



## Arsenic retention in erythrocytes and excessive erythrophagocytosis is related to low selenium status by impaired redox homeostasis

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### ABSTRACT

Arsenic (As) contamination in drinking water is a global public health problem. Epidemiological studies have shown that selenium (Se) deficiency is associated with an increasing risk of arsenism. However, the association between Se status and As retention in erythrocytes and mechanisms underlying this association have not been fully investigated. In the present study, a total of 165 eligible subjects were recruited and As was found to accumulate in blood mainly by retention in erythrocytes. Retention of As in erythrocytes was negatively correlated with Se status, antioxidant parameters related to Se and As methylation capacity, but positively correlated with the protein-binding capacity of As. Additionally, erythrocytes isolated from subjects with low Se status exhibited cellular damage along with lower protein levels of CD47, which could be aggravated by hydrogen peroxide treatment. Consistent with the human study, the erythrocytes from mice with sub-chronic As exposure exhibited similar cellular damage and shown to be phagocytosed by splenic macrophages, and these effects were mitigated by dietary Se supplementation. Furthermore, hydrogen peroxide treatment induced excessive phagocytosis of erythrocytes with As exposure by splenic macrophages, while co-treating erythrocytes with the reducing agent, N-Acetyl-L-cysteine, mitigated this excessive erythrophagocytosis. Hyperactivation of the NFκB pathway was also detected in splenic macrophages after excessive erythrophagocytosis. In conclusion, this study found that low Se status involving impaired redox homeostasis increased As retention in erythrocytes, which were subsequently phagocytosed by splenic macrophages and led to an increased inflammatory status of splenic macrophages. These findings provide insight into physiological features of arsenism related to Se status and redox homeostasis.

### 1. Introduction

Arsenic (As), a prevalent contaminant in the environment, enters the body mainly through drinking water [1]. It is estimated that more than 200 million people worldwide may live in the areas with As-contaminated groundwater at levels above the World Health Organization (WHO) acceptable levels (10 µg/L), which has emerged as a global public health problem [2]. In China, an estimated 19.6 million people are at risk of chronic exposure to As through contaminated drinking water [3]. Chronic As exposure is associated with several

health disorders [4], and epidemiological studies have linked chronic As exposure to anemia [5] and cardiovascular disease [6].

As an important part of circulatory system, erythrocytes rapidly absorb As after exposure [7] as supported by data showing that the concentration of As in erythrocytes is 4 times higher than that in plasma [8,9]. One mechanistic study has revealed that arsenites exhibit high binding affinity to the reduced cysteine residues in peptides and proteins [10]. Hemoglobin (Hb) is the most abundant erythrocytic protein and has been established as an As-binding target, contributing to the retention of As in erythrocytes [11]. Although As accumulation in

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erythrocytes plays an important role in the distribution, redistribution and elimination of circulating As [12], it promotes the death of erythrocytes and eventually can lead to anemia [13]. Damaged erythrocytes may be phagocytosed by the reticuloendothelial macrophages in the spleen and liver [14], and this process is regulated by cluster of differentiation 47 (CD47), a universally expressed cell surface molecule of the immunoglobulin superfamily involved in self-recognition by phagocytes [15]. Erythrophagocytosis can induce both the inflammatory and metabolic reprogramming of macrophages [16–18]. Thus, understanding the mechanism of As retention in erythrocytes would provide important insight into As metabolism and toxicity related to human health.

Selenium (Se) is an essential micronutrient of fundamental importance to human health and exerts its biological functions mainly through its incorporation into selenoproteins as selenocysteine [19,20]. Selenoproteins participate in antioxidant defense, thyroid hormone metabolism, immune responses, development and reproduction [21]. Se deficiency can reduce selenoprotein levels and increase the risk of vascular diseases, male infertility, neurological disorders, and multiple types of cancers [22]. The selenoprotein family includes glutathione peroxidase 1 (GPX1) that is a major antioxidant enzyme in erythrocytes serving to prevent oxidative damage induced by As stress or other oxidant stimuli [23]. In addition, Se has been shown to participate in the export of As during the physiological elimination of this toxin [24,25]. Se deficiency status is associated with increased risk of arsenism [26, 27], which can be mitigated by Se supplementation [28,29]. However, the role of Se in As retention in erythrocytes is unclear.

In the present study, a total of 165 eligible subjects who were chronically exposed to As and resided in a Se-deficient area were enrolled to investigate the associations between As retention in erythrocytes and Se status, as well as the involvement of redox homeostasis. The effects of As exposure on erythrophagocytosis and the related changes of erythrocytes were evaluated using a mouse model involving Se supplementation, including the analysis of splenic macrophage activation after erythrophagocytosis.

## 2. Methods and materials

### 2.1. Study populations and sample collection

This study was approved by the institutional ethics committee of Jinan University (JNUKY-2017-029). All subjects in this study were enrolled randomly from three contiguous villages in south-west edge of the Datong basin (Shanxi province, China), which is located in the area of both chronic endemic arsenicosis and Se deficiency. Of the 243 enrolled subjects, 165 eligible subjects, who were exposed to As through drinking water from the tube wells without history of other chronic disease, donated blood samples after signing an informed consent form and completing the baseline interview for their information regarding occupation, skin lesions, gender, age, exposure time, exposure level, tobacco smoking and alcohol drinking. The whole blood samples were collected from participants in Vacutainer® Tubes with EDTA (BD, Franklin Lakes, NJ, USA). The erythrocytes and plasma were separated by centrifugation (3000 rpm, 10 min). The packed erythrocytes were washed three times with isotonic phosphate-buffered saline (PBS) and resuspended in Hank's balanced salt solution (Biosharp, Chongqing, China) with CPDA-1 solution (10%; Leagene, Beijing, China) to generate 10% (v/v) of erythrocyte suspensions for further study.

### 2.2. Mice and expose protocol

Animal experiments were approved by the Animal Care and Ethics Committee of Jinan University (20180309001). Six-week-old male C57BL/6J mice were purchased from the Experimental Animal Center of Southern Medical University (Guangzhou, China). All mice were housed five mice per cage in a controlled environment with a 12 h light/dark

cycle in the Institute of Laboratory Animal Science (Jinan University). Mice were randomly assigned to 4 groups (n = 10 per group): (1) the control group were received normal diet and tap water; (2) the As group were received normal diet and tap water supplemented with 50 ppb As supplied by arsenic trioxide (ATO, As<sub>2</sub>O<sub>3</sub>; Sigma-Aldrich, St. Louis, MO, USA); (3) the Se group were received a diet supplemented with Se (0.2 mg Se/kg of diet) by sodium selenite (Na<sub>2</sub>SeO<sub>3</sub>; Sigma-Aldrich, St. Louis, MO, USA) and tap water; (4) the Se+As group were received a diet supplemented with Se (0.2 mg Se/kg of diet) and tap water supplemented with As (50 ppb), and all mice were fed ad libitum for 8 weeks.

### 2.3. Total arsenic and selenium detection

The samples of whole blood, plasma, and erythrocytes (n = 3 per group) were digested in 1.5 mL concentrated nitric acid (HNO<sub>3</sub>; 67%; Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 30 min and heated at 100 °C for 4 h with 1 mL concentrated hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; 30%; Sigma-Aldrich, St. Louis, MO, USA) as described in previous studies [30]. After diluting to a total volume of 10 mL with deionized water and passing through the syringe filters (0.22 μm; Pall Corporation, NY, USA), samples were subjected to inductively coupled plasma mass spectrometry with collision/reaction cell technology (ICP-CCT-MS; iCAP™ RQ; Thermo Fisher Scientific, Waltham, MA, USA) for total As and total Se detection. Standard solutions of As and Se were obtained from National Certified Reference Materials (Beijing, China) and used for standard curve graphing, and 2% (v/v) nitric acid was used as solution control.

To detect the protein-binding of As in erythrocytes (PB-As, PB-eAs), erythrocytes were hemolyzed with 0.5 mL of hypotonic Tris-NO<sub>3</sub> (10 mM) on ice, and the concentrations of Hb in hemolysates were measured by Hemoglobin Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) for adjustment. Proteins in homogenates were precipitated by precooled acetone, and collected by centrifugation (14,000 rpm, 20 min). The protein pellets were then dissolved in Tris-NO<sub>3</sub> (50 mM) for detection.

