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Hydrogen gas inhalation enhances alveolar macrophage phagocytosis in an ovalbumin-induced asthma model

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ABSTRACT

Background: Maintaining an airway clear of bacteria, foreign particles and apoptotic cells by alveolar macrophages is very essential for lung homeostasis. In asthma, the phagocytic capacity of alveolar macrophages is significantly reduced, which is thought to be associated with increased oxidative stress. Hydrogen (H_2) has been shown to exert potent antioxidant and anti-inflammatory effects, yet its effects on phagocytosis of alveolar macrophages are unknown. This study is aimed to evaluate the beneficial effects of hydrogen gas inhalation on alveolar macrophage phagocytosis in an ovalbumin (OVA)-induced murine asthma model.

Methods: Female C57BL/6 mice were intraperitoneally sensitized with OVA before they were subject to airway challenge with aerosolized OVA. Hydrogen gas was delivered to the mice through inhalation twice a day (2 h once) for 7 consecutive days. Phagocytic function of alveolar macrophages isolated from bronchoalveolar lavage fluid was assessed by fluorescence-labeled *Escherichia coli* as well as flow cytometry.

Results: Alveolar macrophages isolated from OVA-induced asthmatic mice showed decreased phagocytic capacity to *Escherichia coli* when compared with those of control mice. Defective phagocytosis in asthmatic mice was reversed by hydrogen gas inhalation. Hydrogen gas inhalation significantly alleviated OVA-induced airway hyperresponsiveness, inflammation and goblet cell hyperplasia, diminished T_H2 response and decreased IL-4 as well as IgE levels, reduced malondialdehyde (MDA) production and increased superoxide dismutase (SOD) activity. Concomitantly, hydrogen gas inhalation inhibited NF-κB activation and markedly activated Nrf2 pathway in OVA-induced asthmatic mice.

Conclusions: Our findings demonstrated that hydrogen gas inhalation enhanced alveolar macrophage phagocytosis in OVA-induced asthmatic mice, which may be associated with the antioxidant effects of hydrogen gas and the activation of the Nrf2 pathway.

1. Introduction

Asthma is a chronic airway disease characterized by reversible airflow obstruction, airway hyperresponsiveness and airway inflammation. Accumulating evidence has demonstrated that the phagocytic ability of alveolar macrophages is decreased in asthma patients [1–3]. As one of the primary cells maintaining lung homeostasis and host defense, alveolar macrophages play an important role in

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eliminating foreign particulates, bacteria and apoptotic cells. Defects in alveolar macrophage phagocytosis reduce the clearance of bacteria and apoptotic eosinophils in the airway, both of which contribute critically to asthma pathogenesis. It is well documented that impaired phagocytosis of bacteria would result in facilitated bacterial colonisation and increased susceptibility to infection that may cause recurrent exacerbations of asthma [4,5]. Additionally, when removal of apoptotic eosinophils by alveolar macrophages is not done in a timely manner, which is the most important way for clearing eosinophils in the airway [6,7], secondary necrosis of accumulating apoptotic eosinophils will occur and toxic proteins and proinflammatory contents in the cells will be released into the surrounding tissues to cause an enlarged inflammatory reaction [5], ultimately leading to airway hyperresponsiveness and airway remodeling [8]. Therefore, increasing the phagocytic capacity of alveolar macrophages can be a potential strategy for asthma treatment.

Oxidative stress is emerging as a crucial mechanism that may alter alveolar macrophage function [9–11]. Oxidative products are generated in response to exogenous toxicants or endogenous inflammatory cells. In asthma, excessive oxidant production overwhelms the endogenous antioxidant defense system [12], triggering a vast array of signaling pathways that ultimately lead to cell injury as well as alveolar macrophage phagocytic capacity impairment [13]. Decreased expression of macrophage surface recognition receptors has also been suggested as one mechanism underlying phagocyte dysfunction [13,14]. Recent studies indicate that activation of nuclear erythroid-related factor 2 (Nrf2) signaling pathway improved phagocytic ability of alveolar macrophages by increasing surface recognition receptors expression [15]. The identification of these underlying mechanisms for the defect phagocytosis may present novel therapeutic opportunities for the treatment of asthma.

Hydrogen (H_2), which exhibits antioxidative, anti-inflammation and signaling-regulating effects, has been extensively studied as a therapeutic medical gas in clinical and experimental models of numerous diseases. In 2007, Ohsawa, I et al. demonstrated that hydrogen gas can alleviate oxidative stress by selectively neutralizing hydroxyl radicals (·OH) and antagonizing peroxynitrite (ONOO-), which are the most cytotoxic reactive oxygen species (ROS) leading to cell injury and tissue damage [16]. However, hydrogen does not affect metabolic oxidation-reduction reactions or scavenge ROS needed for normal cellular physiological signaling [17]. Recently, hydrogen has also been proved to increase the expression of antioxidative enzymes such as Nrf2 and SOD [18,19], subsequently ameliorating oxidative stress and inflammatory responses. Increasing evidences demonstrated that many diseases, including ischemia-reperfusion injury [20], rheumatoid arthritis [21], sepsis [22] and Parkinson's disease [23], benefited from or were protected by hydrogen. Furthermore, inhalation of hydrogen gas effectively reduces hyperoxic lung injury in rat [24] and protects against cigarette smoke induced COPD in mice [25]. But the effects of hydrogen gas on phagocytic capacity of alveolar macrophages in asthma still remain unclear. Here, we conducted an investigation to detect whether hydrogen gas inhalation can restore alveolar macrophage phagocytosis in OVA-induced asthma mouse model. We also assessed the effects on allergic inflammatory response.

2. Methods and materials

2.1. Experimental animals

Female C57BL/6 mice (wild-type, aged 6–8 weeks) were purchased from Guangdong Medical Laboratory Animal Center. All the mice were housed in a specific pathogen-free animal facility under 12 h light/dark cycle condition and were fed with sterile water and irradiated food ad libitum. Mice were acclimated for several days prior to any experimental procedures. All experimental protocols in this study were approved by the Ethics Committee for Animal Studies of The First Affiliated Hospital of Guangzhou Medical University, China.

2.2. Reagents

Ovalbumin and methacholine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hematoxylin and eosin (H&E) kit, Periodic acid–Schiff (PAS) kit, MDA detection kit as well as SOD detection kit were purchased from Nanjing Jianchen Biological Institute (Nanjing, China). ELISA kits for IL-4, IL-13 and IgE were obtained from eBioscience (San Diego, CA, USA). ELISA kit for IL-5 was purchased from R&D Systems (Minneapolis, MN, USA). Fluorescence-labeled *Escherichia coli* was obtained from Vybrant Phagocytosis Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Primary antibodies for nuclear erythroid-related factor 2 (Nrf2, ab31163), Heme Oxygenase 1 (HO-1, ab13248) were purchased from Abcam Inc. (Cambridge, MA, USA). NF-κB p65(#8242) and Phospho-NF-κB p65(#3033) were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse $T_H1/T_H2/T_H/17$ phenotyping Kit was purchased from BD Biosciences (San Diego, CA, USA). Sulforaphane (#S5711) was purchased from Selleck Chemicals (Houston, TX, USA). DAB peroxidase substrate kit was purchased from ZhongshanJinqiao (Beijing, China). The nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) was purchased from Beyotime Institute of Biotechnology (Shanghai, China).

