

## Research Article

# Simvastatin Inhibits NLRP3 Inflammasome Activation and Ameliorates Lung Injury in Hyperoxia-Induced Bronchopulmonary Dysplasia via the KLF2-Mediated Mechanism

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Received 3 March 2022; Accepted 4 April 2022; Published 25 April 2022

Academic Editor: Massimo Collino

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Bronchopulmonary dysplasia (BPD) is a chronic lung disease commonly found in premature infants. Excessive inflammation and oxidative stress contribute to BPD occurrence and development. Simvastatin, as an inhibitor of HMG-CoA reductase, has been reported to have antioxidative and anti-inflammatory effects. However, its effect and possible mechanisms in hyperoxia-induced lung injury are rarely reported. In this study, *in vivo* and *in vitro* experiments were conducted to investigate whether simvastatin could ameliorate hyperoxia-induced lung injury and explore its potential mechanism. For the *in vivo* study, simvastatin could improve alveolar development after hyperoxic lung injury and reduce hyperoxic stress and inflammation. The *in vitro* study revealed that simvastatin can reduce inflammation in A549 cells after high-oxygen exposure. Simvastatin suppressed NLRP3 inflammasome activation and played anti-inflammatory and antioxidant roles by increasing KLF2 (Krüppel-like factor 2) expression. *In vitro* experiments also revealed that these effects of simvastatin were partially reversed by KLF2 shRNA, indicating that KLF2 was involved in simvastatin effects. In summary, our findings indicate that simvastatin could downregulate NLRP3 inflammasome activation and attenuate lung injury in hyperoxia-induced bronchopulmonary dysplasia via KLF2-mediated mechanism.

## 1. Introduction

Bronchopulmonary dysplasia (BPD) is a chronic lung disease of preterm infants [1]. According to prior research, genetic susceptibility, premature delivery, mechanical ventilation, infection, oxidative stress injury, and lung inflammation may jointly participate in BPD occurrence and development [2]. In recent years, as neonatal intensive care has improved, the survival rates of very low-birth weight infants (<1500 g)/extremely low-birth weight infants (<1000 g) have increased but BPD incidence has also significantly increased [3]. Although BPD has been clinically prevented and treated by controlling the oxygen concentration,

caffeine, and vitamin A, the therapeutic effect and prognosis of BPD are still not satisfactory. As a result, developing other innovative and effective preventive or therapeutic measures to reduce mortality rates is a great challenge for perinatal medicine.

Since premature infants are sensitive to excessive oxidative stress, continuous exposure to a high-oxygen environment produces excessive reactive oxygen species, activating specific inflammatory cells and ultimately causing lung damage and cell death [4, 5]. Studies have indicated that excessive inflammatory factors, such as IL-6, TNF- $\alpha$ , and IL-1 $\beta$ , have been implicated in hyperoxic lung injury pathogenesis [6–9]. Inflammasome is a major component of innate

immunity, and leucine-rich repeat protein 3 (NLRP3) inflammasome is the most studied [10, 11]. NLRP3 inflammasome mainly comprises NLRP3, caspase adaptor (ASC), and caspase-1 [12]. Recent studies have demonstrated that NLRP3 is involved in many common lung diseases, such as acute lung injury and pulmonary fibrosis [13–15]. In addition, Chen et al. found that NLRP3 can be activated in the BPD model exposed to 85% oxygen and plays a critical role in inflammation and alveolarization [16]. Therefore, NLRP3 inflammasome is involved in BPD pathogenesis and is expected to become a new target for treating BPD in the future.

Krüppel-like factor 2 (KLF2), also referred to as lung Krüppel-like factor (LKLF), is a member of the zinc finger Krüppel-like transcription factor family and is involved in cell differentiation and tissue development [17]. KLF2 is mainly expressed in lung tissue and is required for normal lung development [18, 19]. In addition, KLF2 is a known inflammation regulator [20]. KLF2 could negatively regulate the expression of inflammatory cytokines and the production of adhesion molecules [21]. It has recently been discovered to participate in the occurrence and development of various lung diseases, including acute lung injury, asthma, and chronic obstructive pulmonary disease [18, 22–25]. Lung inflammation induced by lipopolysaccharide (LPS) or influenza A H1N1 virus could lead to reduced KLF2 [22]. However, the expression and effects of KLF2 on hyperoxic lung injury have not yet been investigated.

