International Immunopharmacology 71 (2019) 115–131

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Chronic arsenic exposure in drinking water interferes with the balances of T lymphocyte subpopulations as well as stimulates the functions of dendritic cells *in vivo*

Lu Zhao, Shan Yang, Yuanyuan Guo, Guifan Sun, Bing Li*

Environment and Non-Communicable Disease Research Center, Key Laboratory of Arsenic-related Biological Effects and Prevention and Treatment in Liaoning Province, School of Public Health, China Medical University, Shenyang 110122, China

ARTICLE INFO

Keywords:
Arsenic
Immune
T cell differentiation
Dendritic cell
Lung
Spleen

ABSTRACT

The immunomodulatory properties of arsenic are nowadays supposed be associated with pathological injuries of this toxicant and the details have not been clarified. Our objective was to explore inflammation, differentiation of diverse T cell subsets, as well as the phenotypic molecules and functions of dendritic cells (DCs) by chronic arsenic exposure *in vivo*. We exposed different concentrations of arsenic (0, 0.1, 1 and 10 mg/L) in drinking water for 6 and 12 months in C57BL/6 mice. We first confirmed that low levels of arsenic induced excess inflammation evidenced by accumulation of macrophages and lymphocytes in bronchoalveolar lavage fluid (BALF), secretion of pro-inflammatory cytokine IL-1β in BALF and serum, as well as histological analysis. Flow cytometry analysis revealed that arsenic disturbed CD4/CD8 T-cell ratio in isolated pneumonocytes and splenocytes, as well as enhanced IFN-γ and reduced IL-4 in spleen. The mRNA expressions of transcription factors (T-bet, GATA3, RORγt) and cytokines (IFN-γ, IL-4, IL-10, IL-23, IL-22) showed the imbalanced Th1/Th2/Th17 differentiation in arsenic exposed lung and spleen. We further testified that arsenic enhanced the percentages of $CD11c^+$ DCs, and promoted the expressions of antigen presentation molecule MHC II and cytokine IL-12, co-stimulatory molecules (CD86, CD80), and chemokine receptors (CCR7, CCR5) *in vivo*. Moreover, arsenic activated the expressions of immune-related MAPKs and NF-κB. Taken together, our study here demonstrated that chronic arsenic exposure could disrupt the immune homeostasis *in vivo* possibly by interfering with the differentiation of Th1/Th2/Th17 subsets as well as the function of DCs.

1. Introduction

Arsenic is a toxic metalloid and exposure to arsenic *via* drinking water has been a significant environmental issue affecting millions of people around the world [1]. Arsenic has been linked with neurotoxicity [2], immunotoxicity [3], cardiovascular diseases [4], as well as several internal cancers [5,6]. In spite of current efforts, over 200 million population globally have still been chronically exposed to arsenic *via* drinking water at concentrations above the WHO safety standard of 10 μg/L [7,8], and researchers have estimated that 19.6 million people are at risk of being affected by the consumption of arsenic-contaminated groundwater in China [9].

Growing evidences have indicated the immunotoxic effect of arsenic [10]. A recent study investigated 123 rural women chronically exposed to arsenic above 10 μg/L yet not exceeding 50 μg/L in groundwater, and declared the up-regulation of tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-12 and C-reactive protein (CRP) in blood plasma and sera, as well as the activation of protein kinase B phosphorylated at ser473 (pAKTser473)/nuclear factor-κB (NF-κB)/TNF-α axis in the leukocytes of exposed women [11]. In an experimental study, 3 mg/kg arsenic for 30 days decreased T (CD3) and B (CD19) lymphocyte population, while altered the relative frequency of CD8/CD4 subpopulation in mice thymocytes and splenocytes [12]. However, regarding to the complicated responses of this toxicant on the complex networks of immune system, the current information is not enough and more researches are still needed to fully assess the possible long-term immune relevant effects.

More intriguingly, as a unique toxicant, arsenic in drinking water, rather than inhalation, is implicated in impaired lung function, bronchiectasis, increased susceptibilities of various respiratory

* Corresponding author at: Environment and Non-Communicable Disease Research Center, Key Laboratory of Arsenic-related Biological Effects and Prevention and Treatment in Liaoning Province, School of Public Health, China Medical University, No.77 Puhe Road, Shenyang North New Area, Shenyang, Liaoning Province 110122, China.
E-mail address: bli10@cmu.edu.cn (B. Li).

https://doi.org/10.1016/j.intimp.2019.03.022
Received 23 October 2018; Received in revised form 9 March 2019; Accepted 9 March 2019
Available online 16 March 2019
1567-5769/

infections and pulmonary diseases [13]. An epidemiological study in a rural area of Mexico showed that arsenic exposure during early childhood or *in utero* was associated with chronic lung inflammation and the possibly impaired lung function in children [14], and 10 and 100 μg/L arsenite in drinking water for 5 weeks altered many immune-related genes and cellular migration genes in lung by transcriptome microarray analysis [15]. On the other hand, spleen is the most important and complex immune organ combining the innate and adaptive immune system in a uniquely organized way [16]. As far as the immunomodulatory properties of arsenic are concerned, the alterations and potential regulatory roles of spleen need a better and renewed recognition, although it may not be the target and the main affected organ by this metalloid yet. For these aspects, it is interesting to go further to explore the comprehensive immune responses and the potential mechanisms by chronic arsenic exposure *via* drinking water using concentrations relevant to human exposure.

Functionally diverse T cell subsets are helpful to confer immunological protections against pathogens, exogenous substances and cancers [17], and $CD4^+$ T cells orchestrate the immune responses and play a pivotal role during infection, chronic inflammation, autoimmune diseases, and carcinogenesis [18]. As we know, $CD4^+$ T cells can be subdivided into different subsets characterized by a specific network of transcriptional regulators and unique cytokine profiles [18]. In this respect, arsenic exposure was associated with changes in the transcriptome of $CD4^+$ T cells, both genome wide and in specific genes in an epidemiological study [19]. In the meanwhile, dendritic cells (DCs) are the strongest antigen-presenting cells (APCs), presenting major histocompatibility complexes (MHC), co-stimulatory molecules and chemokine receptors on their membrane surfaces [20]. Activated DCs not only trigger the production of pro- or anti-inflammatory cytokines, but also induce their migration into lymph nodes and subsequent activation of T cells [21]. It is preliminarily proposed that arsenic decreased the number of DCs in the mediastinal lymph nodes early in the course of influenza A infection [22], and *in vitro* exposure could decrease the phagocytic activity, down-regulate the expression of MHC II and CD40 of human monocytes derived DCs [23], as well as impair the T helper (Th)1 and Th17 activities by disrupting the expression of co-stimulatory molecules and secretion of IL-12p70 and IL-23 of DCs [24]. Overall, evidences on DCs dysfunctions, as well as its interactions with subsequent T cells outcomes by chronic low dose arsenic exposure *in vivo* have still been insufficient.

In the present study, we set up a chronic arsenic exposure mice model by treating with 0.1, 1 and 10 mg/L $NaAsO_2$ in drinking water for 6 and 12 months. Firstly, we observed the inflammatory cellular profiles and the secretion of pro-inflammatory cytokines in bronchoalveolar lavage fluid (BALF) and serum. We next analyzed the $CD4^+$ and $CD8^+$ T lymphocytes subpopulation differentiation and Th1/Th2/Th17 balance, as well as the numbers, phenotypes and functions of DCs in mice lung and spleen. Moreover, we detected the activation of mitogen-activated protein kinases (MAPKs) and NF-κB. The aim of this study is to further understand how arsenic feeding to mice for a long term influences the balances of T lymphocyte subpopulations and the functions of DCs, as well as the related potential mechanisms *in vivo*.

2. Materials and methods

2.1. Reagents and chemicals

Sodium arsenite ($NaAsO_2$, ≥99.0%) was acquired from Sigma Chemical (MO, USA). IL-1β ELISA kit (#4297869) and TNF-α ELISA kit (#4314355) were all acquired from eBiosciences (Vienna, Austria). RPMI 1640 medium and phosphate buffered saline (PBS) were acquired from Gibco-Invitrogen (Carlsbad, CA). Leukocyte activation cocktail and cytofix/cytoperm kit were acquired from BD Pharmingen (San Jose, CA, USA). Antibodies of fluorescein isothiocyanate (FITC)-conjugated anti-CD3, phycoerythrin (PE)-conjugated anti-CD4, allophycocyanin (APC)-conjugated anti-CD8 peridinin chlorophyll *a* protein (PerCP)-conjugated anti-CD8 and cell staining buffer were obtained from BioLegend (CA, USA). Antibodies of PE-conjugated anti-IFN-γ, APC-conjugated anti-IL-4, FITC-conjugated anti-CD11c and APC-conjugated anti-MHC II were obtained from eBioscience (San Diego, USA). Primary antibodies of NF-κB p65 (#8242), P-ERK1/2 (#9101), ERK1/2 (#4696), P-JNK (#4668), JNK (#9252), P-P38 (#9216), P38 (#8690) were purchased from Cell Signaling Technology (Danvers, USA). β-Actin (sc-58673) was acquired from Santa Cruz Biotechnology (CA, USA). Thiourea, ascorbic acid, nitric acid, hydrogen peroxide and hydrochloric acid were purchased from Sangon (Shanghai, China).

2.2. Animals and experimental treatments

Female C57BL/6 mice (18–22 g) were obtained from the Center of Experimental Animals from China Medical University (Shenyang, China) at 6–8 weeks of age. All animal experiments were approved by the Animal Care and Use Committee at China Medical University, which comply with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Forty-eighty mice were housed in stainless steel cages (6 mice per cage) in a specific pathogen-free environment and maintained on standard mouse chow at a temperature of 22 ± 1 °C and 12 h/12 h light/dark cycles, with water *ad libitum*. Mice were exposed to environmentally relevant concentrations of arsenic (0, 0.1, 1 and 10 mg/L) with free drinking water for 6 and 12 months, respectively. A total 6 mice were kept in each group. The control group was treated with saline only. Drinking water containing indicated doses of arsenic was prepared every other day.

Urine from each mouse was collected 24 h before the end of exposure. At each end point of the treatment, all mice were weighed and deeply anesthetized. Blood was obtained through eye-ball extirpating, and plasma was separated by centrifugation (3500*g*, 10 min, 4 °C) and stored at −80 °C. BALF was obtained after cannulating the trachea and removing the lungs. The entire mouse lung and spleen tissues were rapidly obtained and weighed before stored at −80 °C. About 0.1 g of tissue was isolated for flow cytometry. Appropriate amounts of tissues were fixed in 4% paraformaldehyde for the following histological analysis.