### 2.4. Arsenic speciation analysis

To analyze As species, erythrocytes were isolated and resuspended with 0.5 mL PBS and homogenized by ultrasonication (VCX 130; Sonics & Materials, Inc., Newtown, CT, USA). The supernatants of samples were collected by centrifugation (14,000 rpm, 20 min, 4 °C) in an Amicon® Ultra-0.5 Centrifugal Filter Unit (3 kDa; Merck, Kenilworth, NJ, USA), and then subjected to an Ultimate 3000 series high-pressure liquid chromatography (HPLC) system (Thermo Fisher Scientific, Waltham, MA, USA) with a CAPCELL PAK C18 column (5 μm, 4.6 × 250 mm; Shiseido, Chuo City, Tokyo, Japan) coupled to ICP-MS as described in our previous study [31]. Standard solutions of As species, including sodium arsenite, sodium arsenate, monomethylarsenic disodium, and dimethylarsenic acid, were obtained from National Certified Reference Materials (Beijing, China). To further confirm the existence of PB-eAs, the filtered samples were subjected to HPLC-ICP-MS equipped with a UV detector (SPD-20A; Shimadzu, Kyoto, Kyoto, Japan) for protein detection.

### 2.5. Detections of erythrocytic biochemistry

Erythrocytes were lysed in RIPA buffer (Yeasen, Shanghai, China) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The supernatants of lysates were collected by centrifugation (12,000 rpm, 4 °C, 15 min). Activities of antioxidant enzymes, including GPX, SOD, CAT, and GR, as well as the contents of GSH and MDA were detected by specific assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and normalized to Hb concentrations.

## 2.6. Detection of phosphatidylserine exposure

Erythrocytes (isolated from 0.1 mL of blood) were washed and resuspended in the Annexin V binding buffer, and incubated with recombinant Annexin V-FITC (4A Biotech Co., Ltd., Beijing, China) for 30 min following the manufacturer's instructions. A total of  $5 \times 10^4$  cells were analyzed by flow cytometer (NovoCyte®; Agilent Technologies, Santa Clara, CA, USA) using the FL1 channel (488/530 nm). Unstained cells were used as negative controls, and each group was analyzed in three biological replicates.

## 2.7. In vitro study

Erythrocytes from subjects with low Se status (L-Se,  $n = 10$ ) or moderate Se status (M-Se,  $n = 10$ ) or from mice were collected for the *in vitro* study. After pretreating with  $H_2O_2$  (100  $\mu$ M, 2 h) or N-Acetyl-L-cysteine (NAC; 10 mM, 2 h; Sigma-Aldrich, St. Louis, MO, USA), erythrocytes were then incubated with As (ATO; 50 ppb, 24 h) and used for further detection.

## 2.8. In vivo phagocytosis assay

Erythrocytes from mice ( $n = 3$  per group) were collected via tail vein bleeding and labeled with CFDA-SE (CFSE; 5  $\mu$ M; Yeasen, Shanghai, China) at 37 °C for 60 min. Mice were then injected with the autologous erythrocytes via tail vein. After housing for 2 h, mice were euthanized followed by collection of blood, spleens, hearts, livers and kidneys. Erythrocytes and tissues were lysed and the fluorescence intensity of CFSE in lysates was measured by using a microplate reader (Synergy™ H1M; BioTek, Winooski, VT, USA). For cryosectioning, tissues were embedded in OCT reagent (Sakura Finetek USA, Inc., Torrance, CA, USA), stored at -80 °C, cut into 10  $\mu$ m sections by microtome (CM1950; Leica Biosystems, Wetzlar, Germany), and adhered to glass slides. After air-drying and fixation with 4% paraformaldehyde, sections were sealed with DAPI Fluoromount-G™ (Yeasen, Shanghai, China) and images were captured by EVOS® FL Auto Imaging System (Thermo Fisher Scientific, Waltham, MA, USA). For flow cytometry, spleens were digested with an enzyme cocktail including Collagenase D (1 mg/mL; Roche, Basel, Switzerland), DNase I (100  $\mu$ g/mL; Sigma-Aldrich, St. Louis, MO, USA) and Dispase (0.6 U/mL; Roche, Basel, Switzerland) in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 2% fetal bovine serum (Yeasen, Shanghai, China) at 37 °C for 30 min [32]. After lysing the erythrocytes with Red Blood Cell Lysis Buffer (Yeasen, Shanghai, China), cell suspensions were passed through cell strainers (100  $\mu$ m; Corning, NY, USA) and collected by centrifugation (1500 rpm, 10 min). Cells were incubated with fixation buffer (Biolegend, San Diego, CA, USA) in the dark at room temperature for 30 min, and blocked with TruStain FcX™ (anti-mouse CD16/32) Antibody (101,319; Biolegend, San Diego, CA, USA) for 30 min on ice. Cells were then incubated with APC-conjugated anti-mouse F4/80 recombinant antibody (123,115; Biolegend, San Diego, CA, USA) for 30 min on ice. After passing through cell strainers (100  $\mu$ m), a total of  $5 \times 10^4$  cells were analyzed by flow cytometer (NovoCyte®; Agilent Technologies, Santa Clara, CA, USA) using the FL1 channel (488/530 nm) and the APC channel (640/667 nm), and the unstained cells were used as negative controls.

## 2.9. In vitro phagocytosis assay

To investigate the morphological changes, erythrocytes from mice ( $n = 3$  per group) were collected, labeled with CFSE, plated on confocal dishes (Jet Bio-Filtration Co., Ltd., Guangzhou, China), and captured by FV3000 confocal laser scanning microscope (Olympus, Shinjuku City, Tokyo, Japan). To determine erythrocyte phagocytosis *in vitro*, splenic macrophages of mice ( $n = 3$  per group) were isolated from enzyme-digested spleen tissues and plated on the confocal dishes. Adherent

macrophages were labeled with Hoechst 33258 (1  $\mu$ g/mL; Yeasen, Shanghai, China) and co-cultured with the CFSE-labeled erythrocytes at a ratio of 1:20 for 2 h. After lysis of the non-ingested erythrocytes, images of macrophages were captured by laser scanning microscope (Olympus, Shinjuku City, Tokyo, Japan). For flow cytometry, splenic macrophages were enriched by plating on the 6-well-plates and phagocytosed the CFSE labeled erythrocytes. Macrophages were harvested by Cellstripper™ (Corning, NY, USA) and a total of  $5 \times 10^4$  cells were analyzed by flow cytometer (NovoCyte®; Agilent Technologies, Santa Clara, CA, USA) using the FL1 channel (488/530 nm). Unstained cells were used as negative controls, and each group was analyzed in three biological replicates.

## 2.10. Western blotting

Erythrocytes were lysed in RIPA buffer containing a protease inhibitor cocktail and supernatants of lysates were collected by centrifugation (14,000 rpm, 4 °C, 15 min), with protein concentrations determined by BCA assay. After adjusting to equal concentrations with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA), the proteins were separated by SDS-PAGE and transferred to PVDF membranes (0.22  $\mu$ m; MilliporeSigma, Burlington, MA, USA). The membranes were blocked with 5% skim milk (BD, Franklin Lakes, NJ, USA) for 1 h at room temperature and then incubated with the indicated primary antibodies overnight at 4 °C. After washing three times with tris buffered saline with 0.1% Tween® 20 (TBST; Sigma-Aldrich, St. Louis, MO, USA), the membranes were incubated with the horseradish peroxidase conjugated secondary antibody (S0001; 1:3000; Affinity, Changzhou, China) for 1 h at room temperature. After washing three times with TBST, the bands of proteins were visualized by Tanon 5200 system (Shanghai, China) using electrochemiluminescence assay kits (Yeasen, Shanghai, China).