As an HMG-CoA reductase inhibitor, simvastatin is a widely used drug for treating dyslipidemia and cardiovascular diseases [26–29]. In addition, simvastatin demonstrates vascular protective effects by inducing KLF2 expression [30]. Recent clinical evidence suggests that simvastatin exhibits additional pharmacological effects, such as antioxidant and anti-inflammatory activities, as well as endothelial protection [31]. Liu et al. discovered that simvastatin exerts its therapeutic effect in rats with hepatic ischemia-reperfusion injury via a KLF2-dependent mechanism [32]. In addition, Sun et al. found that simvastatin improved human endothelial cell barrier function and reduced lipopolysaccharide-induced lung and systemic inflammation based on *in vivo* and *in vitro* experiments [33]. Consequently, we hypothesized that simvastatin could protect against hyperoxia-induced bronchopulmonary dysplasia by suppressing NLRP3 activation and acting as an anti-inflammatory and antioxidant agent by upregulating KLF2 expression.

## 2. Materials and Methods

**2.1. Hyperoxia-Induced Lung Injury.** All Sprague-Dawley rats used in animal experiments were obtained from the animal center of the Chinese Academy of Sciences (Shanghai, China), following the guidelines for the use of experimental animal care issued by the National Institutes of Health and approved by the experimental ethics committee of Wenzhou Medical University. Adult rats were housed in the laboratory animal center with humidity and temperature controlled at  $60 \pm 10\%$  and  $23 \pm 2^\circ\text{C}$ , respectively. The pups (no distinc-

tion between males and females) were randomly divided into four experimental groups and were returned to nursing cages within 6 h after birth: normoxia (NO) group, normoxia + simvastatin (NS) group, hyperoxia (HO) group, and hyperoxia + simvastatin (HS) group. Based on the dosages of simvastatin used in other models, the intermediate dose of 5 mg/kg was selected in our study to examine its effects on the hyperoxia lung injury in neonatal SD rats [34–36]. Simvastatin dissolved in corn oil (5 mg/kg, MCE, USA) was intraperitoneally injected into the pups of NS and HS groups on P0–P7. The pups in NO and HO groups received the same volume of vehicle corn oil (Aladdin, Shanghai, China).

The pups in the normoxia group received 21% oxygen for seven days, whereas those in the hyperoxia group received 80–85% oxygen. The plexiglass chamber flow-through system was employed to maintain a constant 80–85% oxygen, and the  $\text{O}_2$  level was monitored using an  $\text{O}_2$  analyzer. For seven days, we rotated the nursing dams between hyperoxia and normoxia groups every 24 h to protect nursing mothers from  $\text{O}_2$  toxicity.

**2.2. Lung Histological and Morphometric Analyses.** The pups were sacrificed using 1% pentobarbital by intraperitoneal injection. After ligating the right bronchus, the left lungs were perfused with PBS and inflated to 20  $\text{cmH}_2\text{O}$  pressure with 4% paraformaldehyde (PFA) and then preserved and fixed in 4% PFA for 48 h. The paraffin-embedded lung tissues were cut into 5  $\mu\text{m}$  sections, stained with hematoxylin and eosin (HE) (Beyotime, China), and morphologically analyzed under a microscope (Nikon, Japan). The radial alveolar count (RAC), mean linear intercept (MLI), and mean alveolar diameter (MAD), which were utilized to determine the alveolarization degree, were blindly assessed by investigators. There were at least 6 rats per group and at least 5 nonoverlapping HE pictures per rat. RAC was obtained by calculating the number of alveoli passing through the vertical line from the edge of terminal bronchioles to the nearest pleura or mediastinum. MAD was defined as the average alveolar diameter. Five lines were randomly drawn in each region, and the number of alveoli passing across each line was MLI.