2.3. Determination of iAs, MMA, DMA and total arsenic (T-As) in urine

Measurement of arsenic speciation was performed as described previously [25,26]. Briefly, 1 mL urine was digested with 2 mol/L NaOH at 100 °C for 3 h. Digested solutions were assayed using the method based on the hydride generation of volatile arsines, followed by cryogenic separation, and iAs, MMA and DMA were detected by atomic absorption spectrophotometry (AAS). The detection limit of each of the three arsenicals (iAs, MMA, DMA) was 1 ng, and the coefficient of variation was < 5%. We acquired an iAs standard of 1000 mg/L from the National Center for Standard Reference Materials (Beijing, China) and a mixed arsenic standard of 1000 mg/L MMA and DMA (Tri Chemical Laboratories Inc., Yamanashi, Japan). Total arsenic (T-As) level was then calculated by summing up the corresponding levels of iAs, MMA and DMA determined in each urine sample. Determination of arsenic in urine was performed by an atomic absorption spectrophotometer (AA-6800, Shimadzu Co., Kyoto, Japan) with an arsenic speciation pretreatment system (ASA-2SP, Shimadzu Co., Kyoto, Japan).

2.4. Calculation of the lung and spleen indexes

Lung and spleen indexes were calculated according to the formulas: (lung or spleen weight / body weight) × 100%.

2.5. BALF preparation

Tracheas were cannulated after the collection of whole blood in mice. About 2 mL BALF was extracted from mice and centrifuged at 3000 rpm for 10 min at 4 °C. The supernatants of lavaged fluid were collected and stored at −80 °C until analyzed for the levels of cytokines of BALF. Precipitates were re-suspended with 100 μL physiological saline, and 20 μL were used for cell counting. The remaining cell suspensions were added another 1 mL sterile saline into a cytospin (Sakura Co, Ltd., Tokyo, Japan) to make cell smears, and further stained with Diff-Quik staining solution (International Reagents Co, Kobe, Japan). A total of 200 cells were counted by oil immersion microscopy, and different cell types were classified and counted according to their cellular morphology and staining.

2.6. ELISA assay

The levels of IL-1β and TNF-α in BALF and serum were measured by mouse IL-1β and TNF-α ELISA kits, respectively. All experimental procedures were in strict conformity to the manufacturer's instructions. Quantification of ELISA results were performed using the automatic microplate reader (SpectraMax M5, Molecular Devices, USA) at 450 nm. The levels of IL-1β and TNF-α were expressed as pg/ml.

2.7. Histological analysis

Lung and spleen tissues were dissected at 6 or 12 months, then immediately fixed in 4% paraformaldehyde for 48 h. Subsequently, the tissues were washed with running water, and infused in the deionized water for about 6 h. Then the tissues were immersed in the 70% ethanol at 4 °C overnight, graded ethanol dehydration and embedded in paraffin, tissue sections (5-μm thickness) were deparaffinized and stained with hematoxylin and eosin (H&E) for histopathological examination using a microtome (Thermo, USA).

2.8. Flow cytometric analysis

Single cell suspensions were prepared from lung and spleen tissues of mice. In brief, lung and spleen tissues were dispersed by repetitive suction and passed through a 70-μm cell strainer (BD Bioscience). Red blood cell lysis buffer (RBCL) was used to lyse erythrocytes. After that, pneumonocytes and splenocytes (1×10^6 cells/mL) were washed in PBS and re-suspended in cell staining buffers for cell surface staining. FITC-CD3, PE-CD4 and APC-CD8 were used to identify $CD4^+$ or $CD8^+$ T cell types. In addition, FITC-CD11c and APC-MHC II were used to detect the surface molecules of $CD11c^+$ DCs. We captured 50,000 events of each sample, dead cells were gated out depending on forward scattering (FSC) and side scattering (SSC). First, $CD3^+$ T cells were gated in basing on the negative blank (only cells) and the positive dye (FITC-CD3). Then, we divided panel into four quadrants depending on negative blank (only cells) and the positive dye (PE-CD4 and APC-CD8). Right quadrants represent $CD3^+$ $CD4^+$ T cells and upper quadrants represent $CD3^+$ $CD8^+$ T cells. Similar, $CD11c^+$ cells were gated in basing on the negative blank (only cells) and the positive dye (FITC-CD11c), and $CD11c^+$ MHC II^+ cells were gated in depending on negative blank (only cells) and the positive dye (APC-MHC II).

For intracellular staining, splenocytes were pelleted and re-suspended in complete medium. Single cell suspensions of the spleen were distributed in a 48-well round bottom plate at a density of 1×10^6 cells/mL. Splenocytes were stimulated with leukocyte activation cocktail for 5 h, splenocytes were then washed with PBS and fixed/permeabilized using the cytofix/cytoperm kit, following by incubation with FITC-CD3, PerCP-CD8, PE-IFN-γ and APC-IL-4 to identify $CD3^+$ $CD4^+$ IFN-γ^+ and $CD3^+$ $CD4^+$ IL-4^+ T cell types, respectively. It's nowadays acceptable that the expression of $CD4^+$ T cells is susceptible to phorbol 12-myristate 13-acetate (PMA) and calcium ionophore, which are the necessary components in the commercial kit of leukocyte activation cocktail. Therefore, we regarded the left quadrants as $CD4^+$ T cells ($CD3^+$ $CD8^-$), as well as the right quadrants as $CD3^+$ $CD8^+$ T cells in our results.

Analysis of cell markers was all performed using a BD FACS Canto™ II flow cytometry system (BD Bioscience, San Jose, CA, USA). Flow cytometry data analysis was finally done and displayed with FlowJo (FlowJo LLC) and GraphPad (GraphPad Prism 5.0, La Jolla, CA).

2.9. Total RNA isolation and real-time PCR

Total RNA of mice lung and spleen tissues were extracted from experimental mice using a Trizol Reagent (Invitrogen) to prepare cDNA. Real-time PCR was conducted using a two step method with a QuantStudio 6 Flex Real-Time PCR System (ABI, USA). Briefly, 500 ng of total RNA was reverse-transcripted to cDNA using GoScript™ Reverse Transcription System (Promega, WI, USA), and PCR amplification was performed by GoTaq® qPCR Master Mix (Promega, WI, USA). PCR amplification conditions were: 1 cycle of hold stage (95 °C for 2 min), PCR stage (95 °C for 15 s) and 40 cycles of melt curve stage (95 °C for 15 s and 60 °C for 1 min). Primers for mouse genes were designed and synthesized by Invitrogen (Carlsbad, CA, USA) as follows: *T-bet* accession number (NM_019507) forward (tcaaccagcaccagacagaga) and reverse (tccaccaagaccacatccac); *Ifn-γ* accession number (NM_008337) forward (aagcgtcattgaatcacacctg) and reverse (tgacctcaacttggcaatactc); *Gata3* accession number (NM_008091) forward (ctcggccattcgtacatggaa) and reverse (ggatacctctgcaccgtagc); *Il-4* accession number (NM_021283) forward (ggtctcaacccccagctagt) and reverse (gccgatgatctctctcaagtgat); *Il-10* accession number (NM_010548) forward (ggggccagtacagccgggaa) and reverse (ctggctgaaggcagtccgca); *Ror-γt* accession number (NM_011281) forward (acggccctggttctcatca) and reverse (ccaaattgtattgcagatgttccac); *Il-23* accession number (NM_031252) forward (cccgtatccagtgtgaagatg) and reverse (ccctttgaagatgtcagagtc); *Il-22* accession number (NM_016971) forward (atgagtttttcccttatggggac) and reverse (gctggaagttggacacctcaa); *Il-12* accession number (NM_001303244) forward (tggtttgccatcgttttgctc) and reverse (acaggtgaggttcactgtttct); *Cd86* accession number (NM_011245812) forward (ctggactctacgacttcacaatg) and reverse (agttggcgatcactgacagtt); *Cd80* accession number (NM_001359898) forward (gcaggatacaccactcctcaa) and reverse (aaagacgaatcagcagcacaa); *Ccr7* accession number (NM_001301713) forward (tgtacgagtcggtgtgcttc) and reverse (ggtaggtatccgtcatggtcttg); *Ccr5* accession number (NM_009917) forward (ctgctgcctaaaccctgtcat) and reverse (tgcaaaagcgtttgaccatgt); *Gapdh* accession number (NM_001289726) forward (tgtgtccgtcgtggatctga) and reverse (ttgctgttgaagtcgcaggag).

Cycle threshold (Ct) values were obtained graphically for both different target genes and Gapdh. $2^{-\Delta\Delta Ct}$ values were calculated to represent the amounts of different target genes.

2.10. Western blot analysis

The total proteins of lung and spleen were extracted and measured by commercial kits. Aliquots of supernatant (30 μg total protein) were boiled for 5 min. Samples were loaded on to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated and then transferred onto 0.20 μM PVDF membranes (Millipore, USA). The blots were placed in blocking solutions (5% BSA in Tris-buffered saline or 5% non-fat milk) for 2 h at room temperature. Followed by incubation overnight with a primary antibody (rabbit anti-NF-κB, 1:1000 dilution; rabbit anti-P-ERK1/2, 1:1000 dilution; rabbit anti-ERK1/2, 1:1000 dilution; rabbit anti-P-JNK, 1:1000 dilution; rabbit anti-JNK, 1:1000 dilution; rabbit anti-P-P38, 1:1000 dilution; rabbit anti-P38, 1:1000 dilution; rabbit anti-β-actin, 1:2000 dilution) at 4 °C overnight, respectively. On the second day, membranes were washed and incubated with a 1:2000 dilution of secondary antibody for 1 h at room temperature. Membranes were washed and developed with

a high-performance luminol substrate solution (Absin, Shanghai, China) and visualized using Electrophoresis Gel Imaging Analysis System (Azure Biosystems C300, USA). Band densities were normalized to β-actin in each sample.

2.11. Statistical analysis

A statistician was consulted before the start of the experiment for the minimum number of mice required to give viable statistical and reproducible data and for statistical analysis. All analyses were performed using SPSS software, version 11.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as the mean ± SEM. Statistical significance was determined by one-way analysis of variation (ANOVA) and Dunnett's posthoc test, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Body weights, tissues indexes, as well as urinary arsenic levels in mice

C57BL/6 female mice were fed with indicated doses of arsenic for 6 or 12 months. The body weights increased from approximately 20 g to 26 g without apparent changes among different groups (Fig. 1A). Mice in all groups grew well, with smooth hair and agile activity. The average daily food intake of each mouse for 12 months was calculated as 3.27 g, 3.05 g, 3.15 g and 2.79 g, respectively (data not shown). These observations suggested that no obvious systemic toxicity was induced by our treatment.

The weights and indexes of immune organs are generally used to evaluate the body immune functions [27]. In our study, both the weights and the indexes of lung and spleen didn't show any obvious changes for 6 months. However, exposure to arsenic for 12 months at 10 mg/L was associated with reduced pulmonic and splenic weights (Fig. 1B, D), as well as pulmonic and splenic indexes (Fig. 1C, E). 1 mg/L arsenic also decreased the spleen index to some extent at 12 months of exposure. The decrease of both the weights and the indexes of lung and spleen in our results indicated that chronic arsenic administration in drinking water might impair the normal growth of lung and spleen tissues.

In our study, T-As content in urine increased in a dose-dependent pattern, and the concentrations of T-As in different arsenic treatment groups were detected as 559.15 ng/mL, 1638.71 ng/mL and 10,943.78 ng/mL, respectively (Fig. 2A). What's more, the concentrations of iAs, MMA and DMA also proved a dose-dependent manner. Methylated metabolites in 0.1, 1 and 10 mg/L arsenic exposed mice were mainly in the form of DMA with the percentage of 53.04%, 80.2% and 69.37%, respectively. The proportions of iAs were 12.52%, 9.48% and 12.51%, as well as MMA were 34.45%, 10.32% and 18.12%, respectively (Fig. 2B).