2.3. Animal experimental protocol

Forty mice were randomly divided into four groups with 10 mice each, as follow: (i) saline-sensitized, saline-challenged, and ambient atmosphere-exposed mice (Control group); (ii) saline-sensitized, saline-challenged, and H_2-exposed mice (H_2 group); (iii) OVA-sensitized, OVA-challenged, and ambient atmosphere-exposed mice (OVA group); (iv) OVA-sensitized, OVA-challenged, and H_2-exposed mice (OVA + H_2 group). Mice were sensitized intraperitoneally with 100 μL of 20 μg of OVA emulsified with Imject alum diluted in endotoxin-free normal saline on days 0 and 14. On days 24, 25 and 26, mice were challenged with aerosolized OVA (1%) in normal saline for 40 min by using ultrasonic nebulization. The control mice were sensitized and challenged with normal saline. After the measurement of airway responsiveness on day 27, all mice were sacrificed for further analysis.

2.4. Hydrogen gas administration

Hydrogen gas was produced by AMS-H-01 hydrogen oxygen nebulizer (Shanghai Asclepius Meditee Corp., Shanghai, China), which simultaneously generates mixed gases consisting of 67% hydrogen (H_2) and 33% oxygen (O_2) by electrolyzing deionised water. In order to eliminate the harmful effects of hypoxia or hyperoxia, the concentration of O_2 in the mixture was steadily adjusted to 21% by persistently inputting appropriate volume of nitrogen (N_2). The mice were placed into a sealed plastic box with inflow and outflow outlets that connect to the outside air. After the dilution with nitrogen, the final mixed gases which consist of 42% H_2, 21% O_2 and 37% N_2 was delivered through a rubber tube to the sealed box. The sealed box was flushed with mixed gases for 20 min to replace the air in the chamber. During the experiment, the concentration of H_2 and O_2 in the chamber was continuously monitored by gas spectrometer (Unisense A/S, Aarhus, Denmark) to confirm the stability of each gas component. The hydrogen gas inhalation was performed twice a day (2 h per time) with an interval of 6 h for 7 consecutive days after each OVA or saline challenge. On days 24, 25 and 26, hydrogen gas inhalation was performed 2 h after each atomisation challenge. The mice in control group were also placed into the sealed plastic chamber and were supported by fresh air.

2.5. Assessment of airway hyperresponsiveness

Airway responsiveness was determined by whole body

plethysmograph (WBP system, Buxco, USA) in unrestrained and spontaneously breathing mice after challenge with increasing concentrations of aerosolized methacholine. The mice were placed into plethysmograph chamber for 30 min for acclimatization and baseline measurement and then expose to aerosolized normal saline for 30s as control. Mice were subsequently challenged every 20 min with increasing doses of aerosolized methacholine (6.25, 12.5, 25 and 50 mg/mL) for 30s. The enhanced pause (Penh) was recorded during each 5-min sequence by barometric plethysmography and used as an index of airway obstruction. The results are expressed as percentage of corresponding baseline value for each concentration of methacholine.

2.6. BAL fluid and differential cell counts

The bronchoalveolar lavage (BAL) fluid cells were collected by slow injection of prewarmed sterile saline into the trachea through a 22-inch intravenous catheter. The lungs were lavaged twice with 0.8 mL prewarmed sterile saline and the recovered fluid was pooled. After centrifugation, pelleted BAL cells were resuspended in PBS and the total number of BAL cells was counted with a Neubauer chamber. Cytospin sample was prepared and stained with hematoxylin and eosin (H&E) for blinded assessment of differential cell percentages in BALF. The remaining fluids were centrifuged and supernatants were stored at −80 °C for further analysis. Total cells counts and differential cell counts were performed at a magnification of 200×. The number of eosinophils, neutrophils, lymphocytes and macrophages in a total of 400 cells were counted and differentiated under light microscopy, according to classical cell morphology.

2.7. Histopathologic examination of lung tissue

Lung tissues were fixed in 4% neutral formalin, paraffin-embedded, cut in 5 μm sections, and stained with H&E and PAS for assessment of lung inflammation and epithelial goblet cell metaplasia. Airway inflammation was semi-quantified according to the method of Yao et al. [26]. Two criteria were scored to quantify the airway inflammation including peribronchial and perivascular inflammation in H&E-stained lung sections.

To visualize airway mucus production, lung sections were stained with PAS. PAS stained tissues were detected under light microscopy for PAS-positive staining in the upper airway epithelium in fields at 200× magnification. Quantification of PAS-positive staining was identified by calculating the percentage of PAS-positive epithelial cells in total epithelial cells. At least 10 image fields of 8 sections from 8 mice per group were analyzed.

2.8. Measurement of serum total IgE, quantitation of cytokines in BALF

One day after determining airway parameters, mice were sacrificed with overdose of pentobarbital, then blood samples were immediately taken, rest at room temperature for 2 h, then centrifuged (3000*g*, 20 min) and supernatants were harvested for measurement of total IgE by ELISA according to the manufacturers' instructions. Levels of IL-4, IL-5 and IL-13 in BAL fluids were also measured by ELISA kits following the manufacturer's protocol.

2.9. Cell isolation and flow cytometry analysis

About 60 mg of right lung tissue was diced and incubated in RPMI 1640 medium with collagenase (200 U/mL) at 37°Cfor 40 min. Lung single-cell suspensions were obtained after filtration with 100-mm nylon filters. After elimination of red blood cells by ACK lysis buffer, lung single-cell suspensions were stimulated with phorbol 12-myristate 13-acetate (5 ng/mL) and ionomycin (1 mg/mL) in the presence of brefeldin A (10 mg/mL) for 5 h. Lung single-cell suspensions were fixed, permeabilized and stained for 30 min with antibodies for CD4, IFN-γ, IL-4 and IL-17A. Data were collected on a BD FACSVerse flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed using FlowJo software. Cell counts were performed before staining for flow cytometry analysis.