**2.3. Water Content in the Lung.** The moisture content of the lungs is determined using lung tissues from the right lower lobe. To obtain the wet weight, the lobes were isolated and wrapped in a preweighted dry and clean tube. The lung tissue was placed at  $80^\circ\text{C}$  electric oven and dried for 24 h to obtain a dry weight. To determine lung tissue edema, the *W/D* ratio was calculated using the formula:  $(\text{wet weight} - \text{dry weight})/\text{humidity} * 100\%$ .

**2.4. Cell Culture.** A549 cell lines were maintained in DMEM/F-12 containing 10% fetal bovine serum (FBS) (Gibco) and cultured in an incubator with 5%  $\text{CO}_2$  at  $37^\circ\text{C}$ . The experiments were performed until the cells filled approximately 80% of the bottle bottom. The normoxia (NO) group continued to be cultured in an incubator with 5%  $\text{CO}_2$ , whereas the hyperoxia group was cultured in a special incubator containing 85%  $\text{O}_2$  and 5%  $\text{CO}_2$ .

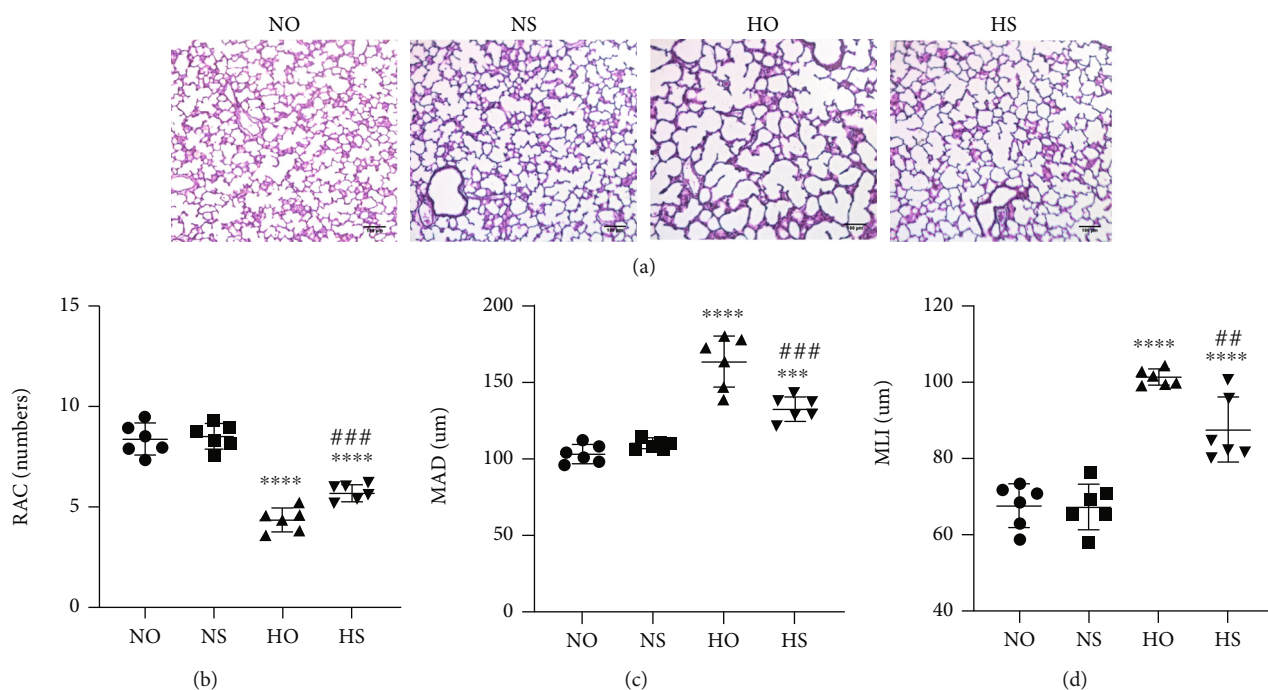


FIGURE 1: HE staining in the images and assessment of RAC, MAD, and MLI revealed that hyperoxia exposure led to marked alveolar simplification. In addition, simvastatin treatment could attenuate lung morphological changes. (a) HE staining (light microscopy,  $\times 100$ ) of lung tissue slides from each group. Scale bar =  $100\ \mu\text{m}$ . (b–d) Semi-quantitative pathology determination of RAC, MAD, and MLI in lung tissues. The values are mean  $\pm$  SD;  $n = 6$ , analyzed by two-way ANOVA with the Bonferroni post hoc test. \*\*\* $P < 0.001$  and \*\*\*\* $P < 0.0001$  versus the normoxia group; ## $P < 0.01$  and ### $P < 0.001$  versus the hyperoxia group.

