also appeared, characterized by the high level of Th17, and corresponding cytokines as well as decreased Tregs and IL-10 [41]. Our results found that the ratio of IL-23 to IL-10 markedly increased in the liver and kidney, which suggested that the homeostasis between Th17 and Tregs was disrupted as well as prone to Th17 in the liver and kidney. In summary, arsenic undermined the dynamic balance of T lymphocyte subpopulation, which may be closely related to arsenic-induced immune-inflammation, immunosuppression, and the occurrence and development of cancer.

Recently, MAPKs, comprising of ERK1/2, JNK, and P38, have been reported to regulate innate and adaptive immune response as well as mediate the expression of inflammatory cytokines including COX-2, TNF- α , and IL-1 β , through the regulation of transcription factors NF- κ B and activator protein 1 (AP-1) [42, 43]. In addition, the MAPK pathway is highly associated with cancers in human, and NF- κ B is a proinflammatory transcription factor and may be involved in many physiological and pathological processes, including proliferation, apoptosis, and oncogenesis [20]. It has been reported that NF- κ B and MAPK activation could participate in CD4⁺ T cell subpopulation Th17 and Treg cell differentiation [44]. Kim et al. found that 0.5 μM As₂O₃ significantly increased the P38MAPK protein expression in BALB/C 3T3 cells (a model widely used to study cancer development) [45]. In our results, all three members of MAPKs, namely, phospho-ERK, phospho-JNK, and phospho-P38, as well as downstream target NF- κ B were upregulated remarkably in arsenic-treated mice. This implied the persistent inductive effect on MAPK and the NF- κ B pathway in the liver and kidney after the subchronic arsenic exposure.

Generally, Nrf2 is a transcription factor that regulates the expression of NAD(P)H quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HMOX1), glutamate-cysteine ligase (GCL), and glutathione S transferases (GST) under oxidative stress, which could then counteract the oxidative damage of the metalloid [46]. Besides its antioxidant func-

tions, many recent studies have demonstrated that Nrf2 could regulate the expression of immune molecules and inflammatory factors and then play an important role in immune and inflammatory diseases [47]. In terms of cancer, Nrf2 has emerged as somewhat a double-edged sword, because it is not only involved in cancer development but also in cancer treatment [48]. In our result, subchronic arsenic exposure has been shown to potently upregulate the expression of hepatic and renal Nrf2 and its downstream genes GST and GCLM. Li et al. found that acute arsenic exposure resulted in a clear increase of Nrf2, GST, and GCLC protein in both the liver and kidney in vivo [49]. Activation of the Nrf2 pathway by acute arsenic is regarded as a biological defense mechanism and helpful response. However, constitutive Nrf2 activation is an excessive response, which is involved in increasing cancer chemoresistance and enhancing tumor cell growth [50]. It is therefore suggested that one of the main mechanisms involved in the hepatic and nephritic immune-inflammatory abnormalities of arsenic is associated with activating the Nrf2 pathway.

In conclusion, we demonstrated that subchronic arsenic exposure may induce hepatonephric toxicity through the modulation of inflammation and CD4⁺ T cell differentiation, which may be regulated through the activation of the MAPK/NF- κ B and Nrf2 pathways.

Abbreviations

ALT:	Alanine aminotransferase
AST:	Aspartate aminotransferase
AP1:	Activator protein 1
COX-2:	Cyclooxygenase 2
ERK1/2:	Extracellular-signal-regulated kinases1/2
GCL:	Glutamate-cysteine ligase
GST:	Glutathione S transferase
HMOX1:	Heme oxygenase 1
IL-1 β :	Interleukin 1 β
JNK:	C-Jun N-terminal kinases
MPO:	Myeloperoxidase
MAPK:	Mitogen-activated protein kinase
NF- κ B:	Nuclear factor kappa B
Nrf2:	Nuclear factor E2-related factor 2
NQO1:	NAD(P)H quinone oxidoreductase 1
ROS:	Reactive oxygen species
TNF- α :	Tumor necrosis factor- α
T-As:	Total arsenic
Th1:	T-helper 1
Treg:	Regulatory T cells.

Data Availability

No data were used to support this study.

Additional Points

Highlights. Arsenic induced persistent inflammatory responses in the liver and kidney. Arsenic damaged Th1/Th2/Th17/Treg balance in the liver and kidney. Arsenic

activated the MAPK/NF- κ B and Nrf2 pathway continuously in the liver and kidney.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Xiaoxu Duan designed the research. Guowei Xu, Jinlong Li, and Nan Yan performed the experiment. Xin Li and Xuping Liu analyzed the data. Xiaoxu Duan and Bing Li wrote the article.

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