2.10. Phagocytosis assay and confocal microscopy

In order to obtain adequate primary alveolar macrophages for phagocytosis assay and other detections. The lungs were gently lavaged eight times with sterile saline in total. Alveolar macrophages isolated from BAL fluids of the experimental mice were plated onto 24-well plates (5×10^4 cells/well) and incubated with fluorescence-labeled *Escherichia coli* at a ratio of 100 bacteria per cell for 2 h at 37 °C in a humidified atmosphere of 5% CO_2. After incubation, alveolar macrophages were washed with Dulbecco-phosphate buffered saline (D-PBS) and the fluorescence of extracellular bacteria was quenched with 1% (*v*/v) trypan blue for 1 min at room temperature. After removing the trypan blue suspension, alveolar macrophages were rinsed again with D-PBS, trypsinized by trypsin, and resuspended in ice-cold PBS. The phagocytosis of fluorescent-labeled bacteria by alveolar macrophages was measured and analyzed using a BD FACSVerse flow cytometer. Data were expressed as mean fluorescence intensity (MFI). For bacterial binding assay, alveolar macrophages were treated with cytochalasin D (5 μg/mL) for 30 min prior to incubation with FITC-*E coil* and analyzed by flow cytometry for the acquisition of fluorescence as an indication of macrophage association with bacteria. Confocal microscopy was used to ascertain whether the bacteria were internalized into the alveolar macrophages. Cells were viewed on a confocal microscope with a kryptonargon laser fluorescence detector (Zeiss LSM880, Oberkochen, Germany).

2.11. Cell culture ex vivo

Alveolar macrophages isolated from OVA-exposed mice and OVA asthmatic mice treated with hydrogen gas inhalation were cultured in 24-well plates with RPMI 1640 at a density of 1×10^5/mL for 16 h with 10 mM sulforaphane (Nrf2 Agonists) respectively. Cells were then collected for further analysis.

2.12. Measurement of MDA content and SOD activity

Lung tissue homogenates were used to investigate both MDA content and SOD activity, which were measured by detection kits according to the manufacturer's instructions.

2.13. Western blot and immunohistochemistry

Pulmonary expression of Nrf2, HO-1 and NF-κB was detected by western blot. Lung tissues from mice were homogenized in ice-cold homogenization buffer. After centrifugation, the supernatants of homogenates were harvested and mixed with 5 × SDS loading buffer. Whole lung extracts were separated by 10% SDS–polyacrylamide gel and transferred onto PVDF membranes. Membranes were then probed with anti-Nrf2, anti-HO-1 and anti-NF-κB antibodies with indicated dilutions. After incubation with the secondary antibodies, immunoreactive bands were exposed to Tanon 5200 Chemiluminescence Imaging System (Shanghai Tanon Science & Technology, Shanghai, China) for image capture. Data analysis was performed with Image J software.

For immunohistochemistry of Nrf2 and HO-1, lung sections were deparaffinized and then submerged in antigen retrieval. Samples were treated with 3% H_2O_2 for 15 min to quench activity of endogenous peroxidase, and then incubated with PBS containing 5% BSA for 30 min to block nonspecific bindings. After that, the sections were incubated overnight at 4 °C in recommended dilutions of anti-Nrf2 and anti-HO-1 antibodies respectively. After washing with PBS for three times,

samples were incubated with secondary antibodies for 30 min at room temperature. Signals were visualized with DAB peroxidase substrate kit. Finally, the lung tissue sections were counterstained with hematoxylin and examined under microscope.

2.14. Immunofluorescence analysis of Nrf2 and HO-1

The cytospin slides were fixed with 4% paraformaldehyde for 15 min and then permeabilized with 0.1% TritonX-100 for 30 min at room temperature. After washing with PBS three times, the specimens were blocked with antibody diluents of 5% BSA in PBS for 30 min. Then the samples were incubated with anti-Nrf2 antibodies and anti-HO-1 antibodies with indicated dilutions at 4 °C overnight. After that, sections were washed 3 times with PBS and incubated with secondary antibody dilutions at room temperature for 30 min. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole and the results were viewed and photographed under a fluorescence microscope.

2.15. Statistic analysis

Statistic analysis was performed using SPSS version 22.0 (SPSS Inc., Chicago, Illinois, US). Data are presented as mean ± SEMs, and comparisons among groups were analyzed by one-way analysis of variance (ANOVA) accompanied by *Bonferroni* post hoc test (equal variances assumed) or Dunnett's T3 (equal variances not assumed) post hoc *tests for multiple* comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Hydrogen gas inhalation decreased airway hyperresponsiveness in asthma mice model

An OVA-induced asthma model was used in this research and the experimental procedure was shown in Fig. 1, Penh value was detected 24 h after the last challenge for evaluating airway responsiveness. As can be seen in Fig. 2A, OVA sensitization and challenge induced significant increases airway responsiveness to inhaled methacholine ($p < 0.05$ at doses of 12.5, 25 and 50 mg/mL), which was markedly inhibited after treatment with hydrogen gas inhalation. This indicates that hydrogen gas is effective in decreasing airway hyperresponsiveness.

3.2. Hydrogen gas inhalation ameliorated airway inflammation induced by OVA

Histologic analysis revealed that numerous inflammatory cells infiltrated around the bronchus in OVA-induced asthmatic mice. While OVA-exposed mice treated with hydrogen gas exhibited marked reduction of inflammatory cells extravasating in the peribronchiolar and perivascular regions (Fig. 2D, E). The evident goblet cell hyperplasia in bronchial epithelia induced by OVA was also attenuated after treatment with hydrogen gas (Fig. 2D, F). Analysis of the inflammatory cells in BAL fluids also proved that sensitization and challenge with OVA resulted in a significant increase in the numbers of total cells and eosinophils ($p < 0.01$), and both were decreased after hydrogen gas intervention (Fig. 2B, C).

3.3. Hydrogen gas inhalation diminished OVA-induced T_H2 responses

We investigated the percentage of $T_H1/T_H2/T_H17$ cells in the lung tissue by flow cytometry, and found that T_H2 response is dominant in OVA-induced asthma model. We adopted CD4 to mark all the T cells roughly, and then different markers (IFN-γ, IL-4, IL-17A) were used to mark $T_H1/T_H2/T_H17$ cells in CD4 positive cells. Dysregulation of T_H2 and T_H17 responses were observed in OVA-sensitized and challenged mice, with significantly increased percentages of CD4 IL-4+ (T_H2) and slightly raised percentages of CD4 IL-17A+ (T_H17) cells in the lung. Treatment with hydrogen gas dramatically suppressed the elevated cell percentages of CD4 IL-4+ cells in lung of OVA-exposed mice, while it had no significant effect on T_H17 cells (Fig. 3A–C). In addition, T_H2 associated cytokines (IL-4, IL-5, IL-13) in BAL fluids and serum IgE were also determined. As expected, OVA exposure dramatically up-regulated the levels of T_H2-related IL-4, IL-5 and IL-13 in BAL fluid as well as serum IgE when compared with control (Fig. 2G–J.). Hydrogen gas inhalation notably suppressed the release of BAL fluid IL-4 and serum IgE ($p < 0.01$) (Fig. 2G, J), but showed no significant effects on the levels of IL-5 and IL-13 in BAL fluids (Fig. 2H, I).