3.2. Chronic arsenic exposure orally induces infiltration of inflammatory cells in vivo

To investigate whether oral chronic arsenic exposure could initiate inflammatory changes, the cellular profiles of BALF were examined first. Different cell types in BALF were shown in Fig. 3A, and 10 mg/L arsenic-treated group at 6 months increased total numbers of inflammatory cells remarkably, which were more convincing at 12 months in all arsenic-treated groups (Fig. 3B). The cells in BALF were mainly macrophages, lymphocytes and neutrophils. Among them, the numbers of macrophages at 6 and 12 months were both significantly increased. Particularly, 0.1 mg/L, 1 mg/L and 10 mg/L arsenic raised macrophages populations by 2.4 folds, 1.8 folds and 2.0 folds at 6 months, as well as 1.8 folds, 2.2 folds and 2.0 folds at 12 months (Fig. 3C). Similar to macrophages, lymphocytes populations were raised 4.5 folds, 2.3 folds and 3.5 folds by 0.1 mg/L, 1 mg/L and 10 mg/L arsenic at 6 months, as well as 3.4 folds, 5.2 folds and 3.9 folds at 12 months (Fig. 3D). As to neutrophils, they were also somehow increased among experimental groups (Fig. 3E).

Pro-inflammatory cytokines, including IL-1β and TNF-α, promote abnormal inflammatory immune responses and homeostatic imbalances [28]. In this paper, we determined the secretion of the representative pro-inflammatory cytokines in both BALF and serum by ELISA. Our results only found a significant increase of IL-1β in BALF at 6 and 12 months by 10 mg/L arsenic treatment (Fig. 4A, C).

Furthermore, we focused on the pathological abnormalities in both lung and spleen tissues. Our results revealed that progressive inflammatory cells accumulated around the pulmonary perivascular cells (arrows) at 6 months (Fig. 5A2–4) and 12 months (Fig. 5B2–4) in arsenic-treated groups, and inflammatory cells were with denser staining at 12 months than 6 months between the same doses. In addition, modern amounts of lymphocytes and macrophages containing hemosiderin (brown pigment) were found to be dispersed and scattered in spleen of experimental groups (Fig. 5C2–4).

3.3. Chronic arsenic exposure orally regulates T lymphocyte subpopulations and impairs the Th1/Th2/Th17 balance in vivo

T lymphocytes and T lymphocyte subpopulations display critical and diverse roles in the establishment and suppression of inflammation [29]. CD4/CD8 ratio keeps dynamic balance and plays an important role in regulating cell immunity and humoral immunity [30]. In our study, it was observed that the number and proportion of $CD4^+$ T lymphocytes elevated in spleen tissues at 12 months (Fig. 6E, F), and CD4/CD8 ratio increased to some extent in all arsenic-treated groups (Fig. 6H). However, the number and proportion of $CD8^+$ T lymphocytes rose at 0.1 and 1 mg/L groups in lung tissues (Fig. 6A, C), resulting in the decrease of CD4/CD8 ratio in 1 mg/L arsenic-treated mice (Fig. 6D).

To further examine whether chronic arsenic exposure regulated the $CD4^+$ T cells subpopulation differentiation, we performed flow cytometry to examine Th1 and Th2 subpopulation in lung and spleen single cell suspensions at 12 months. The percentage of $CD4^+$ T cells expressing IFN-γ, regarded as Th1 cells, notably increased at 1 mg/L and 10 mg/L arsenic-treated group (Fig. 7A, C). On contrast, the percentage of $CD4^+$ T cells expressing IL-4, regarded as Th2 cells, decreased remarkably (Fig. 7B, D). As a result, the ratio of Th1/Th2 in spleen was elevated to approximately 2.9 folds and 3.5 folds by 1 mg/L and 10 mg/L of arsenic, respectively (Fig. 7E). In our experimental conditions, we failed to detect the $CD4^+$ T lymphocyte subsets due to the small proportion of T cells in lung tissues.

What is more, we next focused on the expressions of specific master transcription factors and the corresponding signature cytokines of different types of Th cells in tissues. mRNA levels of *T-bet*, the master transcription factor for Th1 cells, were up-regulated in arsenic exposed groups in both lung and spleen (Fig. 8A, B), while the Th2 master transcription factor *Gata3* slightly decreased (Fig. 8E, F). As to the expression of cytokines, both *Il-4* and *Il-10* levels of Th2 were strikingly inhibited by 1 and 10 mg/L arsenic in lung tissues (Fig. 8G, I), and *Ifn*-γ of Th1 increased by 213.86% and 194.40% in the spleen (Fig. 8D), which were consistent with our results of flow cytometry in Fig. 7 showing the higher percentages of IFN-γ^+ T cells and lower percentages of IL-4. These results suggested the likely imbalance of Th1/Th2 differentiation and Th1-dominant responses of $CD4^+$ T cells by arsenic orally.

It is also reported that arsenic might repress ROR-γt, a key transcription factor in fully differentiated Th17 cells, and imbalanced Th17 responses are implicated in many immune disorders [31]. Our experiments further found that the mRNA levels of *Il-23* and *Il-22*, specific cytokines of Th17, decreased evidently in lung tissues by 1 and 10 mg/L arsenic treatment (Fig. 9C, E), while *Ror*-γ*t* remained changeless (Fig. 9A). However, as to the spleen, both the transcription factor *Ror*-γ*t* (Fig. 9B), as well as the cytokines *Il-23* and *Il-22* (Fig. 9D, F) were all remarkably increased to some extent. Taken together, these results

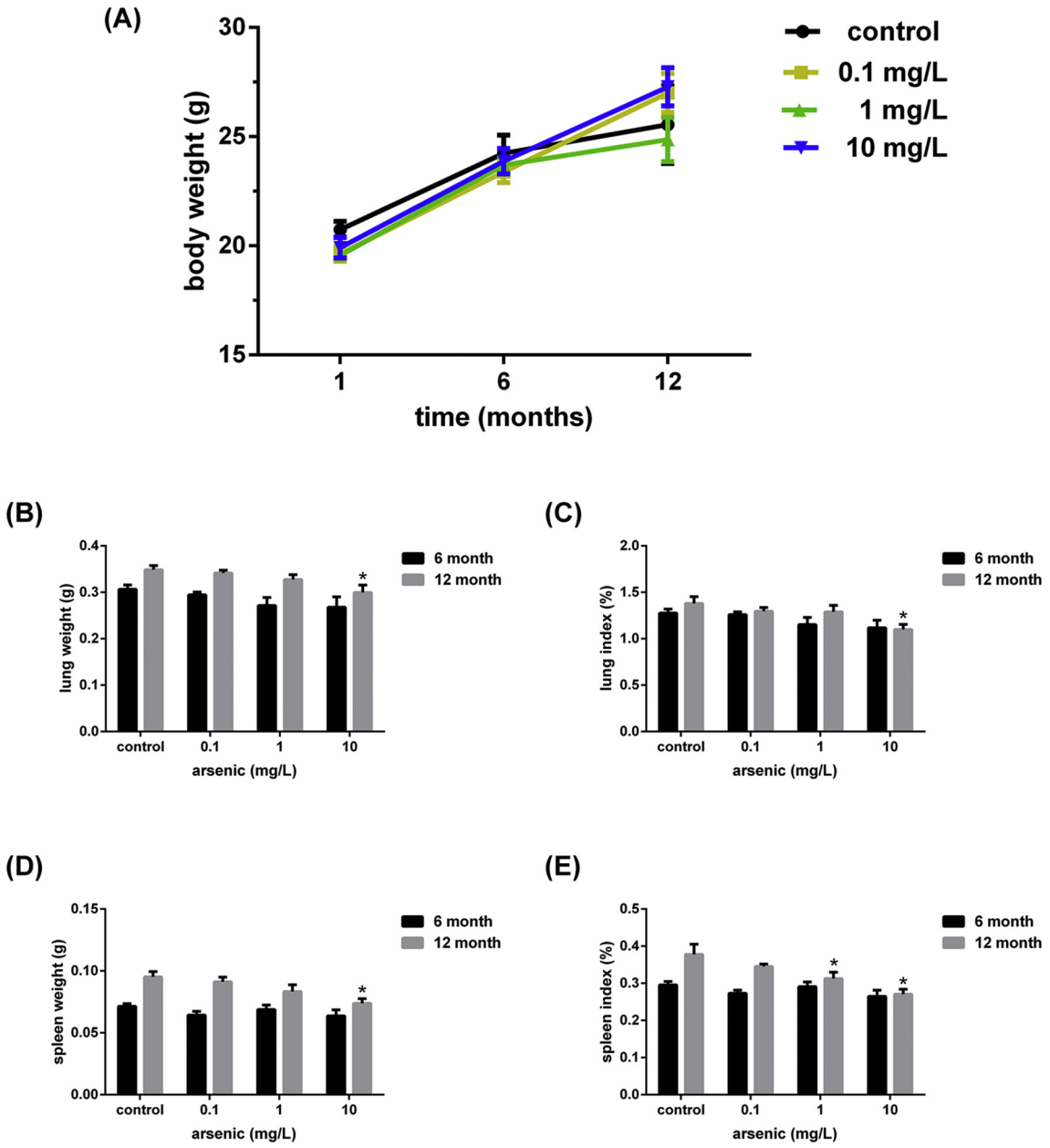

Fig. 1. Alterations of body weights, pulmonic and splenic weights/indexes by chronic arsenic exposure. C57BL/6 mice drank water with indicated doses of arsenic freely for 6 or 12 months. Body weights within 12 months (A), pulmonic and splenic weights (B, D), pulmonic and splenic indexes (C, E) were calculated, respectively. Data were presented as mean ± SEM (n = 4–6). * denoted $p < 0.05$ compared with control group.

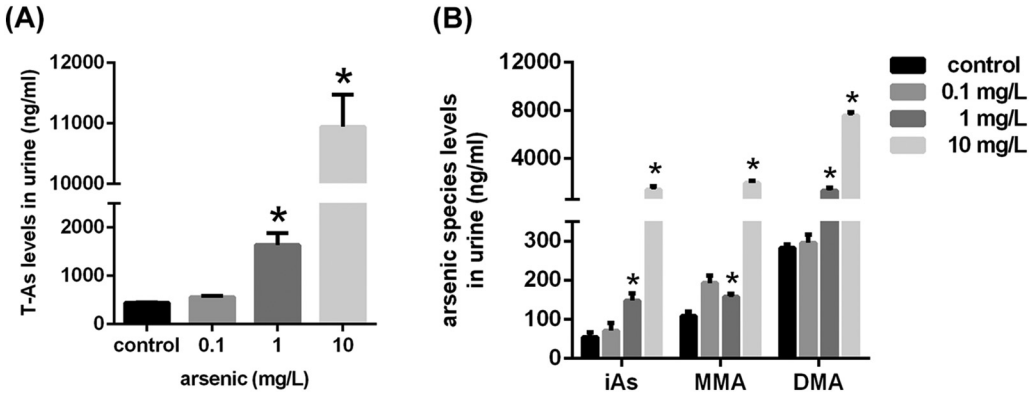

Fig. 2. Urinary total arsenic (T-As), iAs, MMA and DMA levels in chronic arsenic-exposed mice. C57BL/6 mice drank water with indicated doses of arsenic freely for 12 months, urinary (A) T-As, (B) iAs, MMA and DMA levels were determined by AAS as described in materials and methods. Results were expressed as mean ± SEM (n = 4). * denoted $p < 0.05$ compared with control group.