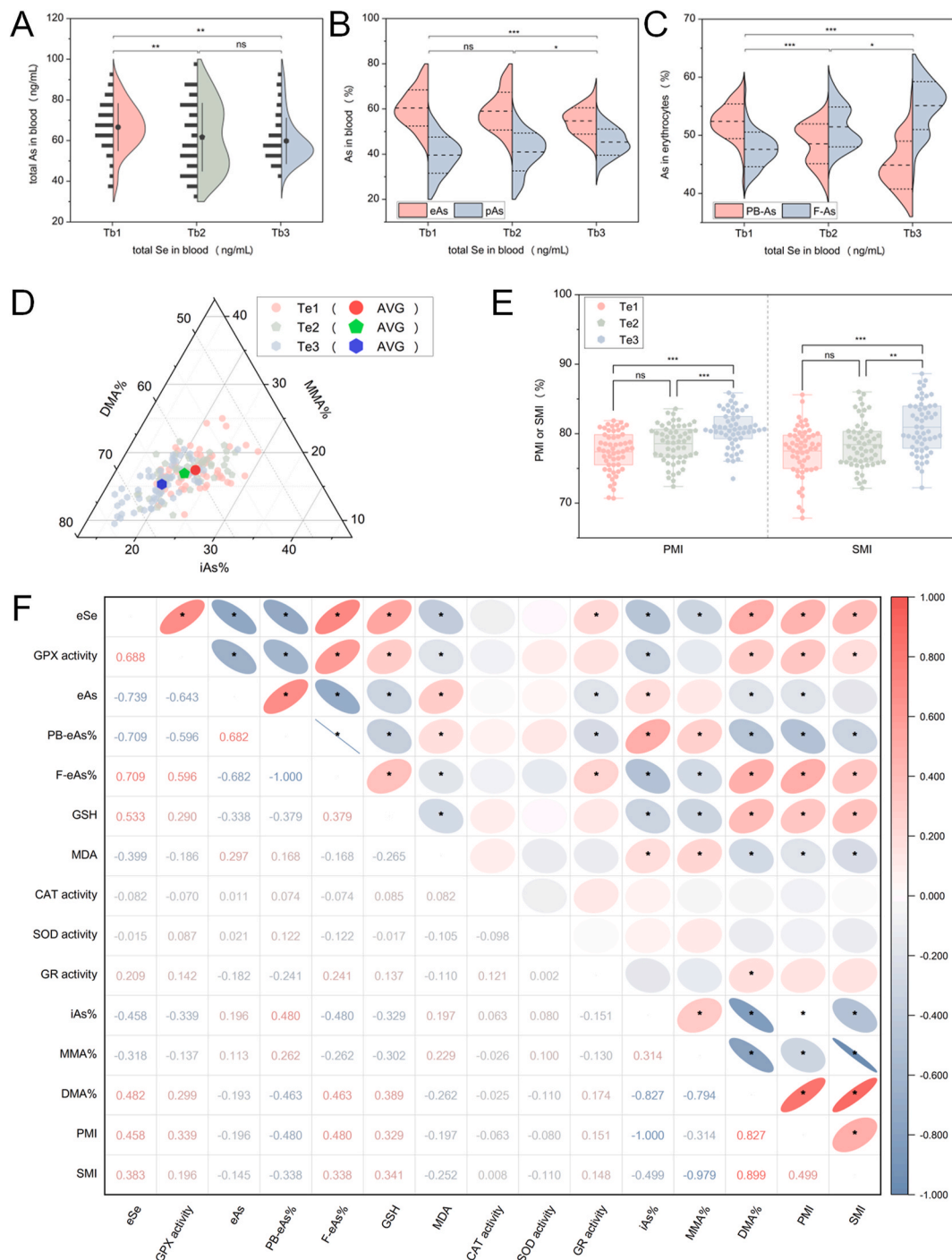
Primary antibodies, including anti-GPX1 (DF6249; 1:1000), anti-GPX4 (DF6701; 1:1000), anti-CD47 (DF6649; 1:1000), anti-IKK $\alpha/\beta$  (AF6014; 1:1000), anti-p-IKK $\alpha/\beta$  (AF3013; 1:1000), anti-NF $\kappa$ B (AF5006; 1:1000), anti-p-NF $\kappa$ B (AF2006; 1:1000), anti-I $\kappa$ B (AF2002; 1:1000), anti-p-I $\kappa$ B (AF5002; 1:1000), anti-iNOS (AF0199; 1:1000), anti-Cox-2 (AF7003; 1:1000), and anti-GAPDH (AF7021; 1:1000), were obtained from Affinity (Changzhou, China). Primary antibodies and secondary antibody were diluted in TBST with 5% bovine serum albumin (Yeasen, Shanghai, China).

## 2.11. RNA extraction and real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from macrophages by using the MolPure® Cell RNA Kit (Yeasen, Shanghai, China) and a total of 1  $\mu$ g isolated RNA for each sample were reversely transcribed to cDNA using Hifair® III 1st Strand cDNA Synthesis SuperMix (Yeasen, Shanghai, China). The qPCR was performed by utilizing 2X RealStar Green Fast Mixture (Genstar Technology Co., Ltd., Beijing, China) and the CFX96 Touch Real-Time PCR detection System (Bio-Rad Laboratories, Hercules, CA, USA) under the following parameters: 95 °C for 5 min, 40 cycles at 95 °C for 10 s, and 55 °C for 30 s. The mRNA levels of target genes were normalized to the levels of GAPDH and calculated by the  $2^{-\Delta\Delta CT}$  method and three biological replicates were analyzed for each group. The qPCR primers listed in Table S1 were synthesized from Sangon Biotech Co., Ltd. (Shanghai, China).

## 2.12. Immunohistochemistry

For immunohistochemical procedures, spleen sections ( $n = 3$  per group) were air-dried, fixed, heated with citrate buffer (10 mM, pH 6.0) for antigen retrieval, and blocked with 5% normal goat serum (Yeasen, Shanghai, China). Sections were then incubated with anti-p-NF $\kappa$ B (AF2006; 1:100; Affinity, Changzhou, China), anti-iNOS (AF0199; 1:100; Affinity, Changzhou, China), or anti-Cox-2 (AF7003; 1:100;