**2.5. Cell Transfection and Grouping.** When cells reached an  $\sim 80\%$  confluence, various plasmids (Sangon Biotech, Shanghai, China) were transfected into A549 cells according to instructions of using the Lipofectamine™ 2000 Reagent (Invitrogen, California, USA). After adding the mixture of transfection reagent, plasmid, and Opti culture medium, the cells were cultured in a  $37^\circ\text{C}$  incubator for 30 min before replacing the old medium with a normal medium. For siRNA transfection, the corresponding scrambled sequence was employed as a control in the normal group to eliminate the impact of transfection on the cells. The complementary RNAi oligos were annealed and ligated into pSilencer-GFP, which is a bicistronic plasmid that expresses shRNA and eGFP, as previously described by Konishi et al. [37]. In addition, the transfection efficiency was  $40\% \sim 45\%$ . Accordingly, cells were grouped into the normoxia group (NO), normoxia + KLF2 shRNA group (NO + KLF2 shRNA), hyperoxia group (HO), hyperoxia + KLF2 shRNA group (HO + KLF2shRNA), normoxia + simvastatin group (NS), hyperoxia + simvastatin group (HS), and hyperoxia + simvastatin + KLF2 shRNA group (HS + KLF2shRNA).

**2.6. Cell Counting Assay.** To determine the appropriate high-oxygen treatment time and doses of simvastatin, we exposed the cells to  $85\% \text{O}_2$  for 4, 8, 12, 24, 36, and 48 h and added different simvastatin doses (2.5, 5, 10, 15, and  $20\ \mu\text{M}$ ) before high-oxygen treatment. The cell viability was measured using Cell Counting Kit-8 assays (CCK8) (C0038, Beyotime, China). After 48 h incubation,  $10\ \mu\text{L}$

CCK-8 solution was added to each well and incubated at  $37^\circ\text{C}$  for 1 h in the dark. The OD value was measured at 450 nm, and cell viability of each group was calculated with reference to the normoxia group.

**2.7. Western Blotting.** First, the tissues were homogenized using protein lysates consisting of RIPA lysis buffer (P0013B; Beyotime) and a protease and phosphatase inhibitor cocktail (P1048; Beyotime) and centrifuged at 12000 rpm for 30 min at  $4^\circ\text{C}$ . We then quantified the proteins using the BCA kit (P0010S; Beyotime). Equal mass proteins (50ug) were separated by 10% or 12.5% Tris-glycine gels in SDS-PAGE. After being transferred to PVDF membranes (Merck KGaA, Darmstadt, Germany) at 300 mA for 2 h, the membranes were blocked with 5% skim milk for 3 h at room temperature and incubated with appropriate primary antibodies, including KLF2 (1:1000, A16480, ABclonal, Wuhan, China), NLRP3 (1:1000, A12694, ABclonal, Wuhan, China), caspase-1/P20/P10 (1:1000, 22915-1AP; ProteinTech), IL-1 $\beta$  (1:1000, A1112, ABclonal, Wuhan, China), ASC (1:1000, 340097, ZEN-BIO, Chengdu, China), and  $\beta$ -actin (1:5000, AF7018, Affinity Biosciences, Cincinnati, OH, USA) overnight at  $4^\circ\text{C}$ . On the second day, the membranes were washed three times in Tris-buffered saline and Tween 20, followed by their incubation with appropriate secondary antibodies for 2 h at room temperature: goat anti-rabbit IgG (1:5000, SE134, Solarbio, Beijing, China) or goat anti-mouse IgG (1:5000, SE131, Solarbio, Beijing, China). The protein bands were detected using enhanced chemiluminescence reagents (Epizyme Biotech, Shanghai,