3.4. Hydrogen gas inhalation increased alveolar macrophage phagocytosis

The phagocytic ability of alveolar macrophages was assessed by means of fluorescence-labeled bacteria and flow cytometry analysis. Primary alveolar macrophages isolated from OVA-induced asthmatic mice displayed significant decreased phagocytic capacity to fluorescence-labeled *Escherichia coli* compared with those from control mice. Surprisingly, after hydrogen gas inhalation, the phagocytic ability of macrophage was obviously recovered ($p < 0.01$) (Fig. 3E, F). There was no difference in the phagocytosis of bacteria between control group and H_2 group. In order to better speculate the possible mechanistic role of hydrogen gas, in vitro experiment was performed. Alveolar macrophages isolated from OVA-exposed mice and OVA asthmatic mice treated with hydrogen gas inhalation were incubated with sulforaphane respectively. Results showed that hydrogen gas and sulforaphane both significantly increased phagocytosis of *E coil* (Fig. 4A).

In order to further confirm that the bacteria were being internalized by alveolar macrophages, phagocytosis experiments were also performed in the absence and presence of cytochalasin D. Cytochalasin D is an inhibitor of actin filament polymerization, which cause an inhibition of phagocytosis with *E coil* unable to enter the cells, localizing to the outer cell membrane of alveolar macrophages. After the stimulation with Cytochalasin D, phagocytosis of *E coil* was absolutely suppressed in alveolar macrophages from all groups as indicated by the difference in mean fluorescence intensity. There was a significant difference between non-stimulated and Cytochalasin D treated cells for all groups (Fig. 3G).

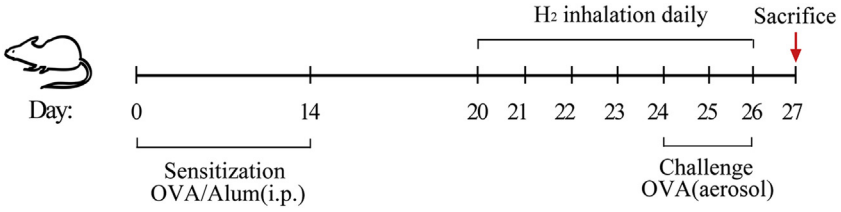

Fig. 1. Animal experimental procedures. Mice were sensitized intraperitoneally with 100 μL of 20 μg of OVA emulsified with Imject alum diluted in endotoxin-free normal saline on days 0 and 14. On days 24, 25 and 26, mice were challenged with aerosolized OVA (1%) in normal saline for 40 min by ultrasonic nebulization. The control mice were sensitized and challenged with normal saline. The hydrogen gas inhalation was performed twice a day (2 h per time) with an interval of 6 h for 7 consecutive days after each OVA or saline challenge. On days 24, 25 and 26, hydrogen gas inhalation was performed 2 h after each atomisation challenge. After the measurement of airway responsiveness on day 27, all mice were sacrificed for further analysis.