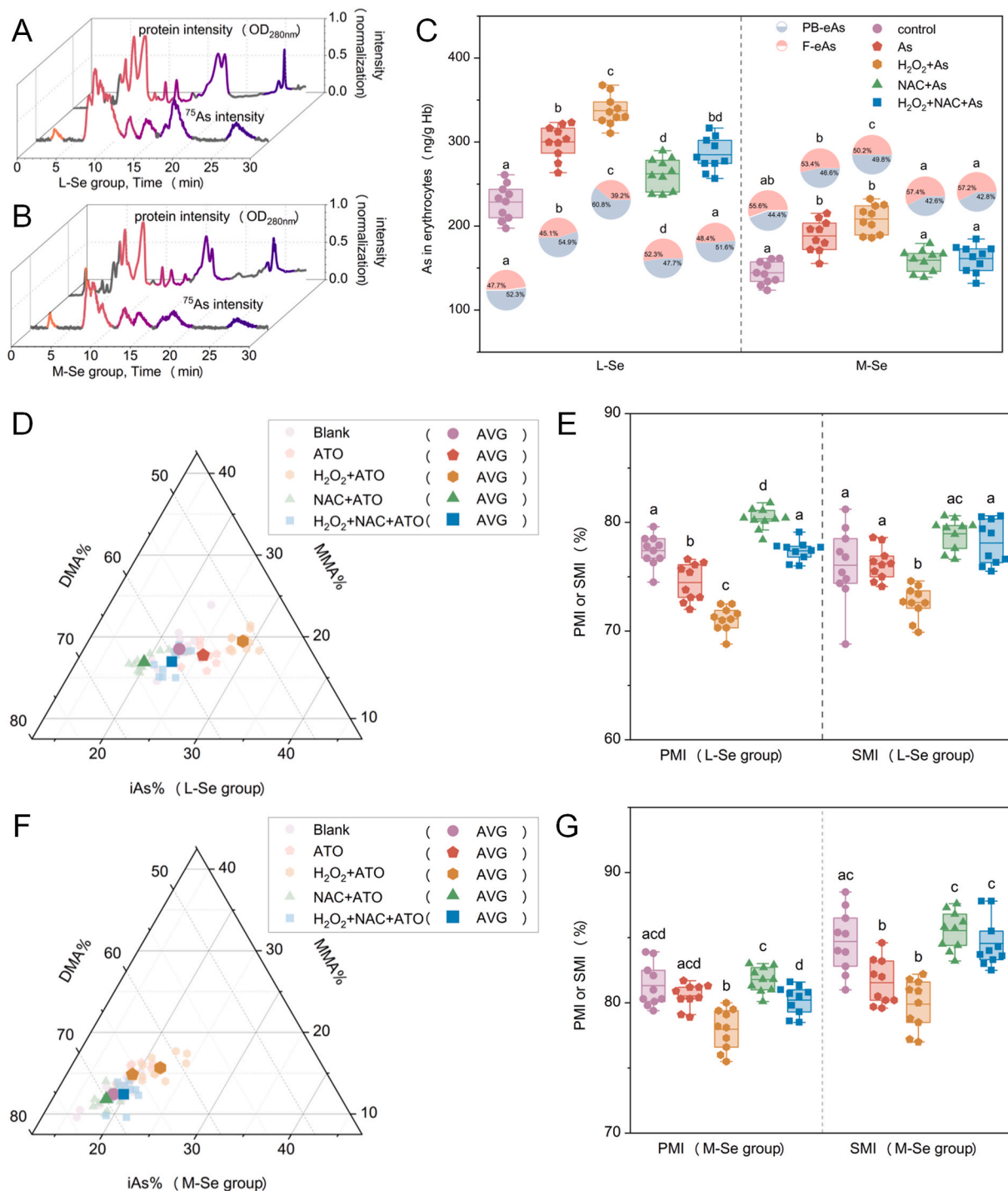


**Fig. 1.** As retention in erythrocytes of the As-exposed subjects with different Se status. The eligible subjects were divided into tertiles according to bSe (A-C; Tb1: bSe < 106.5 ng/mL, Tb2: bSe = 106.5–124.5 ng/mL, Tb3: bSe > 124.5 ng/mL) or eSe (D-F; Te1: eSe < 247.0 ng/g Hb, Te2: eSe = 247.0–302.9 ng/g Hb, Te3: eSe > 302.9 ng/g Hb). The dose-dependent associations among bSe and (A) bAs, (B) eAs% and pAs%, or (C) PB-eAs% and F-eAs% are shown. (D) The distribution of eAs species and the compositional means (AVG) are shown. (E) The PMI and SMI among tertiles are shown. (F) Spearman's correlation matrix with the Spearman's correlation (*r*) and significant difference (\**p* < 0.05) are shown. \*\*\**p* < 0.001, \*\**p* < 0.01, or \**p* < 0.05 indicates significant difference among tertiles as determined by the Kruskal-Wallis test.

Affinity, Changzhou, China) overnight at 4 °C followed by incubation with HRP conjugated secondary antibody (S0001; 1:200; Affinity, Changzhou, China) for 1 h at room temperature. Then, the target proteins in spleen sections were visualized using a DAB kit (ZSGB-Bio, Beijing, China) and the nuclei were counterstained with hematoxylin (Leagene, Beijing, China). After sealing, the slides were scanned by using a EasyScan Pro 6 (Motic, Xiamen, China).

### 2.13. Statistical analysis

Statistical analysis was performed using SPSS software (version 21.0; IBM, NY, USA) and Origin 2021b (OriginLab, Northampton, MA, USA) was used for graphing. Grouped results from at least three independent experiments were presented as mean ± standard deviation (SD). Unpaired Student's *t* tests were used to compare the means of two groups, while one-way analysis of variance (ANOVA) was used for comparison



**Fig. 2.** Effects of redox status on As retention in erythrocytes. The normalized protein intensity and the <sup>75</sup>As intensity in L-Se group (A) and M-Se group (B) are shown. (C–G) After pretreating with H<sub>2</sub>O<sub>2</sub> (100 μM) or NAC (10 mM), erythrocytes in L-Se group and M-Se group were treated with As *in vitro*. (C) Total As, PB-As% and F-As% in erythrocytes with different treatments were detected. Triplots describe the distribution of As species and the compositional means (AVG) in erythrocytes of (D) L-Se group and (F) M-Se group. PMI and SMI among erythrocytes with different treatments in (E) L-Se group and (G) M-Se group are shown. Different lower-case letters indicate significant difference between groups (*p* < 0.05) as determined by One-way ANOVA and Tukey post-test.

among three or more groups. When ANOVA was significant, post-hoc testing of differences between groups was performed using the Tukey's honestly significant difference (HSD) test. For population studies, the 165 eligible subjects were stratified into tertiles (n = 55 for each tertile) according to the Se concentrations in blood (bSe) or the Se concentrations in erythrocytes (eSe). Differences among tertiles were analyzed by using the Kruskal-Wallis test. A *p*-value of < 0.05 was considered statistically significant. Linear regression analysis and partial

correlation analysis adjusted by the variables (occupation, skin lesions, gender, age, As exposure time, As exposure level, tobacco smoking and alcohol drinking) were used to define the correlations between As retention and the Se status or the redox status.

### 3. Results

#### 3.1. As retention by binding to erythrocytic proteins was related to the Se status of the populations

Among the 165 eligible subjects, 80.6% had been exposed to As above the guideline values of 10  $\mu\text{g/L}$  and 87.3% had experienced As exposure for more than a decade (Table S2). Additionally, the Se dietary intake levels of these subjects were lower than the recommended dietary allowance (RDA) suggested by China or WHO (Table S3). To investigate associations between the Se status of subjects and the accumulation of As in blood, these subjects were divided into tertiles (Tb1, Tb2 and Tb3) according to the contents of bSe. Among tertiles, the contents of bAs were decreased with the increasing concentrations of bSe (Fig. 1A). Similar trends were also observed when the percentage of eAs in bAs (eAs%) and the percentage of PB-As in eAs (PB-eAs%) were evaluated, while the percentage of plasma As in bAs (pAs, pAs%) and the percentage of free As in eAs (F-As, F-eAs%) exhibited opposed trends (Fig. 1B–C and Table S4). Besides, the eAs occupied the major part of bAs (Fig. 1B and Table S4).

Thus, to define the effects of Se status on the As retention in erythrocytes, these subjects were then re-stratified into tertiles (Te1, Te2 and Te3) according to eSe. In erythrocytes, decreasing levels of iAs% (inorganic arsenic) and MMA% (monomethylarsonic acid), accompanied by the increasing levels of DMA% (dimethylarsinic acid), PMI (primary methylation index) and SMI (secondary methylation index), were observed among tertiles of eSe (Fig. 1D–E). Additionally, the associations observed above were further confirmed by the Spearman's correlation matrix. As expected, eAs, PB-eAs%, iAs% and MMA% showed significantly negative correlations with eSe, while F-eAs%, DMA%, PMI and SMI showed significantly positive correlations (Fig. 1D, Fig. S1 and Table S5). Interestingly, significant correlations were also observed between the As related parameters and the redox parameters that related to eSe (including GPX activity, GSH and MDA). However, the redox parameters that unrelated to eSe (including CAT, SOD and GR activity) showed no significant correlations with eAs (Fig. 1D and Table S5). Moreover, similar correlations were also observed when partial correlation analysis was performed (Table S6).

#### 3.2. Low Se status involving impaired redox homeostasis accelerated As retained in erythrocytes *in vitro*

To further investigate the effects of the Se status involving the redox status on the retention of As in erythrocytes, individuals with L-Se status and individuals with M-Se status were matched (Table S7). Firstly, the synchronizing signals of As and proteins observed both in L-Se group and in M-Se group confirmed the existence of PB-eAs in erythrocytes of L-Se group and M-Se group, but relatively lower intensity of As and weaker synchronization were observed in M-Se group (Fig. 2A–B). Next, erythrocytes were treated with  $\text{H}_2\text{O}_2$  or NAC to evaluate the effects of oxidative stress on As retention *in vitro*. In comparison with the As-treated erythrocytes, erythrocytes pre-treated with  $\text{H}_2\text{O}_2$  in L-Se group exhibited higher levels of eAs, PB-eAs% and MDA, but lower levels of GSH and GPX activity (Fig. 2C, and Table S8). Additionally, these erythrocytes also exhibited higher iAs% and MMA%, but lower DMA%, PMI and SMI (Fig. 2D–E, and Table S8). Although similar and moderate changes were observed in M-Se group, only PB-eAs%, iAs%, DMA% and PMI showed significant differences (Fig. 2C, F and G, and Table S8). Simultaneously, when comparing with the  $\text{H}_2\text{O}_2$  pre-treated erythrocytes, opposite changes in As related parameters (including eAs, PB-eAs%, iAs%, MMA%, DMA%, PMI and SMI) and redox parameters (GSH, GPX activity, and MDA) were observed in erythrocytes co-treated NAC both in L-Se group and M-Se group (Fig. 2C–G, and Table S8).