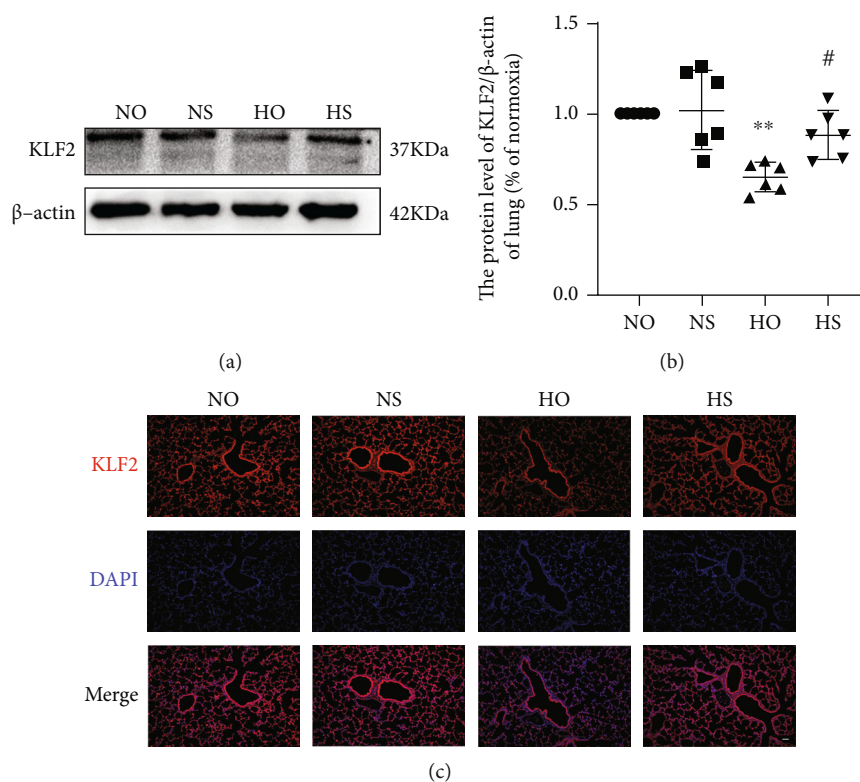


FIGURE 2: KLF2 levels in lung were decreased after hyperoxia. Simvastatin treatment significantly reversed KLF2 reduction after hyperoxia-induced lung injury. (a) KLF2 levels in lung tissues as determined by Western blotting. (b) Analyses of KLF2 levels based on Western blot results (normalized to  $\beta$ -actin levels). The values are the mean  $\pm$  SD;  $n = 6$ , analyzed by two-way ANOVA with the Bonferroni post hoc test,  $**P < 0.01$  versus the normoxia group;  $\#P < 0.05$  versus the hyperoxia group. (c) IF staining image of KLF2 in lung tissue (light microscopy,  $\times 100$ ). Scale bar represents 200  $\mu$ m.

China) through the ChemiDoc XRS+Imaging System (Bio-Rad, Hercules, CA, USA). All protein bands were calculated using Image Lab 5.0 software (Bio-Rad, Hercules, CA, USA).

**2.8. Immunofluorescence.** After drying overnight at 37°C, the 5  $\mu$ m lung tissue sections were deparaffinized using a gradient series of xylene and ethanol. Antigen retrieval was performed by microwave heating the sections for 20 min in 10 mM citric acid buffer (pH 6.0). After three washes with 1x PBS, the sections were blocked using 10% goat serum albumin for 1 h. KLF2 (1:100, A16480, ABclonal, Wuhan, China) and Ki67 (1:100, A2094, ABclonal, Wuhan, China) were diluted in 10% goat serum albumin, and 30  $\mu$ L was added to the sections (overnight at 4°C). The following day, the sections were incubated at room temperature for 2 h with Alexa Fluor-488 goat anti-rabbit IgG (1:200; AB150077; Abcam) and Alexa Fluor-555 goat anti-rabbit IgG (1:200; AB150078; Abcam). Finally, the sections were treated with a mounting medium containing 4',6-diamidino-2-phenylindole (Solarbio, Beijing, China) and the images were obtained using a scanning microscope (C1; Nikon, Tokyo, Japan).