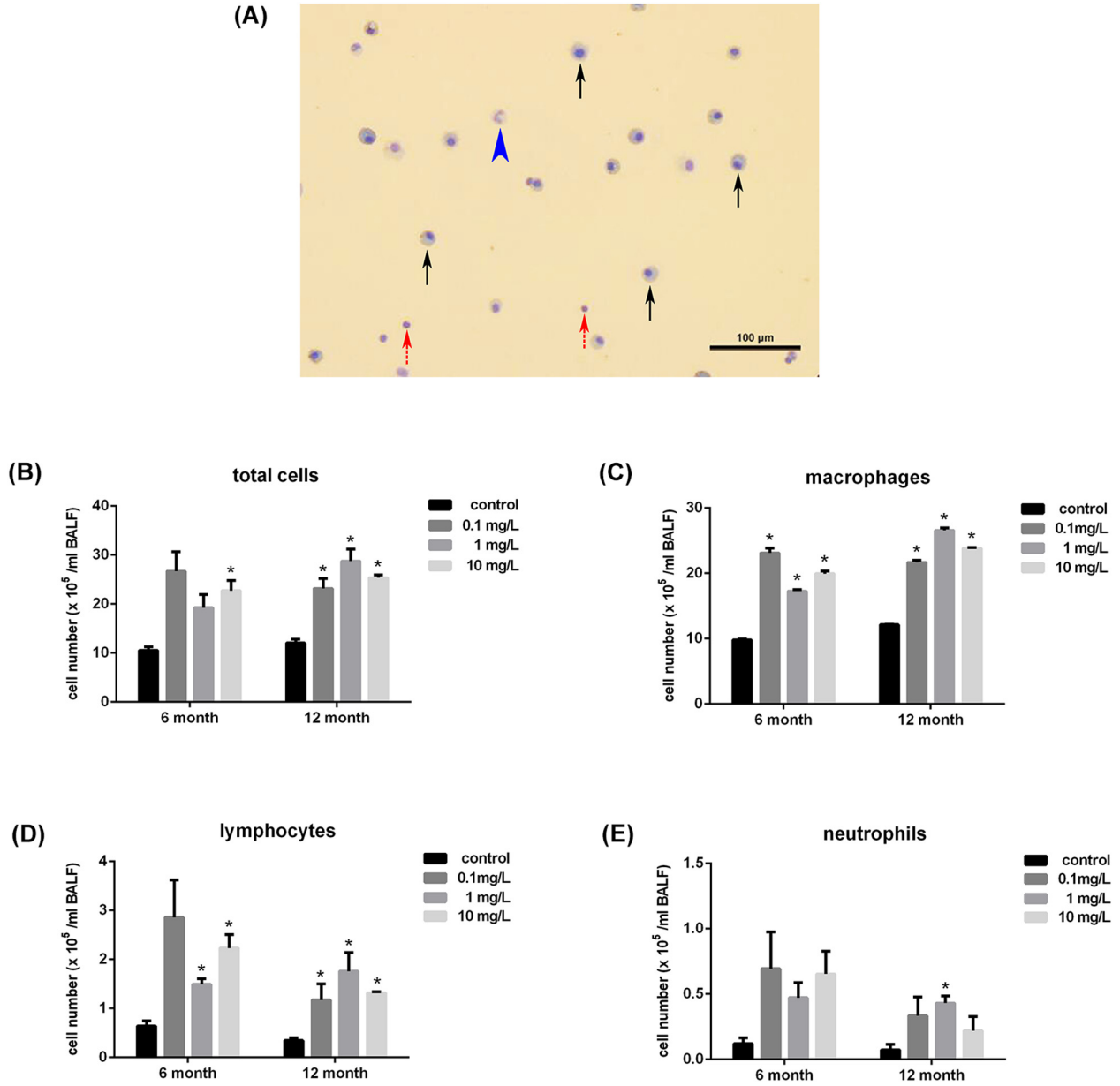

Fig. 3. Inflammatory cells in BALF by chronic arsenic exposure. C57BL/6 mice drank water with indicated doses of arsenic freely for 6 or 12 months. BALF of the mice were prepared as described in materials and method, and the inflammatory cells were observed and counted immediately. The morphology of different cell types (macrophages: solid arrows; lymphocytes: dotted arrows; neutrophils: arrowheads) were shown in (A). Scale bar = 100 μm. The numbers of total inflammatory cells (B), as well as different cell types of fresh fluid specimen (C–E) were counted. Data were presented as mean ± SEM ($n = 4$). * denoted $p < 0.05$ compared with control group.

suggested that chronic low dose of arsenic exposure could regulate the $CD4^+$ and $CD8^+$ T lymphocytes proportion, as well as interfere with the balance of Th1/Th2/Th17 differentiation.

3.4. Chronic arsenic exposure orally enhances both the phenotypes and functions of DCs in vivo

DCs are the most powerful APCs in the body which possess the ability to activate T lymphocytes, further affecting the body's immune responses [32]. In this study, we also identified the effects of chronic arsenic exposure in drinking water on the quantity of DCs. Our results showed that not only the proportion, but also the absolute cell numbers of DCs in lung increased notably in 10 mg/L arsenic-exposed mice (Fig. 10A, B). What's more, the percentage of DCs in spleen consistently elevated in all arsenic-treated groups, even at the low dose of 0.1 mg/L (Fig. 10D).

We simultaneously investigated the expression levels of surface marker MHC II in lung and spleen single cell suspensions, respectively. MHC II are cell surface glycoproteins on APCs which are involved in the binding and presentation of peptide antigens to the T lymphocyte receptors (TCRs) of $CD4^+$ T lymphocytes [33]. As compared with the control group, the percentage of splenic $CD11c^+$ MHC II^+ DCs notably increased in all arsenic-exposed groups (Fig. 10F).

Cytokines are critical in the regulation of DCs function as well as their capacity to prime T cell responses [34]. Particularly, cytokine IL-12 secreted mainly by mature DCs could specifically control the differentiation of Th1 lymphocytes [35]. In our experiments, we found that arsenic exposure significantly increased the transcription levels of *Il-12* not only in spleen (Fig. 11B) but also in the lung tissues of mice (Fig. 11A). In addition, surface molecules CD86 and CD80 are two key

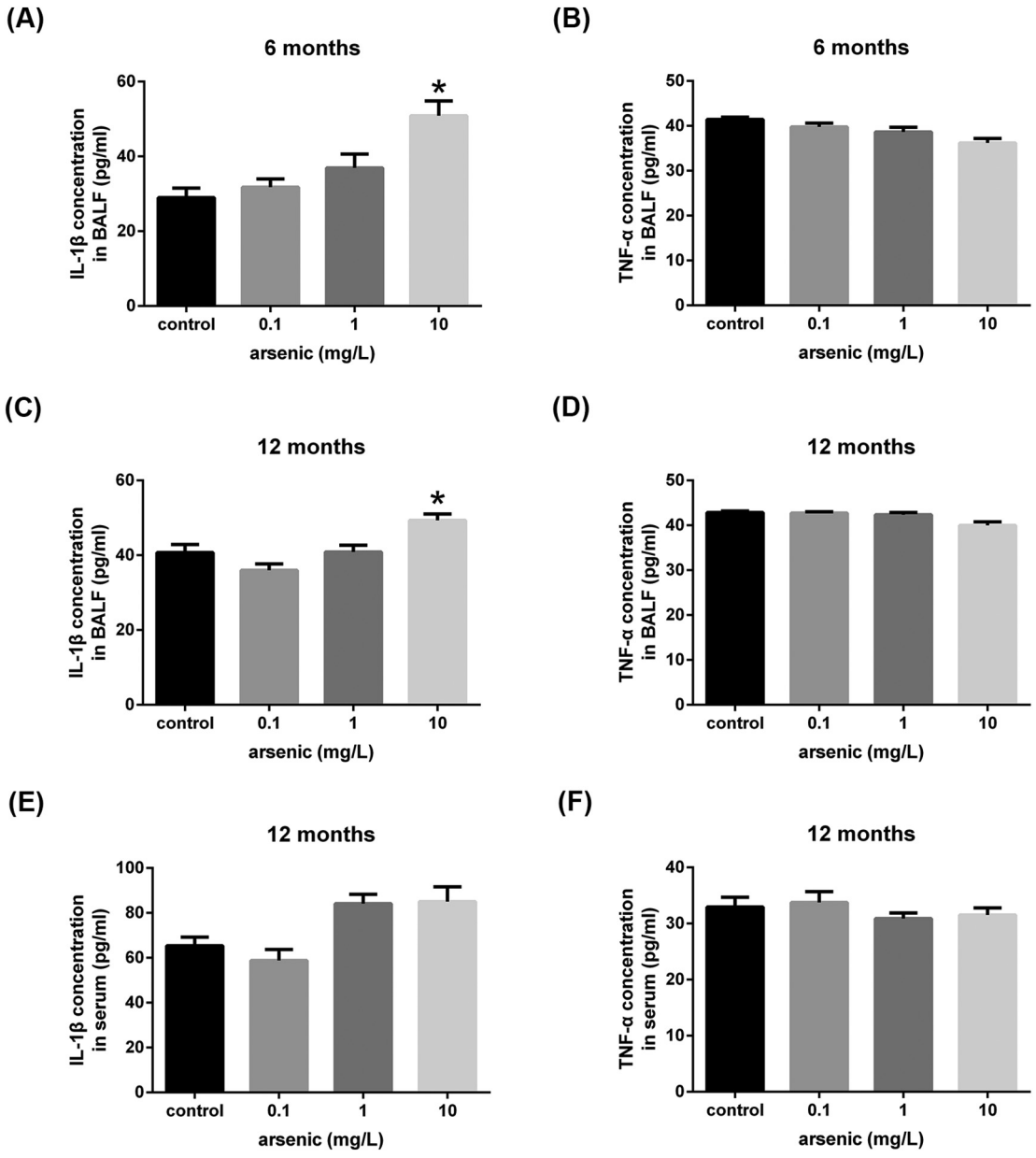

Fig. 4. Inflammatory cytokine levels in BALF and serum by chronic arsenic exposure. C57BL/6 mice drank water with indicated doses of arsenic freely for 6 or 12 months. The concentrations of IL-1β (A, C, E) and TNF-α (B, D, F) in BALF and serum were measured by ELISA. Data were presented as mean ± SEM (n = 4–6). * denoted $p < 0.05$ compared with control group.

co-stimulatory factors of DCs, which are requisite in T cell activation and survival [36]. We next determined the effect of chronic arsenic exposure on the expression of CD86 and CD80. In comparison with control group, the mRNA levels of *Cd86* were increased by 179.35% and 199.82% in spleen with 1 and 10 mg/L arsenic treatment, respectively (Fig. 11D). Besides, *Cd80* in both lung (Fig. 11E) and spleen (Fig. 11F) was also enhanced to some extent.