#### 3.3. Low Se status modulates changes of erythrocytes induced by As exposure

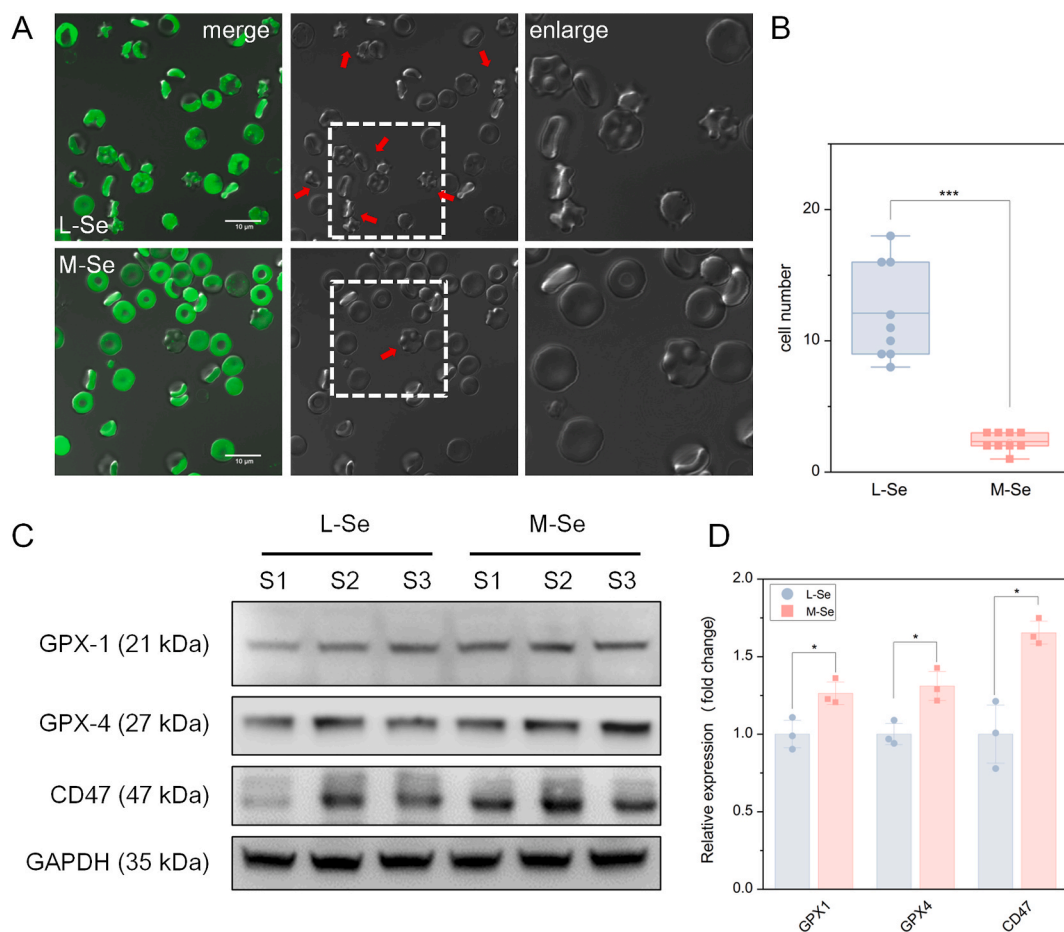
Given that erythrocytes incur damages during blood storage and As exposure, blood samples were refrigerated to further evaluate the effects of Se status on As-exposed erythrocytes. After a one-week refrigeration, more erythrocytes with morphological changes were observed in L-Se group when compared with the M-Se group (Fig. 4A–B). Then, autolysis was evaluated in erythrocytes labeled with CSFE after a 12-h culture in a humidified incubator. Compared with the M-Se group, higher absorbance at 450 nm and fluorescence intensity of CSFE were detected in the homogenates obtained from the L-Se group (Figs. S2A–C). Moreover, protein levels in erythrocytes with different Se status were detected for selenoprotein enzymes GPX1 and GPX4 as well as CD47, a molecular switch for erythrophagocytosis. In comparison with the M-Se group, lower protein levels of CD47, GPX1 and GPX4 were detected in the erythrocytic lysates of L-Se group (Fig. 4C–D). Interestingly, lower protein levels of CD47 were also detected in erythrocytes with a further As-exposure *in vitro* (Figs. S2D–G).

#### 3.4. The Se status and redox status modulates erythrophagocytosis

The red pulp of the spleen is the primary site where damaged or senescent erythrocytes are removed through phagocytosis by the reticuloendothelial macrophages. To further evaluate erythrophagocytosis *in vivo*, a mouse model of sub-chronic As exposure was established. Firstly, similar assays were performed to determine the effects of As exposure on mouse erythrocytes, where erythrocytes from As-exposed mice exhibited higher levels of morphological change (Fig. 4A–B), autolysis (Figs. S3A–C), and phosphatidylserine exposure (Fig. 4C), but lower protein levels of CD47 (Fig. 4D and Fig. S3D) in comparison with the erythrocytes from control mice. However, lower levels of changes in morphology, autolysis, and phosphatidylserine exposure were observed in erythrocytes from As-exposed mice with Se supplementation when compared with the erythrocytes of the As-exposed mice (Fig. 4A–C and Figs. S3A–C).

Next, levels of erythrophagocytosis were evaluated by autologous injection of CSFE-labeled erythrocytes. In comparison with the control groups, higher fluorescence intensity of CSFE was detected in the spleen homogenates (Fig. 4E) and in the spleen sections (Fig. 4F, arrows) of As-exposed mice. Additionally, more CSFE<sup>+</sup> cells and higher proportions of CSFE<sup>+</sup> cells in the subpopulation of F4/80<sup>+</sup> macrophages were detected in spleen cell suspensions of As-exposed mice (Fig. 4G). Similar to the lower levels of erythrocytic changes, lower levels of erythrophagocytosis, as indicated by the lower fluorescence intensity of CSFE in spleen homogenates and sections and the lower proportions of CSFE<sup>+</sup> cells, were found in As-exposed mice with Se supplementation in comparison with the As-exposed mice (Fig. 4G). Importantly, data showed no significant differences in fluorescence intensity of CSFE in erythrocytes and tissues (including hearts, livers and kidneys) between groups (Figs. S3E–G).

To further evaluate the effects of redox status on erythrophagocytosis, mouse erythrocytes were pretreated with an oxidative agent ( $\text{H}_2\text{O}_2$ ) or a reducing agent (NAC) before As exposure *in vitro*. In comparison with As-exposed erythrocytes, As-exposed erythrocytes with  $\text{H}_2\text{O}_2$  pretreatment were found to undergo higher levels of morphological changes (Fig. 5A–B), autolysis (Figs. S4A–C) and phosphatidylserine exposure (Fig. 5C), and more of these erythrocytes were phagocytosed by splenic macrophages *in vitro* (Fig. 5D–E). As expected, lower levels of cellular damage were observed in the As-exposed erythrocytes with NAC co-pretreatment and less levels of erythrophagocytosis were detected when compared with the As-exposed erythrocytes with  $\text{H}_2\text{O}_2$  pretreatment alone (Fig. 5 and Fig. S4).



**Fig. 3.** Effects of Se status on erythrocytes of the subjects with As exposure. (A) Representative images of erythrocytes labeled with CFSE are shown (scale bars: 10 μm) and arrows indicate erythrocytes with morphological changes. (B) The number of erythrocytes with morphological changes were counted (nine images per group). (C) Proteins levels of targets were analyzed by western blotting. (D) Relative protein levels normalized to GAPDH are shown (n = 3 per group). Differences between L-Se group and M-Se group were analyzed by using the unpaired Student's t tests. \*\*\* $p < 0.001$  or \* $p < 0.05$  indicates significant difference.