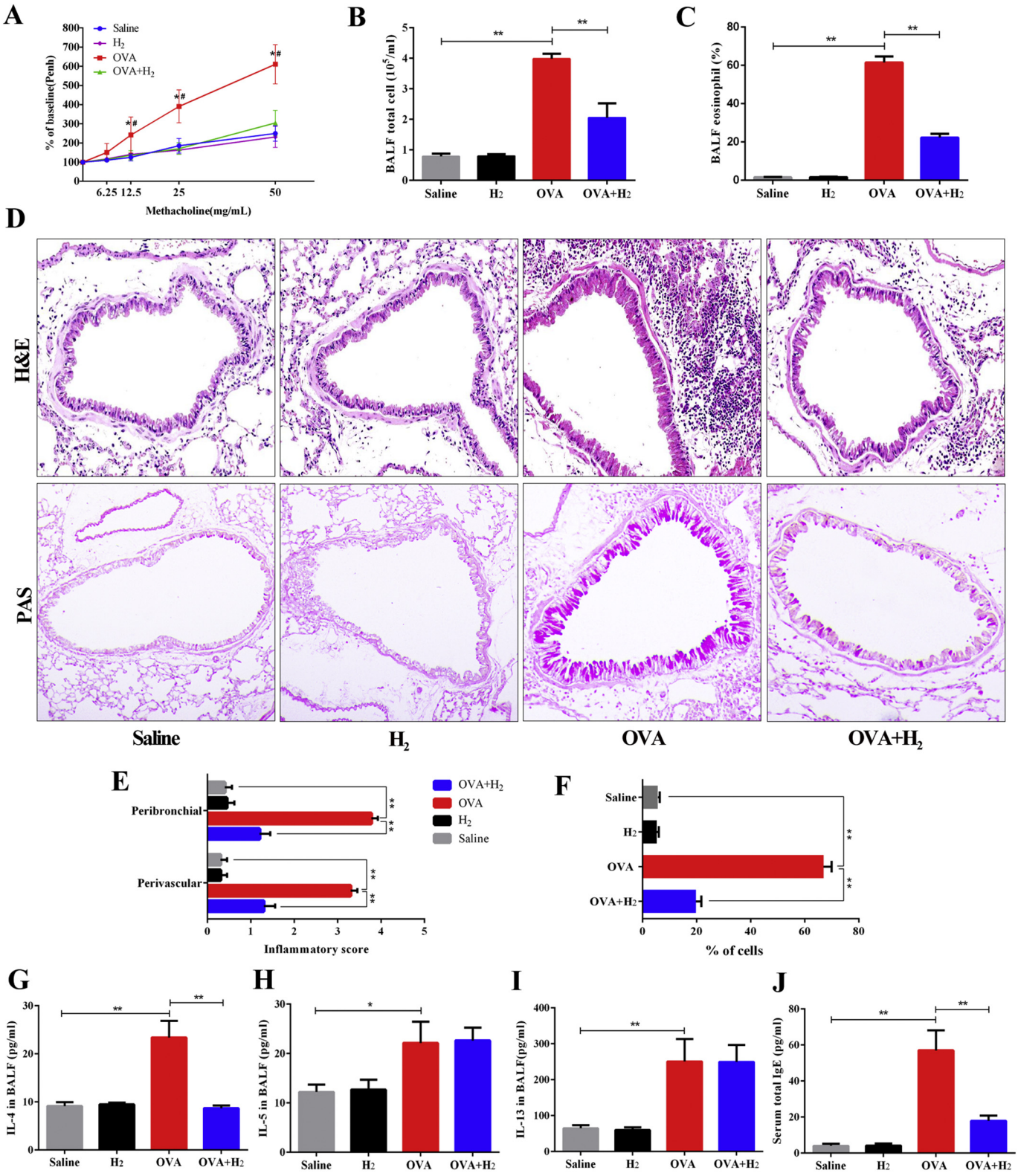

Fig. 2. The effects of hydrogen gas on airway hyperresponsiveness and airway inflammation. (A) Airway hyperresponsiveness was measured by Penh. Results are expressed as percentage of baseline value for each concentration of methacholine. *$p < 0.05$ compared with control group, #$p < 0.05$ compared with OVA + H_2 group, $n = 4$–6. (B) Total inflammatory cell counts in BAL fluids, $n = 8$–10. (C) Percentage of eosinophil in BAL fluids, results are expressed as percentages of total cells by counting a total of 400 cells in H&E-stained cytospin samples, n = 8–10. (D) Representative H&E-stained and PAS-stained lung sections were used to identify inflammatory cells infiltration and epithelial goblet cell metaplasia respectively. Images were at 200× original magnification. Semiquantification of airway inflammation (E) and PAS staining (F) was performed in different groups, n = 8–10. Quantification of PAS-positive staining was identified by calculating the number of PAS-positive epithelial cells. (G–I) Levels of IL-4, IL-5 and IL-13 in supernatants of BAL fluids were measured by ELISA, n = 8–10. (J) Total serum IgE was quantified by ELISA, n = 8–10. *$p < 0.05$ and **$p < 0.01$.

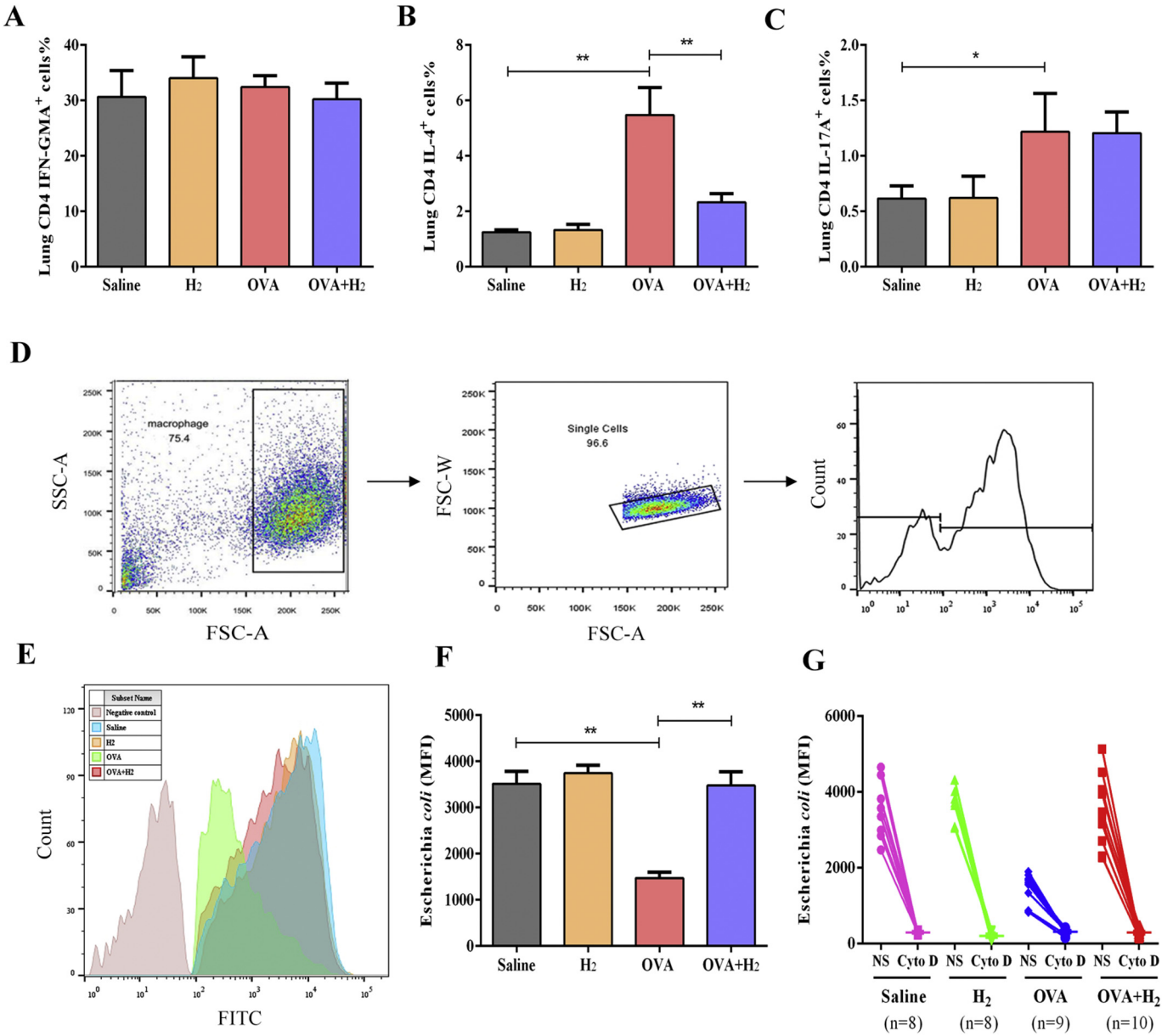

Fig. 3. Hydrogen gas inhalation robustly attenuated OVA-induced T_H2 responses and increased alveolar macrophage phagocytosis. (A–C) Numbers of CD4 IFN-γ+, CD4 IL-4+, CD4 IL-17A+ cells in lung single cell suspensions. Data were presented as percentages of CD4+ cells, $n = 6$. (D) Strategies for identification of alveolar macrophages by means of FACS (FSC, Forward scatter; SSC, Side scatter). (E) The capacity to phagocytose fluorescence-labeled *E coli* of different groups was analyzed by Flowjo software. (F) Phagocytosis of fluorescence-labeled *E coli* by alveolar macrophages. (G) The effect of Cytochalasin D on phagocytosis. NS = non stimulated, Cyto D = Cytochalasin D stimulated. Data were expressed as mean fluorescence intensity (MFI), n = 8–10. *$p < 0.05$ and **$p < 0.01$.

3.5. Hydrogen gas inhalation decreased MDA level and increased SOD activity presented in lung homogenates

To further confirm that the protective effects of hydrogen gas inhalation were related to its anti-oxidative property, two oxidative stress markers MDA and SOD were measured. MDA is the end product of lipid peroxidation. While SOD is an important antioxidative enzyme that catalyzes the dismutation of the superoxide radical into either ordinary molecular oxygen or hydrogen peroxide, sequentially eliminating deleterious ROS. Results showed that pulmonary MDA increased significantly in OVA-induced asthmatic mice, but was inhibited to almost control level (saline or H_2 group) after hydrogen gas inhalation (Fig. 4B). Meanwhile, we also detected decreased activity of SOD in OVA sensitized and challenged mice, which was dramatically restored by hydrogen gas inhalation (Fig. 4C). These suggested that hydrogen gas inhalation can protect OVA asthmatic mice from oxidative damage.