Given to DCs migration is crucial for antigen specific activation of T cells, we further investigated the expressions of chemokine receptors (CCR) 7 and CCR5 in the presence of arsenic. Our results showed that the transcription levels of *Ccr7* increased significantly in both lung (Fig. 11G) and spleen (Fig. 11H) of mice treated with 1 and 10 mg/L arsenic at 12 months, respectively. Similarly, the mRNA levels of *Ccr5* were also up-regulated in both lung (Fig. 11I) and spleen (Fig. 11J). These results together suggested that not only the quantities, but also the phenotypes and functions of DCs might be affected by chronic arsenic exposure *in vivo*.

3.5. Chronic arsenic exposure orally activates nuclear translator factor NF-κB and its up-stream MAPKs

NF-κB is an important transcription factor that regulates the expressions of a variety of inflammatory mediators [37]. What's more, it is widely reported that MAPKs play important roles in the regulation of several genes involved in immune and inflammatory responses, through the regulation of transcription factor NF-κB [38]. We therefore examined the expressions of NF-κB and its up-stream MAPKs in lung and spleen tissues. In our results, the expression of NF-κB protein was notably up-regulated in lung by 0.1, 1 and 10 mg/L arsenic-treated groups at 12 months. Consistently, phosphorylated forms of ERK1/2, JNK and P38 were also induced to different degrees (Fig. 12A). What's more, arsenic also raised the NF-κB protein and phosphorylated forms of JNK and P38 in spleen at 12 months, except that phosphorylated ERK1/2 was slightly elevated (Fig. 12B).

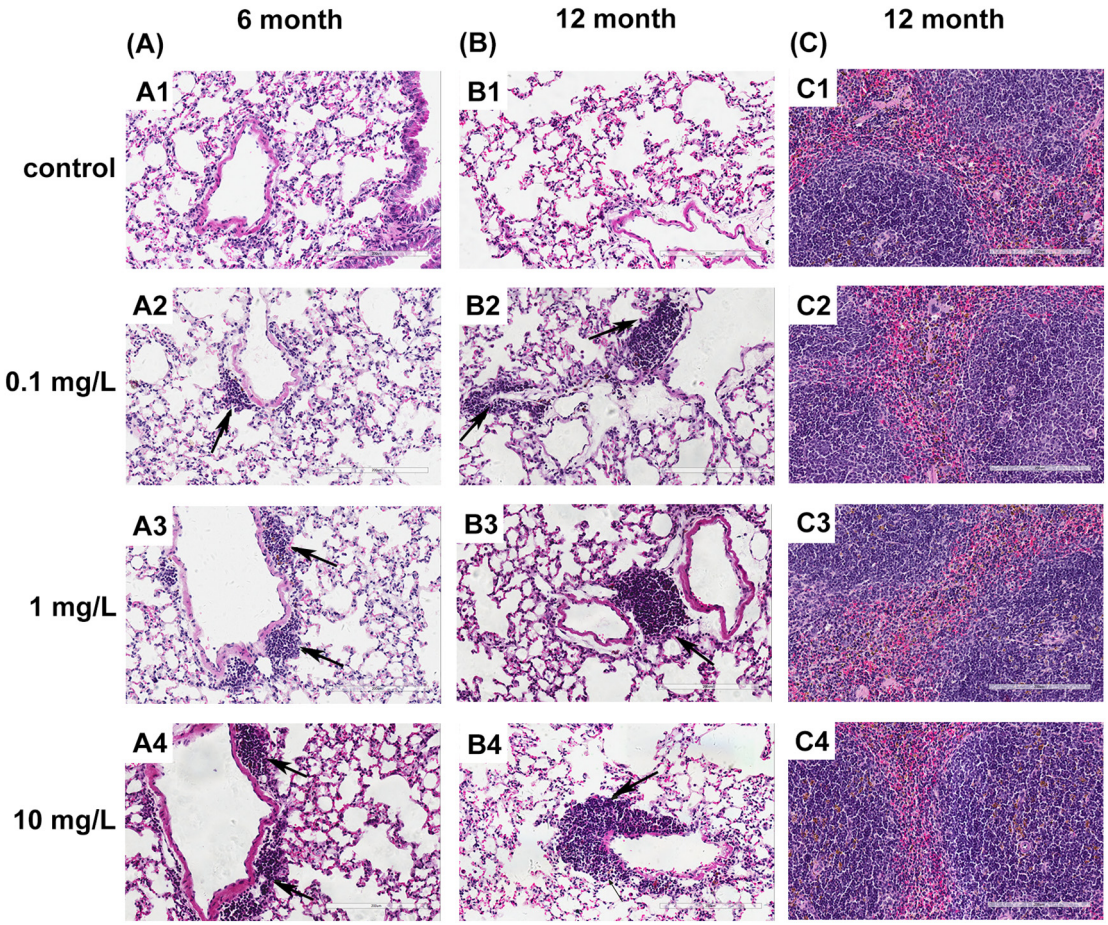

Fig. 5. Pathological changes in lung and spleen by chronic arsenic exposure. C57BL/6 mice drank water with indicated doses of arsenic freely for 6 or 12 months. Lung (A and B) and spleen (C) tissues were paraffin-embedded and stained with H&E to observe the histopathological changes. Morphological characteristics of sections were shown in the lung of mice at 6 months (A1–A4), 12 months (B1–B4), as well as in the spleen at 12 months (C1–C4). Arrows indicated inflammatory cells accumulation around pulmonary venules. Scale bar = 200 μm.

4. Discussion

Recent studies have suggested that arsenic potentially impairs vital immune responses thus leading to the increased risk of infections and chronic diseases [39]. The present study mainly focused on the possible immunological homeostasis in both immune and non-immune organs upon chronic low doses of arsenic exposure. Our results firstly confirmed the inflammatory alterations by arsenic orally. What's more, we explored the impairment of T lymphocyte subpopulations and Th1/Th2/Th17 balance in mice lung and spleen. In the meantime, arsenic *via* drinking water was also testified to affect the quantities and the antigen presenting functions of DCs *in vivo*.

Some studies have shown that arsenic could restrain the weights and indexes of immune organs in mice [40], rats [41] and chicken [42]. Ahmed et al. [43] has indicated a nonlinear association in a Bangladeshi cohort between prenatal arsenic exposure and the reduced thymic size in infancy, probably *via* oxidative stress and apoptosis. In the present study, the weights and indexes of lung and spleen decreased significantly after arsenic treatment for 12 months, confirming that chronic arsenic exposure might cause damages to both the immune and non-immune organs.

Evidences have attributed various arsenic-related chronic diseases to the altered body inflammatory responses [44]. An epidemiological study in Bangladesh has shown the arsenic-related secretion of IL-1β, TNF-α and IFN-γ in cord blood [3], and consecutively exposure orally for 180 days enhanced TNF-α, IL-1β and IL-6 in mice serum [28]. In this study, we observed the evident accumulation of macrophages and lymphocytes in BALF particularly in 0.1 mg/L and 1 mg/L groups, which is corresponding with a mice model of influenza A (H1N1) infection containing 100 μg/L of arsenic in drinking water [22]. Inflammatory changes were further confirmed by histopathological examinations after exposure for 6 months, which was more evident and convincing at 12 months. As to the pro-inflammatory cytokines, we detected the notable secretion of serum IL-1β in 10 mg/L arsenic-treated group at 6 and 12 months. However, we did not find any significant changes on TNF-α, although the IL-1β levels were pronounced under our observation, and the inconsistent increase or decline trends have been suggested in several other studies [22]. Collectively, arsenic-exposed mice in the present study displayed excessive inflammatory responses, mainly by infiltration and aggregation of inflammatory cells in BALF and lung tissues.

Unbalanced T lymphocyte subpopulations by arsenic have been paid more attention regarding to the immunotoxicity of this metalloid. The present flow cytometry results indicated a certain enhancement of $CD8^+$ T cells and decreased CD4/CD8 ratio in mice lung of 1 mg/L arsenic, consistent with an *in vivo* study showing slightly increased $CD8^+$ in human peripheral blood mononuclear cells by 5 μM sodium arsenite in drinking water [45]. On contrast, we also detected that the number and proportion of $CD4^+$ T cells were elevated moderately in spleen tissues, and CD4/CD8 ratio increased to some extent in all arsenic-treated groups at 12 months. On the other hand, $CD4^+$ T cells are generally subdivided into different subsets characterizing by specific master transcriptional factors and signature cytokines, which are still instrumental in clarification of various immunological responses. As to