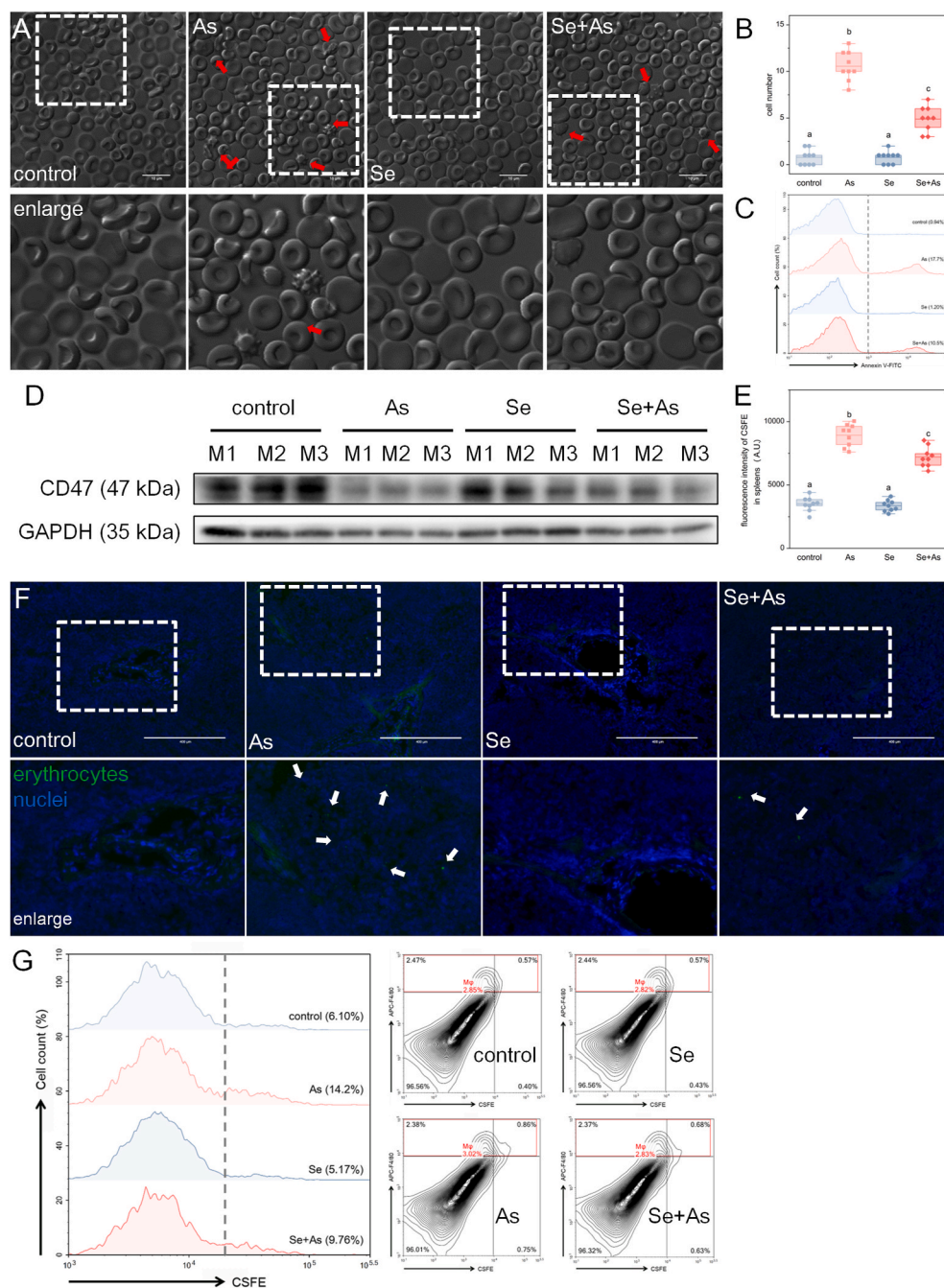
### 3.5. Phagocytosis of As-exposed erythrocytes induced activation of splenic macrophages

To further evaluate the effects of phagocytosis of As-exposed erythrocytes on macrophages, qPCR and western blotting were employed to assess mRNA and protein levels for inflammatory markers. Compared with macrophages phagocytosed control erythrocytes, macrophages phagocytosed As-exposed erythrocytes exhibited higher mRNA levels of ICAM-1, VCAM-1, iNOS, Cox-2, CXCL-9, TNF $\alpha$ , IL-1 $\beta$ , IL-8, IL-12 and IL-12p40, which related to the M1 polarization of macrophages, with no significant differences in the mRNA levels of IL-10 and MCP-1, which related to the M2 polarization (Fig. 6A–B). Along with the higher protein levels of iNOS and Cox-2, higher phosphorylation levels of IKK $\alpha/\beta$ , NF $\kappa$ B and I $\kappa$ B $\alpha$  were detected in macrophages phagocytosed the As-exposed erythrocytes (Fig. 6C and Fig. S5A). Additionally, higher levels of iNOS, Cox-2, p-IKK $\alpha/\beta$ , p-NF $\kappa$ B and p-I $\kappa$ B $\alpha$  were also detected in macrophages that phagocytosed erythrocytes with short As-exposure or long As-exposure (Figs. S6B–C). To further confirmed the activation of macrophages in the mouse, the levels of p-NF $\kappa$ B, iNOS and Cox-2 were detected in the mouse spleen sections. Compared with the control group, the red pulp of mice with As exposure exhibited higher levels of p-NF $\kappa$ B and iNOS, accompanied by no significance in the levels of Cox-2 (Fig. 6D). Interestingly, compared with the As-exposure group, lower levels p-NF $\kappa$ B and iNOS were observed in the red pulp of As-exposed mice with Se supplementation (Fig. 6D).

## 4. Discussion

Se supplementation has been shown to reduce health risks of chronic As exposure in animals and humans [28,29], and this protective effect has suggested to occur through detoxification and elimination of As [12]. The association between As retention in blood and the Se status, and the underlying mechanisms remain unclear, which prompted the study herein. We found that As was retained in blood mainly by retaining in erythrocytes, and this excessive retained As induced cellular damage. Moreover, excessive retained As caused decreased levels of CD47 in erythrocytes, inducing erythrophagocytosis and activation of splenic macrophages. Se status influenced this process by attenuating cellular damage and modulating the involving redox status.

As a well established environmental pollutant [33,34], As contaminates groundwater and is a global problem associated with several different health disorders [2]. In the present study, 243 subjects were enrolled from three villages in Datong basin, which is one of the As affected regions in China. Among the enrolled subjects in the present study, a total of 165 eligible subjects had suffered from As exposure above the guideline values for more than a decade (Table S2). The estimated Se dietary intake of these eligible subjects was lower than the RDA value (Table S3), but closely resembled the intake levels of populations from Se deficient belt in China [35,36]. These two characteristics suggested that these subjects with both chronic As exposure and Se dietary deficiency was suitable for the purpose of our study. Consistent with previous observations [26,27], our results showed that bAs, as well as eAs% and PB-eAs%, were decreased in a dose-dependent manner with