**2.9. Enzyme-Linked Immunosorbent Assays (ELISA).** After ligating the right bronchus, 200  $\mu$ L of PBS was injected through the tracheal tube to lavage the left lung three times.

The lavage fluid was recovered and centrifuged at 3000 rpm for 10 minutes to obtain bronchoalveolar lavage fluid (BALF). Then, the TNF- $\alpha$ , IL-6, and IL-1 $\beta$  levels in BALF were determined by the rat cytokine enzyme-linked immunosorbent assay (ELISA) kit, following manufacturer's instructions (Multi Sciences Lianke Biotech, Hangzhou, China).

**2.10. Assessment of Oxidative Stress in Lung Tissues.** Superoxide dismutase (SOD) and glutathione (GSH) levels in lung tissue were measured using a commercially available kit (BC0170/BC1175, Solarbio, Beijing, China). A certain amount of lung tissue was obtained, the extract was added proportionally, and ice bath homogenization was conducted. The specific operation and result analysis were conducted following kit instructions.

**2.11. Statistical Analysis.** The data from experiments that were performed at least three independent times are presented as mean  $\pm$  SD. GraphPad Prism 8.0 (GraphPad Software, San Diego, USA) and SPSS Statistics version 19.0 (SPSS Inc., Chicago, IL) were used for statistical analysis. Differences between groups were analyzed using one- and two-way ANOVA followed by Bonferroni post hoc test.  $P < 0.05$  values were considered statistically significant.