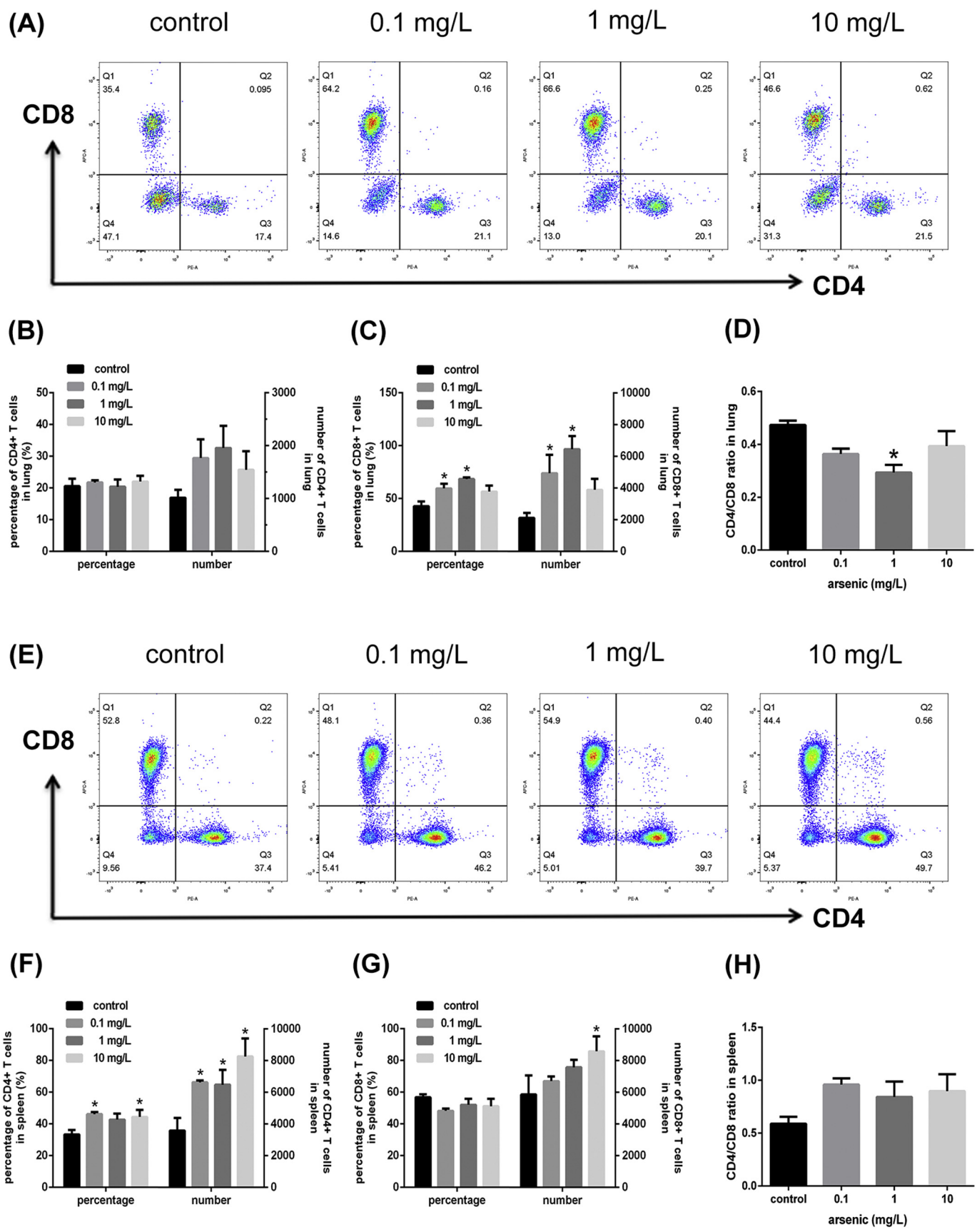

Fig. 6. Alterations of T lymphocyte subpopulation in lung and spleen by chronic arsenic exposure. C57BL/6 mice drank water with indicated doses of arsenic freely for 12 months. Single cell suspensions of lung (A) and spleen (E) were prepared as described in materials and methods, stained with anti-CD3, anti-CD4 or anti-CD8 antibodies, and then measured by flow cytometry, respectively. Quantitative analysis of $CD3^+$ $CD4^+$ T lymphocytes in lung (B) and spleen (F), $CD3^+$ $CD8^+$ T lymphocytes in lung (C) and spleen (G) were shown, and CD4/CD8 ratio in lung (D) and spleen (H) were calculated. Data were presented as mean ± SEM ($n = 3$). * denoted $p < 0.05$ compared with control group.

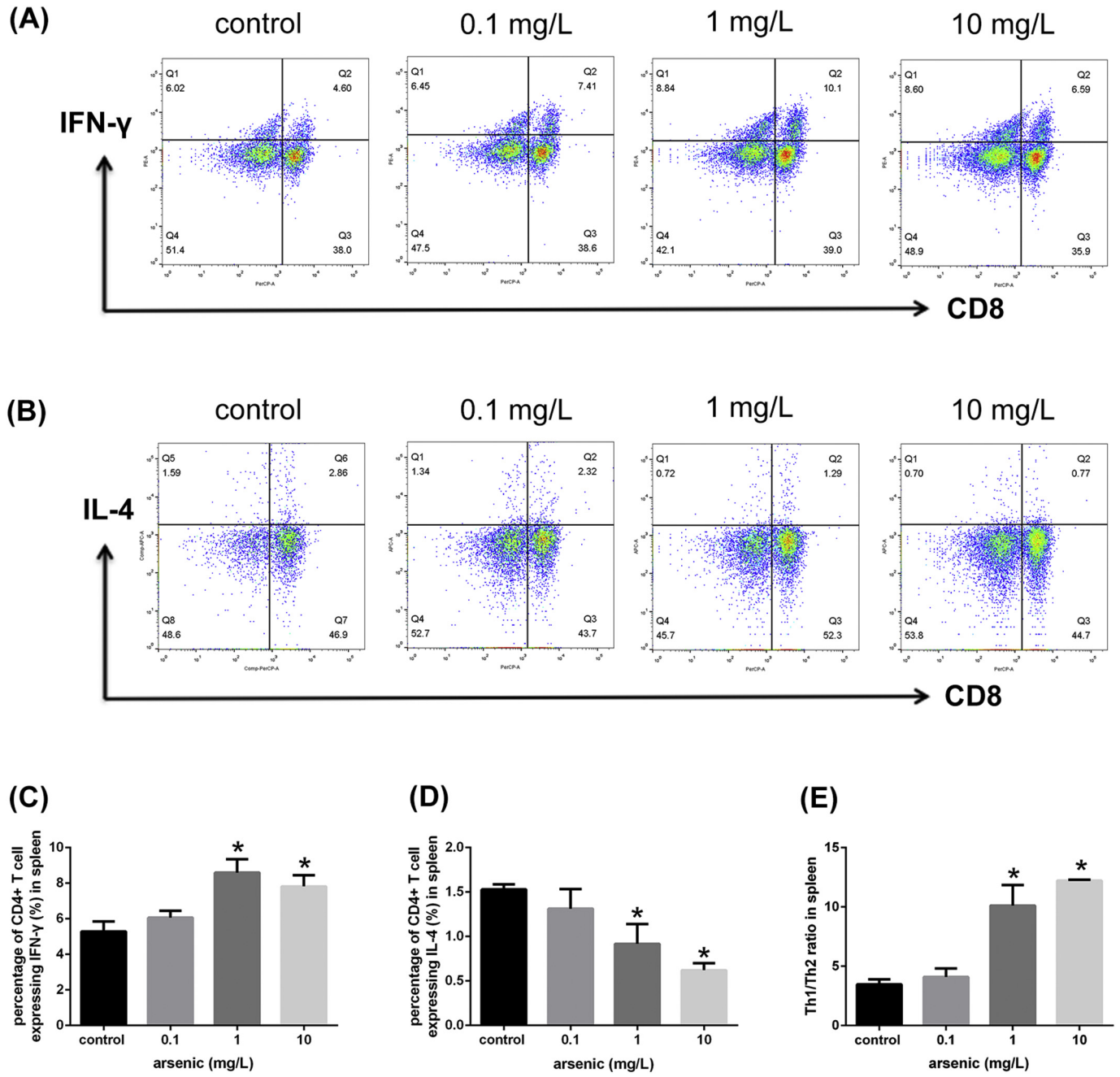

Fig. 7. Percentages of IFN-γ^+ T cells and IL-4^+ T cells in splenocytes by chronic arsenic exposure. C57BL/6 mice drank water with indicated doses of arsenic freely for 12 months. Spleen single cell suspensions stained with FITC-conjugated anti-CD3, PerCP-conjugated anti-CD8, PE-conjugated anti-IFN-γ or APC-conjugated anti-IL-4 were measured by flow cytometry. The left quadrants represent $CD4^+$ T cells ($CD3^+$ $CD8^-$) and the right quadrants represent $CD3^+$ $CD8^+$ T cells (A, B). Percentages of $CD3^+$ $CD4^+$ IFN-γ^+ Th1 cells (C) and $CD3^+$ $CD4^+$ IL-4^+ Th2 cells (D) were quantitative analyzed and Th1/Th2 ratio was calculated (E). Data were presented as mean ± SEM (n = 3). * denoted $p < 0.05$ compared with control group.

the different types of Th cells, we indicated that continuous arsenic exposure strongly enhanced the Th1-associated cytokine IFN-γ while reduced Th2-associated IL-4 in the splenocytes of C57BL/6 mice. What's more, the transcription factor *T-bet* for Th1 were up-regulated in both lung and spleen. As to the expression of cytokines, both *Il-4* and *Il-10* levels of Th2 were strikingly inhibited by 1 and 10 mg/L arsenic while *Ifn*-γ of Th1 increased, which were consistent with our results showing the higher percentages of IFN-γ^+ T cells and lower percentages of IL-4 by flow cytometry.

Furthermore, the discovery of Th17 cells introduces complexity into the existing Th1/Th2 balance paradigm and expands our understanding of T cell differentiation [46]. Our results further found the dramatically down-regulated mRNA levels of Th17 cytokines *Il-23* and *Il-22* in lung homogenates, in agreement with the decreased positive Th17 (ROR-γt) population by 1 to 10 nM arsenic treatment *in vitro* [47]. However, both the transcription factor *Ror*-γ*t* and cytokines *Il-23* and *Il-22* were increased to some extent in spleen tissues. Overall, as to the alterations of T cell differentiation and relevant cytokines by arsenic, there has been no consensus among the literatures yet [48,49]. These inconsistencies may be due to the differences among arsenic doses and duration, exposure patterns, disparate organs and tissues microenvironment involved, as well as species variation between human and laboratory animals [50,51]. Nevertheless, the present study, along with the other researches, suggested conformably that chronic low doses of arsenic