3.6. Hydrogen gas inhalation inhibited OVA-induced NF-κB activation in lung tissue

To reveal the effects of hydrogen gas inhalation on proinflammatory transcription factor, we measured the expression level of NF-κB in the lung. As expected, there's increased phosphorylation of NF-κB in OVA induced asthmatic mice, and was inhibited by hydrogen gas inhalation (Fig. 4D).

3.7. Hydrogen gas inhalation activated Nrf2 and HO-1 expression

To uncover the possible mechanisms involved in phagocytic dysregulation of alveolar macrophages in asthmatic mice, western blot, immunohistochemistry and immunofluorescence were performed to evaluate the relative signaling pathways. The results showed that expression of the antioxidative enzyme Nrf2 as well as one its downstream

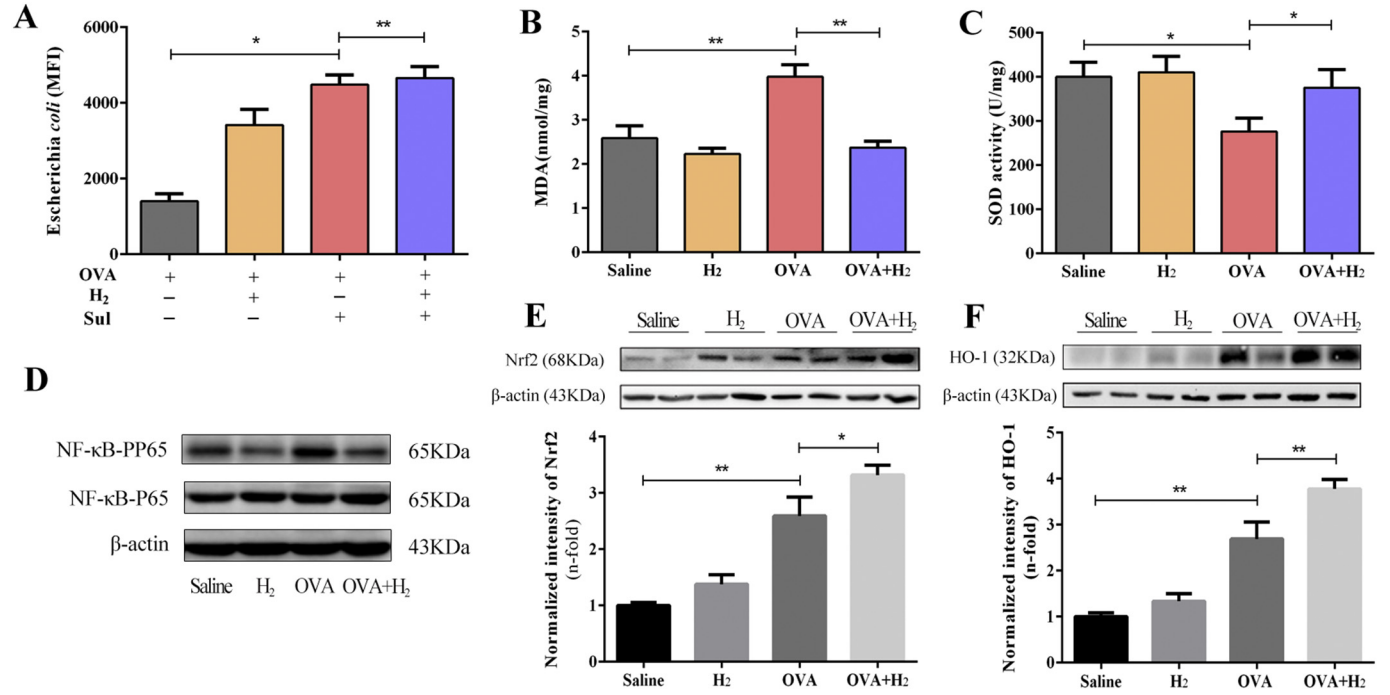

Fig. 4. The effects of sulforaphane on phagocytosis and the influences of hydrogen gas on oxidative stress indicators and Nrf2 signaling pathway. (A) Phagocytosis assay was performed in the absence and presence of sulforaphane, $n = 5–6$. (B and C) Assessment of MDA content and SOD activity in lung tissue homogenates, n = 8–10. (D and F) Protein expression of NF-κB, Nrf2 and HO-1 in lung tissue homogenates was detected by Western blot, Densitometric analysis of Nrf2 and HO-1 were performed in different groups, n = 6, results are expressed as mean ± SEM, *$p < 0.05$ and **$p < 0.01$.

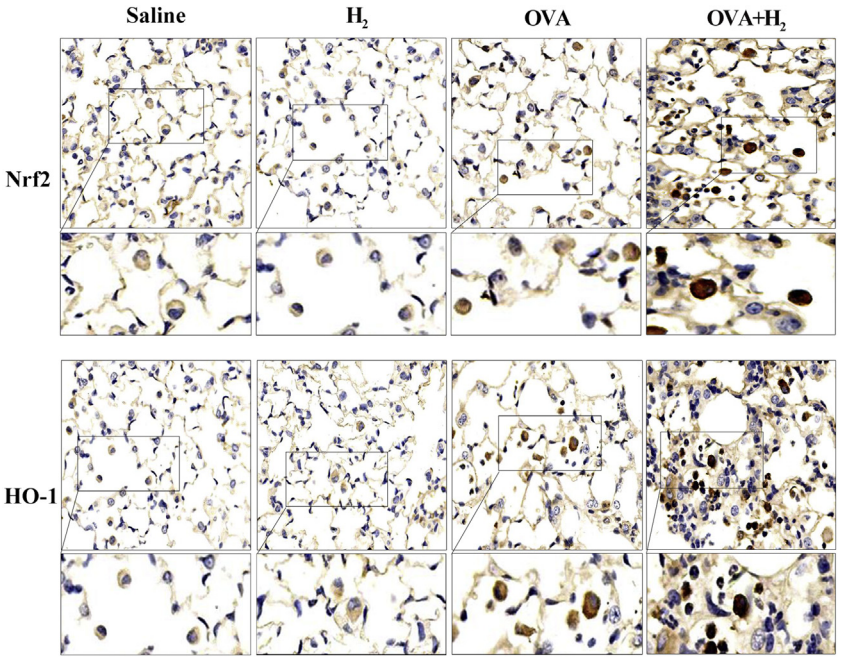

Fig. 5. Hydrogen gas enhanced Nrf2 and HO-1 expression of alveolar macrophages in OVA-induced asthma mice. Representative immunohistochemistry of Nrf2 and HO-1 on alveolar macrophages. Original magnification was 1000×. OVA sensitized and challenged mice had much more positive staining of Nrf2 and HO-1 in lung sections when compared with the saline-exposed. Treatment with hydrogen gas significantly increased Nrf2 and HO-1 expressions.