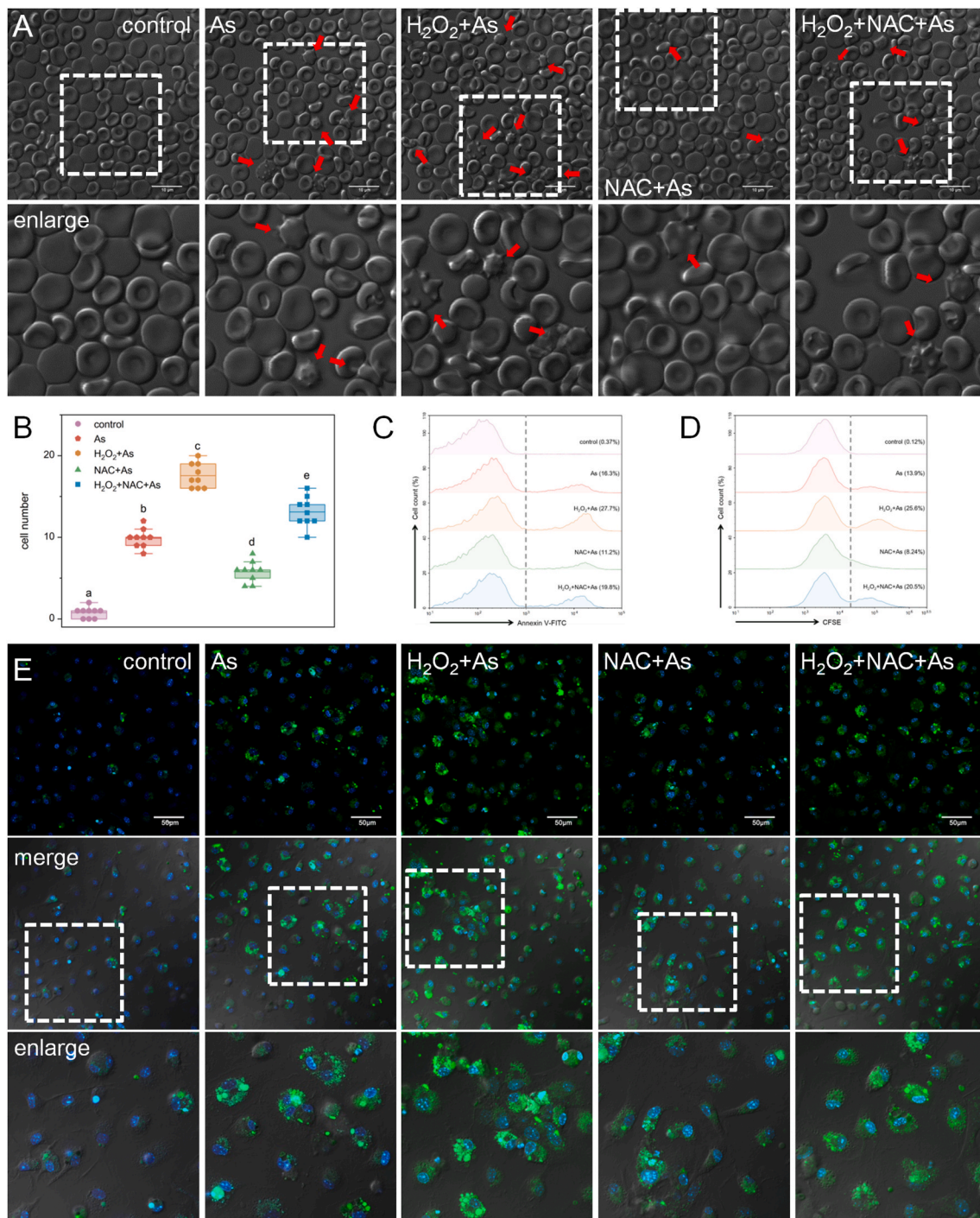
**Fig. 4.** Effects of Se status on erythrophagocytosis in mice with As exposure. The mice were randomly divided into four groups (n = 5 per group; control, As, Se, and Se+As), and housed as indicated in Methods and Materials. **(A)** Representative images of erythrocytes from mice are shown (scale bars: 10  $\mu$ m; arrows indicate erythrocytes with morphological changes), and **(B)** the number of erythrocytes with morphological changes were counted (nine images per group). **(C)** Levels of phosphatidylserine exposure in mouse erythrocytes were detected. **(D)** Proteins levels of targets in erythrocytes were detected by western blotting. **(E)** Fluorescence intensity of CSFE in spleen homogenates were detected. **(F)** Representative images of spleen sections are shown (scale bars: 400  $\mu$ m; arrows indicate the retained erythrocytes labeled with CSFE) and nuclei were counterstained with DAPI. **(G)** Levels of erythrophagocytosis in F4/80<sup>+</sup> cells of splenic cell suspensions were determined by flow cytometry. Different lower-case letters indicate significant differences between groups ( $p < 0.05$ ) as determined by One-way ANOVA and Tukey post-test.

the increasing levels of bSe (Fig. 1A–C). Although the eAs comprised the majority of circulatory bAs (Fig. 1B and Table S4), the ratio of eAs to pAs in our study was lower than that reported in the previous studies [8,9]. Moreover, 48.6% of eAs found in erythrocytes was bound to proteins (Fig. 1C), and the existence of PB-eAs was supported by the results of HPLC-UV-ICP-MS (Fig. 2A–B). Given that eSe was a more reliable marker for Se status across a wide range of Se intake [37], these subjects were further divided into tertiles based on the concentrations of eSe. As expected, eSe was negatively correlated with eAs and PB-eAs%, while positively correlated with F-eAs% (Fig. 1F, Figs. S1A–C and Table S5). These collective data suggest that the processes of As retention and protein binding are associated with the Se status of population.

Se plays an important role in protecting against oxidative stress mainly through the antioxidant selenoproteins [38]. Consistently, eSe exhibited positive associations with the antioxidant parameters that

related to Se status (including GSH and GPX activity), but exhibited a negative association with MDA and no significant association with antioxidant parameters that unrelated to Se status (including CAT, SOD and GR activity), suggesting that low Se status involves an impaired redox status in erythrocytes (Fig. 1F). Additionally, antioxidant parameters related to the Se status showed negative associations with eAs and PB-eAs, while no significant correlations were observed between antioxidant parameters unrelated to the Se status and eAs (Fig. 1D and Table S5). Taken together, these data suggest that As retention in erythrocytes relates to the Se status involving the redox status of the erythrocytes. To further explore this possibility, erythrocytes with different Se status were matched (Table S7) and subjected to oxidative stress *in vitro*. Notably, erythrocytes of subjects with low Se status were more sensitive to the oxidative challenge and As exposure as evidenced by the lower antioxidant capacity and higher levels of PB-eAs, and these