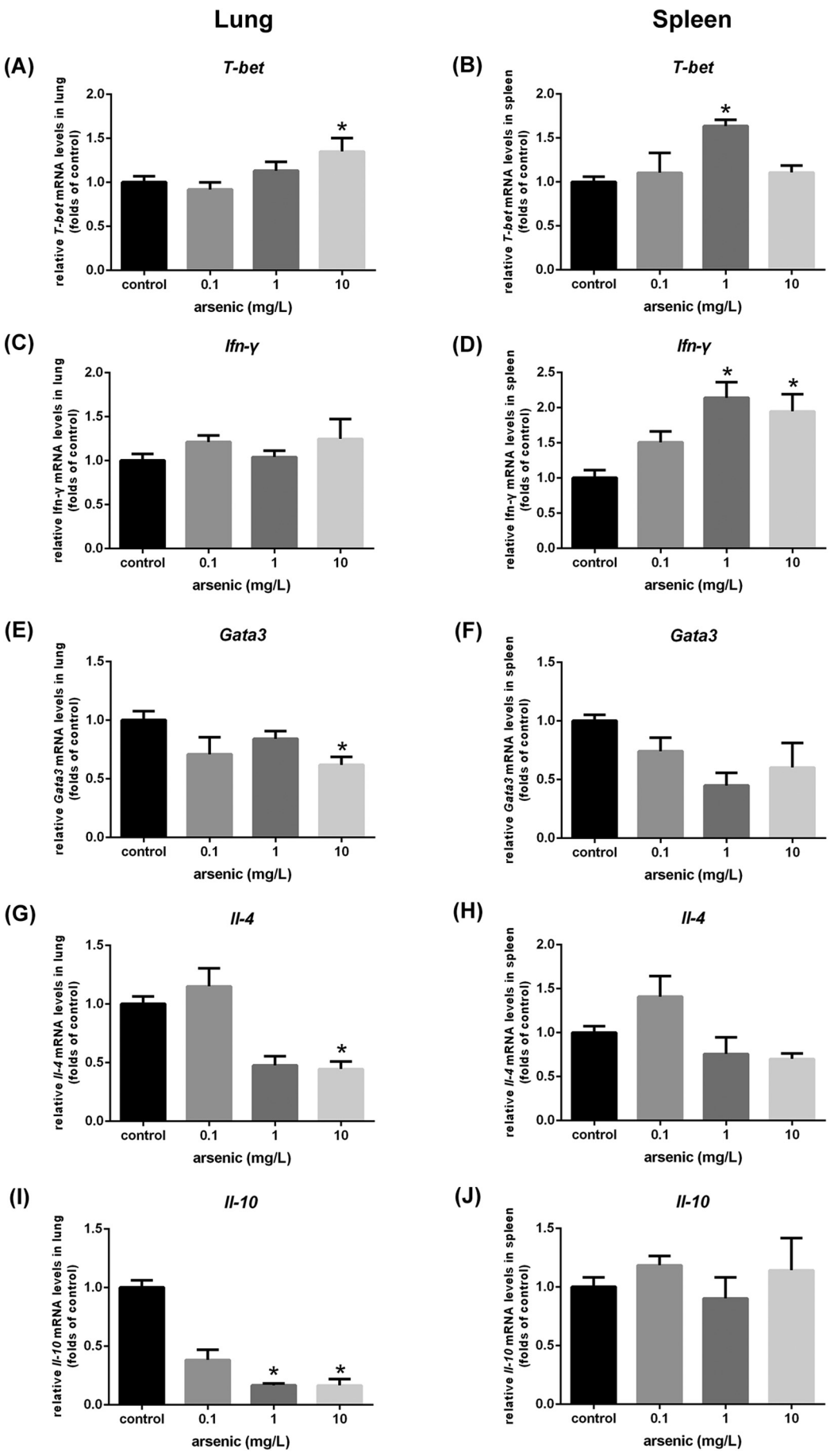

Fig. 8. Alterations of Th1 and Th2 specific transcription factors and cytokines in mice lung and spleen by chronic arsenic exposure. C57BL/6 mice drank water with indicated doses of arsenic freely for 12 months. Total RNA of lung and spleen tissues was isolated and real-time PCR was conducted. The mRNA levels of transcription factors *T-bet* in lung (A) and spleen (B), *Gata3* in lung (E) and spleen (F), as well as the corresponding cytokines *Ifn*-γ in lung (C) and spleen (D), *Il-4* in lung (G) and spleen (H), and *Il-10* in lung (I) and spleen (J) were shown, respectively. Data were presented as mean ± SEM ($n = 4$). * denoted $p < 0.05$ compared with control group.

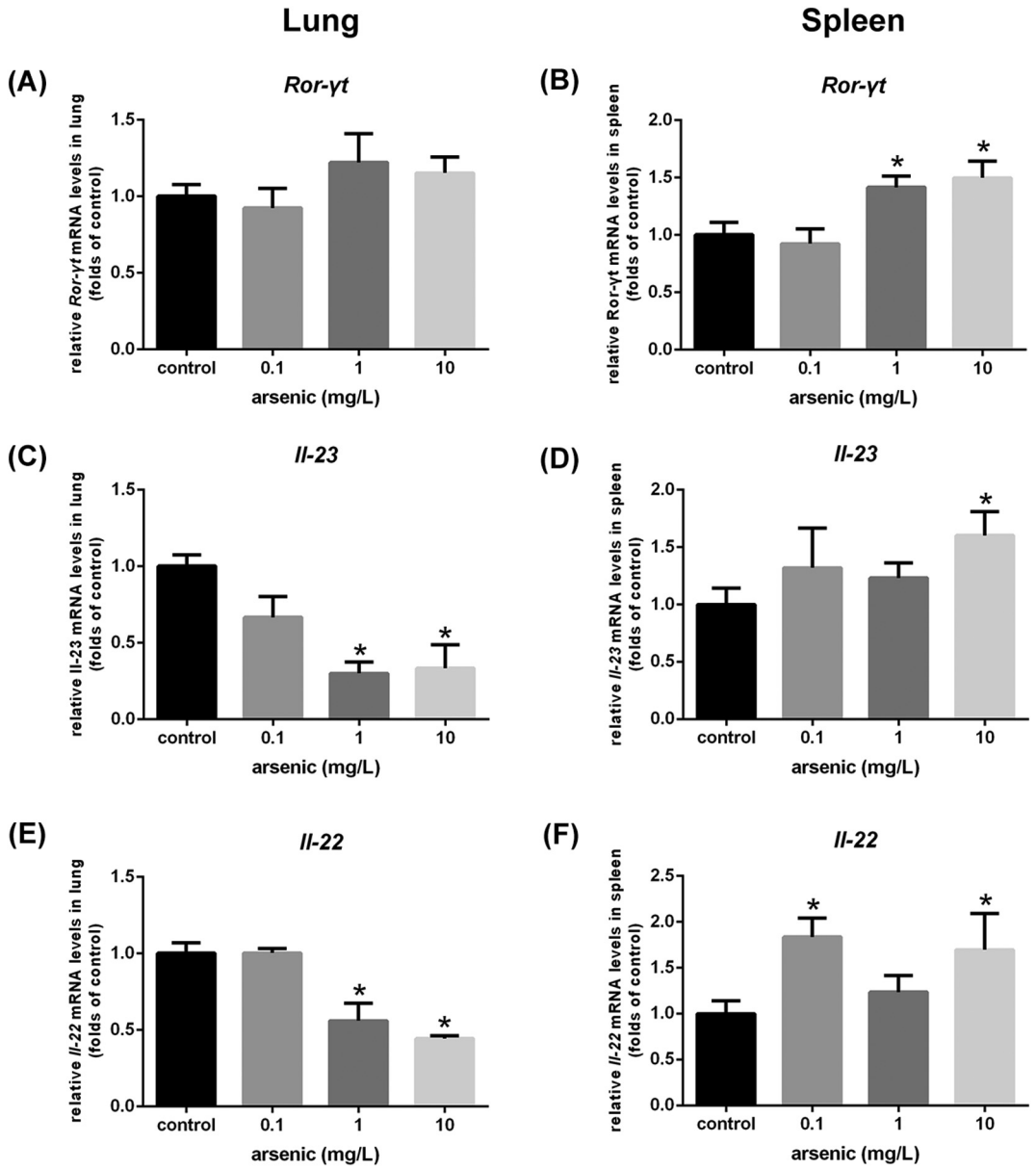

Fig. 9. Alterations of Th17 specific transcription factor and cytokines in mice lung and spleen by chronic arsenic exposure. C57BL/6 mice drank water with indicated doses of arsenic freely for 12 months. Total RNA of lung and spleen tissues was isolated and real-time PCR was conducted. The mRNA levels of transcription factor *Ror-γt* in lung (A) and spleen (B), and the corresponding cytokines *Il-23* in lung (C) and spleen (D), and *Il-22* in lung (E) and spleen (F) were shown, respectively. Data were presented as mean ± SEM (n = 4). * denoted $p < 0.05$ compared with control group.

could be involved in the immune dysfunctions with the imbalanced differentiation of Th1/Th2/Th17 subpopulations.

It is further acceptable that the differentiation of phenotypically distinct Th cells upon antigenic stimulation is predominantly regulated by DCs, the most powerful APCs in our body, and the activation and maturation of DCs is concomitant with the up-regulation of peptide-presenting MHC, co-stimulatory molecules, as well as relevant cytokines [52]. A microarray-based genome study of USA showed that arsenic averaged 0.7–32 μg/L in drinking water was associated with decreased MHC II expression in human peripheral blood mononuclear cells [53]. However, the functionally changes of DCs by chronic arsenic administration are mainly based on *in vitro* experiments and the results are still inconsistent [23,54,55]. In the present study, our data demonstrated the evident elevation of both the numbers and the proportions of $CD11c^+$ DCs. Meanwhile, we also confirmed the over-expression of phenotypic molecules (MHC II, CD86 and CD80), chemokine receptors (CCR7 and CCR5) and the specific cytokine (IL-12) of DCs in both lung and spleen, and these results were also consistent with the aforementioned results of Th1-shift in mice. As T cells and DCs are coordinated to the microenvironmental immune responses, and the expression of co-stimulatory molecules and cytokines by DCs and surrounding cells orchestrates the balance and differentiation of Th cells. We therefore hypothesized that arsenic might play special roles in the interactions between DCs and T cells, thus resulting in the subsequent immune dysfunctions [56,57].

In our study, we also discovered that low levels of arsenic remarkably increased the phosphorylation of ERK1/2, JNK and P38, as well as enhanced the expression of NF-κB in both lung and spleen. Consistently, a new dimension is recently added that low doses of arsenic could govern miR-2909 RNomics and induce sustained expression of Bmi-1 gene for sustained NF-κB activation and predominant Th1 responses [45]. We suspected that under long-term low doses of arsenic exposure, yet not evoking severe tissue damages, arsenic orally may interfere with some imperative physical immune functions of both