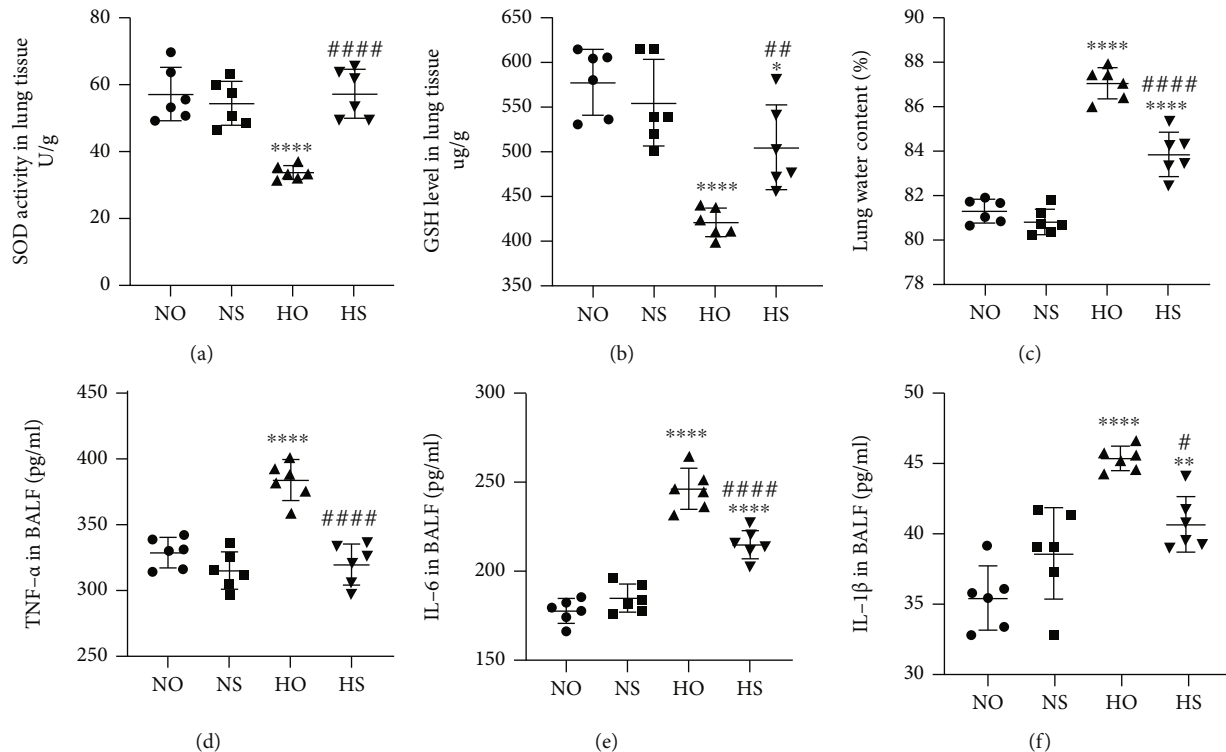


FIGURE 3: Effect of simvastatin on oxidative stress and inflammation in lung tissues. (a) SOD activity in various lung tissues. The values are demonstrated as the mean  $\pm$  SD;  $n = 6$ , analyzed by two-way ANOVA with the Bonferroni post hoc test, \*\*\*\* $P < 0.0001$  versus the normoxia group; ##### $P < 0.0001$  versus the hyperoxia group. (b) GSH activity in different lung tissues. The values are displayed as the mean  $\pm$  SD;  $n = 6$ , analyzed by two-way ANOVA with the Bonferroni post hoc test, \* $P < 0.05$  and \*\*\*\* $P < 0.0001$  versus the normoxia group; ## $P < 0.01$  versus the hyperoxia group. (c) The ratio of wet and dry weight is calculated in each group. The values are illustrated as the mean  $\pm$  SD;  $n = 6$ , \*\*\*\* $P < 0.0001$  versus the normoxia group; ##### $P < 0.0001$  versus the hyperoxia group. (d–f) TNF- $\alpha$ , IL-6, and IL-1 $\beta$  levels in BALF. The values are manifested as the mean  $\pm$  SD;  $n = 6$ , analyzed by two-way ANOVA with the Bonferroni post hoc test, \*\* $P < 0.01$  and \*\*\*\* $P < 0.0001$  versus the normoxia group; # $P < 0.05$  and ##### $P < 0.0001$  versus the hyperoxia group.

### 3. Results

**3.1. Effect of Simvastatin on Pulmonary Alveolar Simplification in the Lung.** Figure 1 illustrates the lung morphology using HE staining. In normoxia and normoxia + simvastatin groups, the lung exhibited complete lung structures with normal alveolar epithelium and alveolar septum (Figure 1(a)). However, after seven days of hyperoxia exposure, the lungs of hyperoxia-exposed rats were significantly simplified, MLI and MAD of per unit area increased, and RAC decreased (Figures 1(b)–1(d)). These data manifested that prolonged exposure to hyperoxia can increase alveolar damage, cause alveolar simplification, and delay lung development. Compared with that in the hyperoxia group, the alveolar simplification in the hyperoxia + simvastatin group was significantly improved. Compared with that of the hyperoxia group, RAC of the hyperoxia + simvastatin group increased, while MLI and MAD decreased. These findings indicated that simvastatin treatment could combat hyperoxia-induced lung injury and partially restore hyperoxia-induced alveolar simplification.

**3.2. Simvastatin Maintains KLF2 Expression.** The KLF2 level in the lung was determined by Western blotting (Figures 2(a) and 2(b)). KLF2 expression was decreased in

the hyperoxia group compared with the normoxia and normoxia + simvastatin groups. However, the hyperoxia + simvastatin group had a higher KLF2 level than hyperoxia group and there was no statistically significant difference between normoxia and normoxia + simvastatin groups in KLF2 levels. These results indicated that hyperoxic injury reduces KLF2 expression in lungs while simvastatin could reverse hyperoxic effects on KLF2. Then, we used immunofluorescence to further explore the changes of KLF2 expression. We found that immunofluorescence results are consistent with those of Western blotting (Figure 2(c)), hyperoxia could reduce KLF2 expression, and simvastatin could reverse this effect.