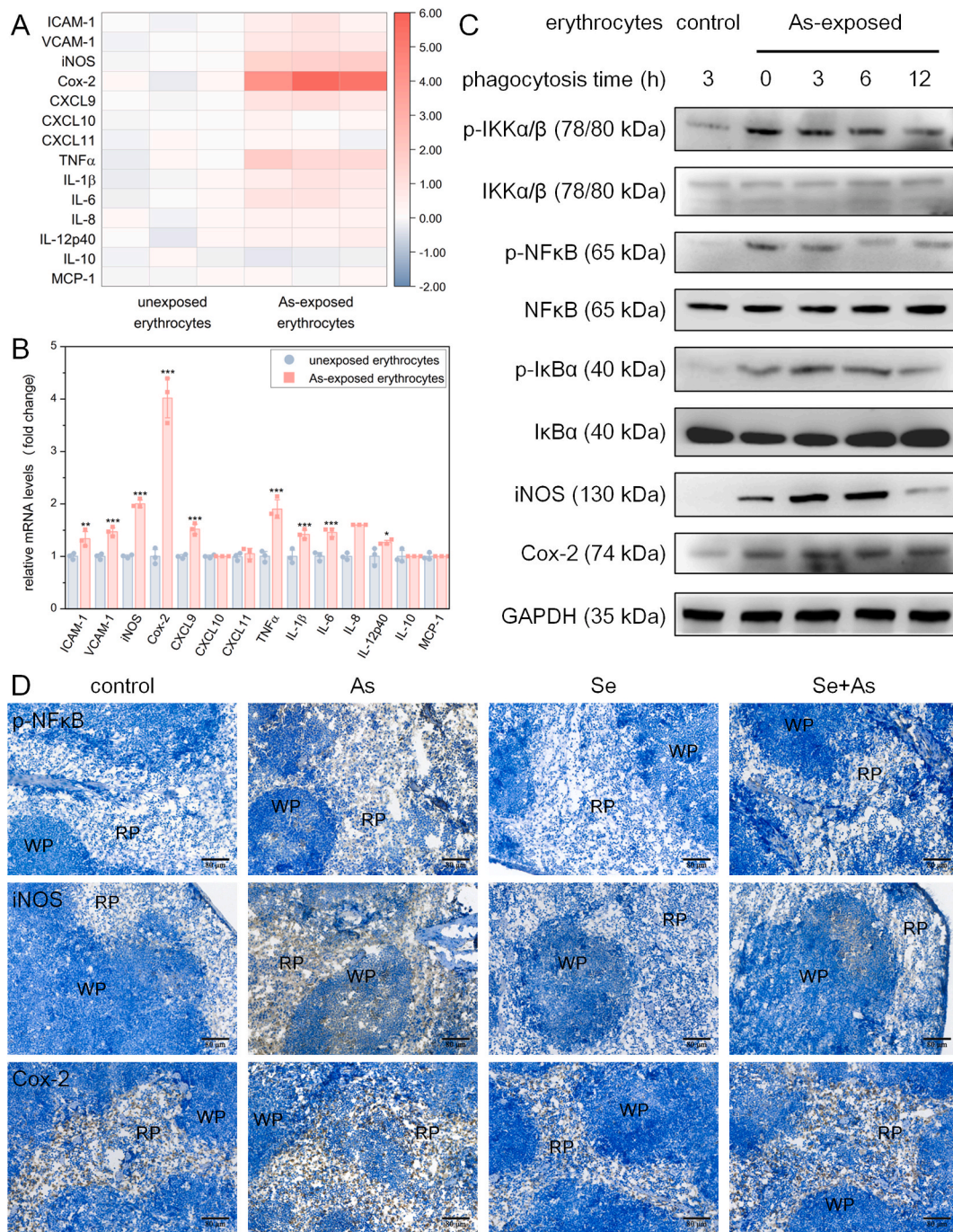


**Fig. 5.** Effects of redox status on erythrophagocytosis *in vitro*. After pretreating with H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M) or NAC (10 mM), mouse erythrocytes were exposed to As *in vitro*. (A) Representative images of erythrocytes are shown (scale bars: 10  $\mu$ m, arrows indicate erythrocytes with morphological changes) and (B) the number of erythrocytes with morphological changes were counted (nine images per group). (C) Phosphatidylserine exposure in erythrocytes were detected. (D) Erythrophagocytosis in splenic macrophages was determined after phagocytosing the erythrocytes with different treatment *in vitro*. (E) Representative images of splenic macrophages phagocytosed erythrocytes are shown (scale bars: 50  $\mu$ m). Different lower-case letters indicate significant difference between groups ( $p < 0.05$ ) as determined by One-way ANOVA and Tukey post-test.

could be partly attenuated by NAC, a widely used antioxidant (Fig. 2C and Table S8). These data suggest that impaired redox status may exacerbate As retention in erythrocytes. Besides, previous study has reported that As could induced protein carbonylation [39], which leads to non-enzymatic modification of proteins, loss of protein functions and oxidative damages [40]. Although dietary Se deficient has been proved to be associated with increased levels of protein carbonylation in certain

mouse tissues [41], further studies focused on protein carbonylation could elucidate the detoxification effect of Se on As in erythrocytes.

Methylation of iAs to MMA and MMA to DMA is established as a facilitator for the elimination and detoxification of As, and indicated by PMI and SMI [42,43]. Erythrocytes with either low Se status or oxidative stress exhibited relatively lower As methylation capacity, as evidenced by the higher levels of iAs% and MMA%, but lower levels of DMA%, PMI



**Fig. 6.** Inflammation in splenic macrophages that phagocytosed As-exposed erythrocytes. (A–B) Relative mRNA levels of targets genes in macrophages that phagocytosed control erythrocytes or As-exposed erythrocytes were determined by qPCR ( $n = 3$  per group) and data are presented. (C) Protein levels of targets in splenic macrophages phagocytosed control erythrocytes or As-exposed erythrocytes were analyzed by western blotting. (D) The levels of p-NF $\kappa$ B, iNOS and Cox-2 in mouse spleen sections were analyzed by immunohistochemistry. Differences between groups were analyzed by using the unpaired Student's  $t$  tests. \*\*\* $p < 0.001$ , \*\* $p < 0.005$  or \* $p < 0.05$  indicates significant difference.

and SMI (Fig. 1D–F, Fig. 2D–G and Table S8). These processes may be due to the limited supply of reductive GSH resulted from the lower levels of GPX activity under low Se status or impaired redox homeostasis.

As the most abundant circulating cells in blood and the primary target for circulating As, erythrocytes can act as an As sink to relieve the distribution and accumulation of this toxin in other tissues, although accumulation of As leads to damage of erythrocytes and may eventually result in anemia [44]. For erythrocytes of human subjects and As-exposed mice, there was an increase in cellular damage, which was influenced by the Se status of human subjects and mice (Fig. 3A–B,

Fig. 4A–C, Figs. S2A–C and Figs. S3A–C) as well as the redox status (Fig. 5A–C and Figs. S4A–C). Lower protein levels of CD47 were detected in these erythrocytes (Fig. 3C–D, Fig. 4D and Fig. S3D), and the protein levels of CD47 were decreased in a dose-dependent and time-dependent manner with further As-exposure *in vitro* (Figs. S2D–G). Interestingly, As exposure led to morphological changes, autolysis, phosphatidylserine exposure and the decreasing protein levels of CD47, which are features related to the process of erythrophagocytosis [14]. Consistent with this notion, erythrocytes of As-exposed mice were phagocytosed by splenic macrophages more easily *in vivo* and *in vitro*, and this process could be

affected by the Se status of mice (Fig. 4E–G) and the redox status of erythrocytes (Fig. 5D–E). These results suggest that As retention induces cellular damages of erythrocytes and promotes erythrophagocytosis by splenic macrophages, both of which could be affected by the Se status and redox status. In addition to the decreased protein levels of CD47, similar trends were detected when evaluating GPX1 and GPX4 (Fig. 3C–D, and Figs. S2D–G). As mature mammalian erythrocytes lack nuclei and are assumed to be void of protein synthesis, decreased protein levels in As-exposed erythrocytes seems likely because As exposure could induce oxidative stress [45] and oxygen radicals have been reported to induce protein degradation in erythrocytes [46], but further investigation will be required to determine the involvement of oxidative protein degradation.

Erythrophagocytosis has been shown to promote metabolic reprogramming of mouse bone marrow derived macrophages [16], induce activation of macrophages after transfusion [17], and induce inflammation of macrophages in plaques [18]. In our study, activation of the NFκB signaling pathway was detected in macrophages after phagocytosis of As-exposed erythrocytes as evidenced by the higher mRNA levels of NFκB target genes and the higher levels of phosphorylated NFκB in macrophages and in spleen sections (Fig. 6 and Fig. S6). Moreover, higher mRNA and protein levels of M1 markers were also detected in macrophages after phagocytosis of As-exposed erythrocytes (iNOS and Cox-2) and in the red pulp of spleen sections (iNOS only), which differed from the observation of M2 polarization in mouse bone marrow derived macrophages [16]. These preliminary results indicated that phagocytosis of As-exposed erythrocytes could induce activation and M1 polarization of splenic macrophages. Although macrophages were proved to participate in iron recycling through erythrophagocytosis and these could induce ferroptosis in splenic macrophages in a mouse model of transfusion [47], further studies are needed to determine whether ferroptosis occurs in splenic macrophages during As exposure as these cells engage in erythrophagocytosis.

In conclusion, our findings suggest that low Se status involving the impaired redox homeostasis increases the As retention in erythrocytes and promotes phagocytosis of these As-retained erythrocytes by splenic macrophages. This excessive erythrophagocytosis is mediated by the increased levels of cellular damages and decreased protein levels of CD47, which further lead to NFκB pathway activation in splenic macrophages. The present data provide insights into the effects of low Se status and impaired redox homeostasis on the occurrence of arsenism and subsequent increased inflammation in spleen.

## Authorship

Z.C., Y.Z., W.Z. J.Y. and Q.L. performed the experiments. Z.C., Y. L., W.Y. and Z.H. designed the study, analyzed the data and wrote the paper. Z. X., S. Z. and P. H. interpreted and contributed to the writing. All authors discussed the results and implications and commented on the manuscript at all stages. All authors have read and approved the final manuscript.

## Declaration of competing interest

The authors declare they have no actual or potential competing financial interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2022.102321>.

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