proteins HO-1, was upregulated in OVA-sensitized and challenged mice. A further increase in the expression of Nrf2 and HO-1 was observed in OVA asthmatic mice treated with hydrogen gas inhalation (Fig. 4E, F). Similar results were found in immunohistochemical lung sections. As shown in Fig. 5, there was more positive staining of Nrf2 and HO-1 in alveolar macrophages in OVA group compared with those in control group. After hydrogen gas inhalation, the immunoreactivity was much more abundant than OVA asthmatic mice without hydrogen gas treatment. These results suggested that OVA allergen challenge induced the expression of Nrf2 and HO-1 that can be enhanced by hydrogen gas inhalation. Immunofluorescence analysis of Nrf2 and HO-1 in alveolar macrophages indicated that hydrogen gas significantly accumulated Nrf2 in the nucleus and dramatically increased HO-1 expression in the cells, like Nrf2 agonists (Fig. 6). Internalization of *E coil* by alveolar macrophages was ascertained by using confocal microscopy (Fig. 7)

4. Discussion

In this study, we demonstrated that hydrogen gas inhalation could protect the phagocytic function of alveolar macrophages in OVA-

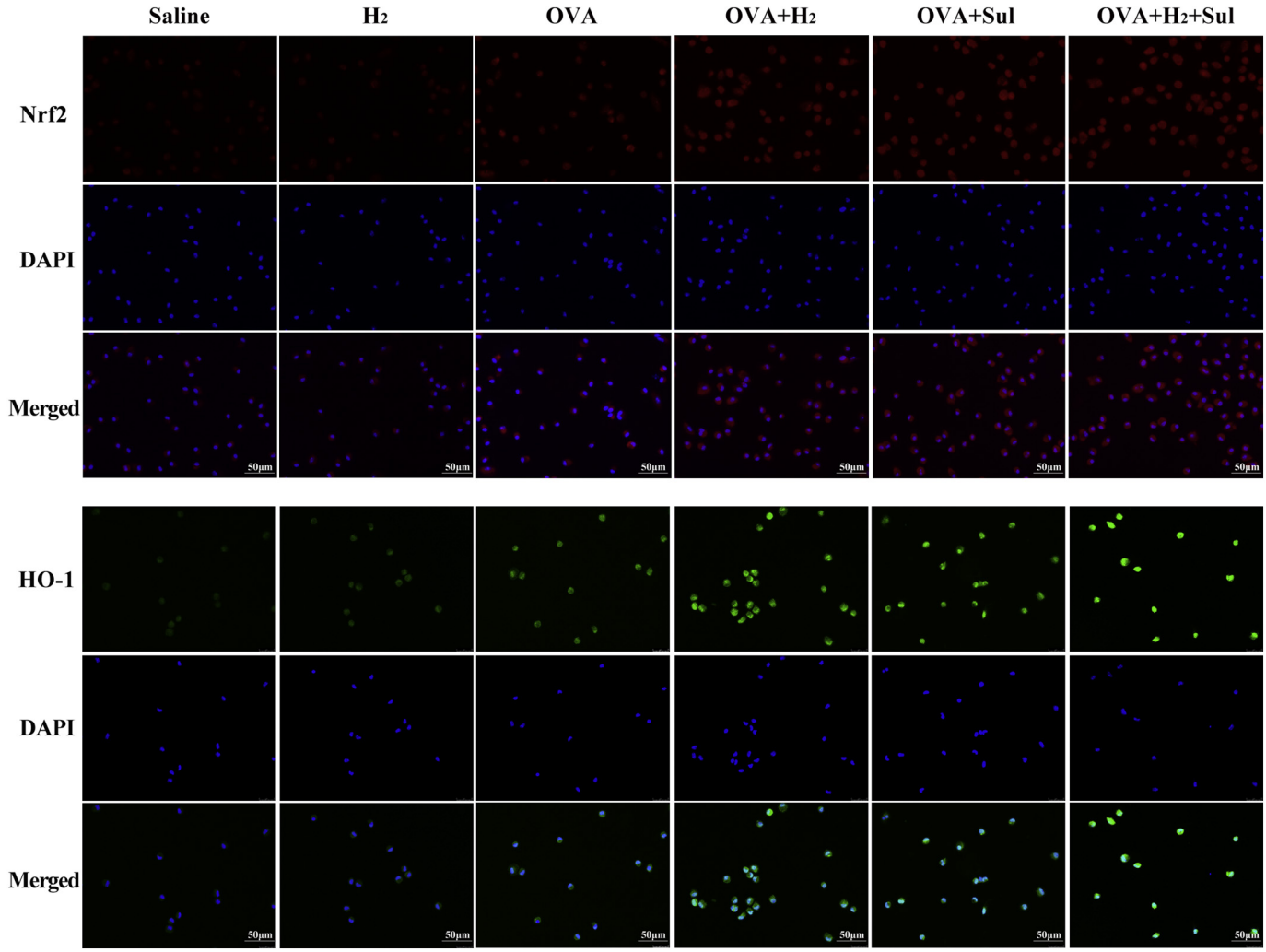

Fig. 6. Hydrogen gas and sulforaphane significantly accumulated Nrf2 in the nucleus and dramatically increased HO-1 expression in the alveolar macrophages. Representative images of immunohistochemical staining for Nrf2 and HO-1 in alveolar macrophages from OVA group mice and OVA + H_2 group mice. Images were obtained under a fluorescence microscope and the original magnification was 400×.

induced asthma model, which may be attributed to mitigation of oxidative damage and activation of Nrf2 signaling pathway.

To mimic the characteristics of clinical asthma patients, we developed an OVA-induced murine asthma model that is dominated by airway hyperresponsiveness, bronchial eosinophil infiltration and goblet cell hyperplasia, elevated levels of BAL fluid IL-4, IL-5, IL-13 and serum IgE. In recent years, researchers have revealed potent anti-inflammatory effects of hydrogen gas in a variety of diseases, including chronic obstructive pulmonary disease. Similarly, in this study, we found that repeated hydrogen gas inhalation in OVA sensitized and challenged mice could reverse most of their asthmatic features [27]. More importantly, we also observed that the OVA-induced impaired alveolar macrophage phagocytosis was recovered by hydrogen gas treatment. Yet the mechanisms involved remained unclear.