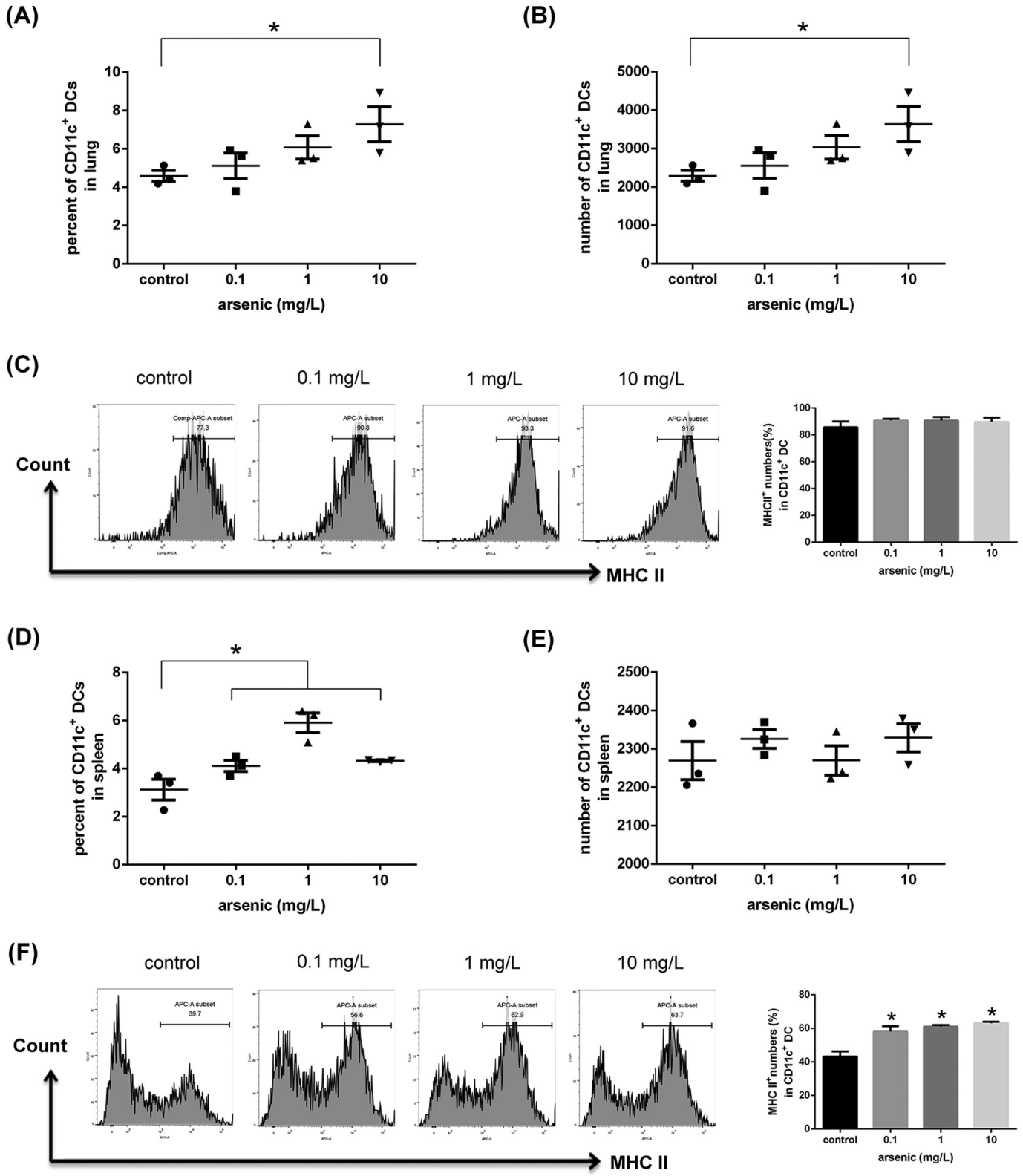

Fig. 10. Effects of chronic arsenic exposure on cell counts and MHC II expressions of DCs in lung and spleen single cell suspensions. C57BL/6 mice drank water with indicated doses of arsenic freely for 12 months. Single cell suspensions of lung and spleen were prepared, stained with FITC-conjugated anti-CD11c or APC-conjugated anti-MHC II, and measured by flow cytometry. Percentages and numbers of $CD11c^+$ DCs in lung (A, B) and spleen (D, E), as well as expressions of MHC II on DCs membrane in lung (C) and spleen (F) were quantitative analyzed. Data were presented as mean ± SEM ($n = 3$). * denoted $p < 0.05$ compared with control group.

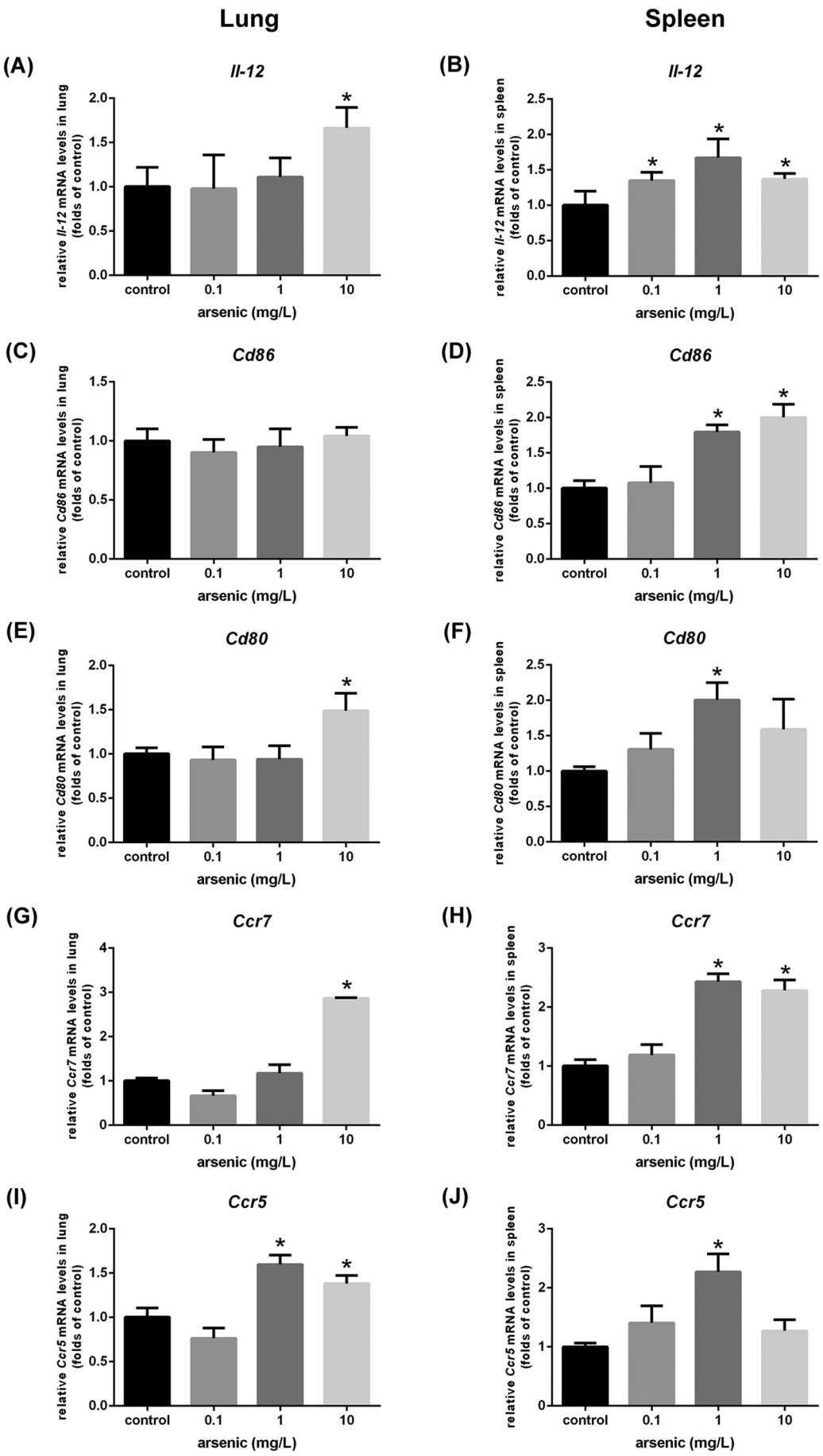

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Fig. 11. Alterations of cytokine, costimulatory molecules and chemokine receptors in mice lung and spleen by chronic arsenic exposure. C57BL/6 mice drank water with indicated doses of arsenic freely for 12 months. Total RNA of lung and spleen tissues was isolated and real-time PCR was conducted. The mRNA levels of *Il-12* in lung (A) and spleen (B), costimulatory molecules (*Cd86* and *Cd80*) in lung (C, E) and spleen (D, F), as well as chemokine receptors (*Ccr7* and *Ccr5*) in lung (G, I) and spleen (H, J) were shown, respectively. Data were presented as mean ± SEM ($n = 4$). * denoted $p < 0.05$ compared with control group.

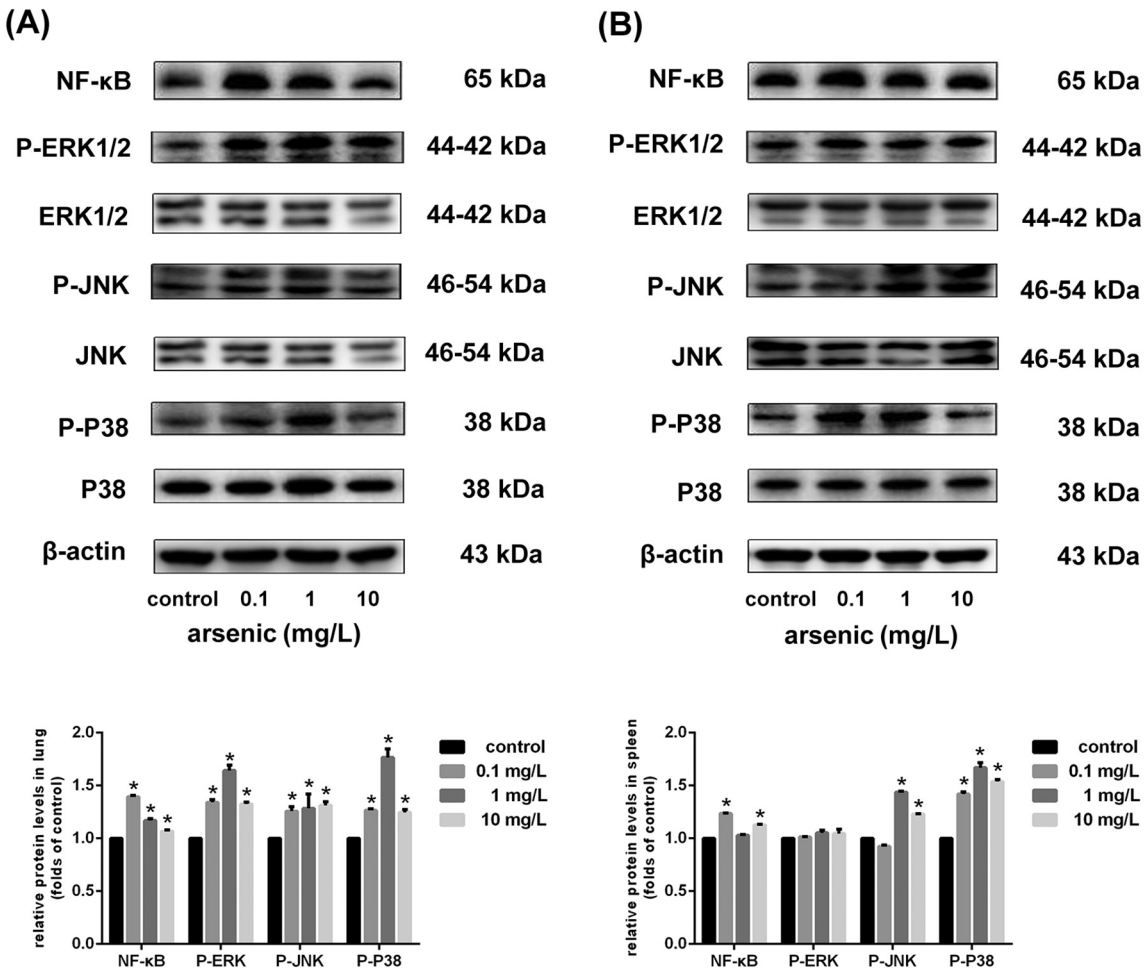

Fig. 12. Activations of NF-κB and MAPKs pathway in lung and spleen by chronic arsenic exposure. C57BL/6 mice drank water with indicated doses of arsenic freely for 12 months, and the extracted proteins were subjected to SDS-PAGE. Expressions of NF-κB, P-ERK1/2/ERK1/2, P-JNK/JNK and P-P38/P38 in lung (A) and spleen (B) were assessed by western blotting, β-actin was blotted as the loading control. Data were presented as mean ± SEM ($n = 3$). * denoted $p < 0.05$ compared with control group.

immune and non-immune organs of arsenic-exposed populations [58]. However, further studies using inhibitors and other innovative technologies are imperative for clarifying the causal connections.

5. Conclusions

The present study provides evidences that chronic low levels of arsenic exposure in drinking water could interfere with multiple immune responses in both immune and non-immune organs *in vivo*, especially the imbalance of Th1/Th2/Th17 subpopulation and enhancement of DCs functions, which might lead to various arsenic-related chronic diseases in general, as well as the increased risk of infections and cancers in particularly.

Compliance with ethical standards

The manuscript does not contain clinical studies or patient data.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This study was supported by grants from National Natural Science Foundation of China (Grant Number: 81673114) and Key Laboratory Basic Research Funds from Liaoning Provincial Education Department (Grant Number: LS201607).

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