Maintaining an intact phagocytic capacity of alveolar macrophages is very important for limiting airway inflammation [28]. As mentioned before, oxidative stress plays a crucial role in the pathogenesis of phagocytic dysfunction by alveolar macrophages [11,29]. It has been reported that oxidative stress reduces the expression of several surface recognition receptors of alveolar macrophages such as mannose receptor [13] and Toll-like receptor [14], leading to decreased phagocytic activity towards bacteria and apoptotic cells. Furthermore, Richens, T.R. et al. found that oxidative stress activates RhoA-Rho kinase pathway, which is known to negatively regulate macrophage phagocytosis [30]. Accordingly, several antioxidants have also been found to rectify alveolar macrophage phagocytosis of bacteria. Hodge, S et al. demonstrated that antioxidant procysteine improved alveolar macrophage phagocytosis by elevating the activity of glutathione, which is essential for maintaining efficient macrophage function [9]. Similarly, another antioxidant *N*-acetyl cysteine has also been shown to promote macrophage phagocytic response by decreasing the production of ROS [31] and increasing glutathione activity [10]. Interestingly, in recent years, hydrogen gas has emerged as a novel antioxidant that exerts various protective effects [32–34]. In the current study, we observed that the impaired macrophage phagocytosis in OVA-exposed mice was accompanied by an increased level of the lipid peroxidation products MDA and decreased activity of the antioxidative enzyme SOD, and that hydrogen gas inhalation recovered macrophage phagocytosis as well as MDA and SOD to almost baseline levels, indicating that hydrogen enhanced macrophage phagocytosis may be associated with anti-perioxidative and anti-oxidative effects. Though there's currently no direct evidence suggesting that scavenging MDA facilitates phagocytic functions, SOD has been reported to take a pivotal part in maintaining the alveolar macrophage phagocytosis and contributing to bacteria clearance [35,36]. Taken together, we suggested that hydrogen gas increases alveolar macrophage phagocytic capacity partly through increasing SOD activity.

Accumulating evidences have demonstrated that Nrf2 plays a

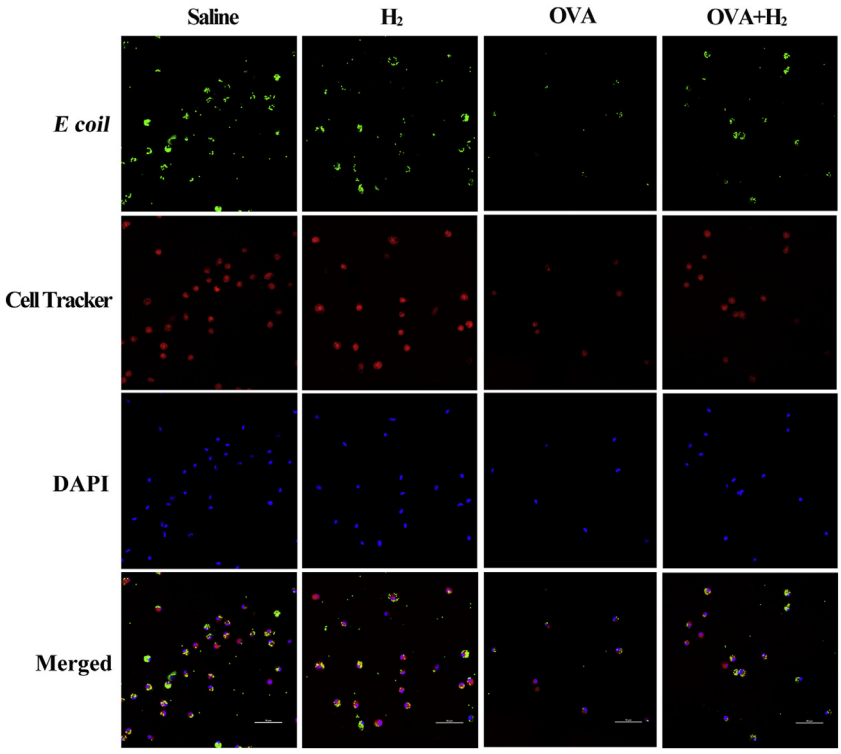

Fig. 7. Hydrogen gas inhalation partly recovered phagocytosis of fluorescence-labeled *E coli* by alveolar macrophages in OVA asthmatic mice. Internalization of *E coil* by alveolar macrophages was confirmed by using confocal microscopy. Cell cytoplasm was stained with Cell Tracker fluorescent probe (red) and the nuclei with DAPI (blue), *E coil* was conjugated with FITC (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

positive role in regulating both nonopsonin phagocytosis and opsonin-dependent phagocytosis of macrophages [15,37,38]. Nrf2 is a redox-sensitive basic leucine zipper transcription factor that regulates the expression of numerous cytoprotective proteins including antioxidants and xenobiotic detoxification enzymes [39]. In 2011, Harvey, C.J. et al. found that activating Nrf2 signaling pathway improved phagocytic ability of alveolar macrophages by increasing surface recognition receptor MARCO expression, which is the major scavenger receptor that mediated nonopsonin phagocytosis [15]. Later, in 2015, Staitieh, B.S. et al. demonstrated that treatment with Nrf2 agonist increased opsonin-dependent recognition receptor FcγR-mediated phagocytosis by regulating transcription factor PU.1 expression and activity [38]. Our present study consistently demonstrated that activation of Nrf2 by sulforaphane increases phagocytic ability of alveolar macrophages. Here in this study, increased expression of Nrf2 and its downstream protein HO-1, especially in alveolar macrophages, was seen after mice were subject to OVA sensitization and challenge. This indicates that OVA triggers the activation of Nrf2/HO-1, one strategy trying to recover macrophage phagocytosis. Of note, hydrogen gas treatment induced a further increase in the expression of Nrf2 and HO-1, implying that hydrogen acts to boost the recovering strategy and finally enhances the phagocytic function of alveolar macrophage. Yet further investigations are still needed to define the precise mechanism responsible for our observations.

Additionally, inflammatory cytokines have also been shown to modulate macrophage phagocytic function [40,41]. IL-4, a T_H2-associated cytokine that mediates important pro-inflammatory functions in asthma, has dual effects upon macrophages phagocytic function, which may be either stimulatory or inhibitory. Bingisser, R.M. and colleagues demonstrated that IL-4 inhibits the production of prostaglandin endoperoxide 2, which impair Fcγ-mediated phagocytosis of alveolar macrophages, thus promoting phagocytosis [42]. On the contrary, Lacraz, S et al. reported that IL-4 inhibits TNF-α production, thereby inhibiting the priming for phagocytosis of macrophage [41]. Here in the OVA-sensitized and challenged mice we found that hydrogen inhalation recovered macrophage phagocytosis along with restricting T_H2 response and suppressing IL-4 release, suggesting that inhibiting IL-4 helps to strengthen phagocytic ability, which agrees with the findings of Bingisser, R.M. et al.

In conclusion, our study demonstrated that inhalation of hydrogen gas can enhance alveolar macrophage phagocytic function in an OVA-induced murine asthma model, which may be partly associated with the activation of Nrf2 signaling pathway and its anti-oxidative effects. Our study proposed a novel therapeutical strategy for asthma treatment. With easy access, cheap price and excellent tolerance, hydrogen gas can be a promising option for clinical asthma patients in the future.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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