**3.3. Simvastatin May Decrease Oxidative Stress and Inflammation Infiltration in Neonatal Rats.** Oxidative stress expression was determined using available kits. As illustrated in Figures 3(a) and 3(b), the hyperoxia group had lower SOD and GSH activities than the normoxia group. However, SOD and GSH activities increased under simvastatin treatment in hyperoxia + simvastatin. To evaluate the effects of simvastatin on hyperoxia-induced inflammation in neonatal rats, we measured the lung water content and used ELISA kits to determine the expression of several inflammatory markers (TNF- $\alpha$ , IL-1 $\beta$ , and IL-6). As

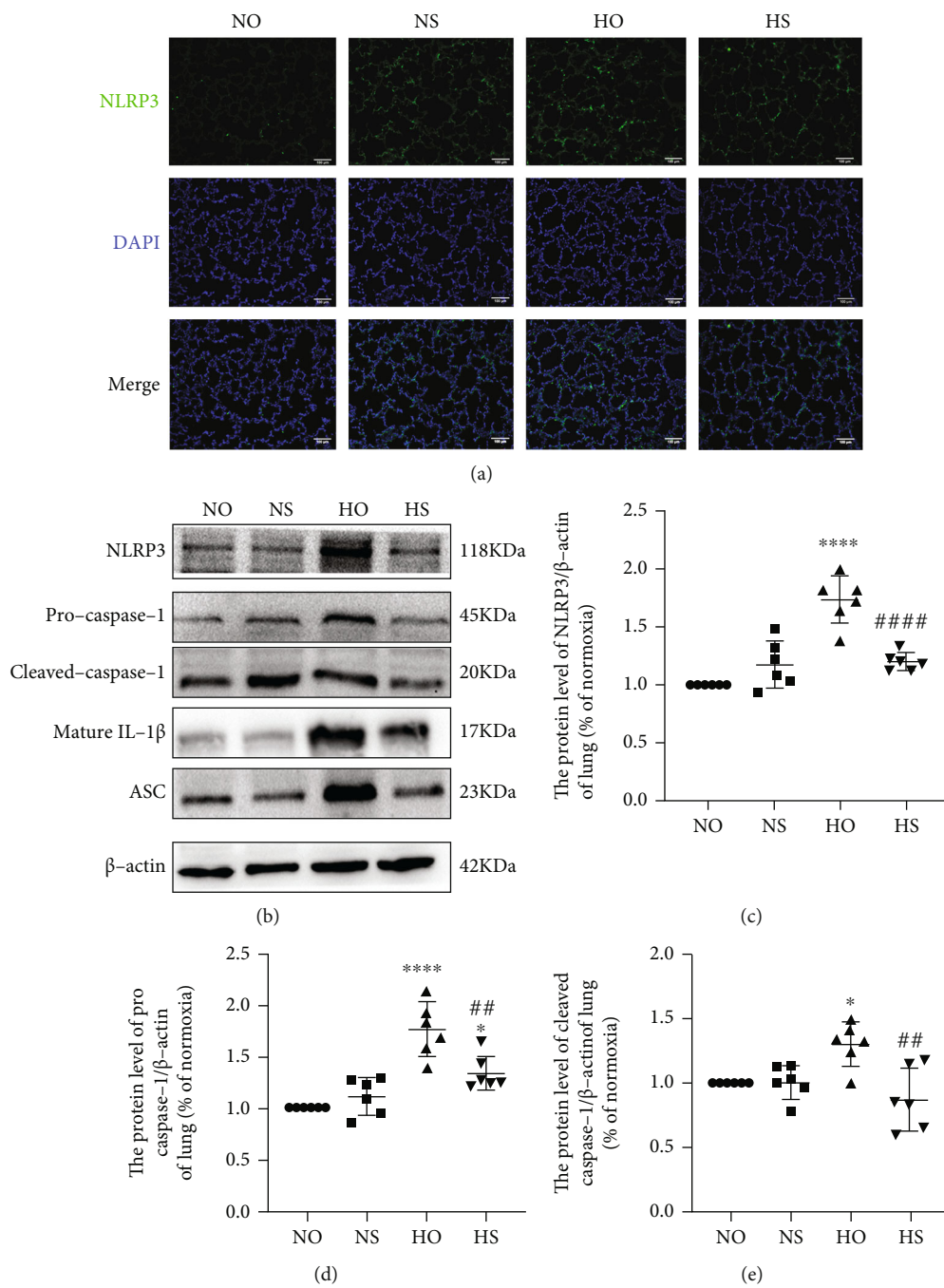


FIGURE 4: Continued.

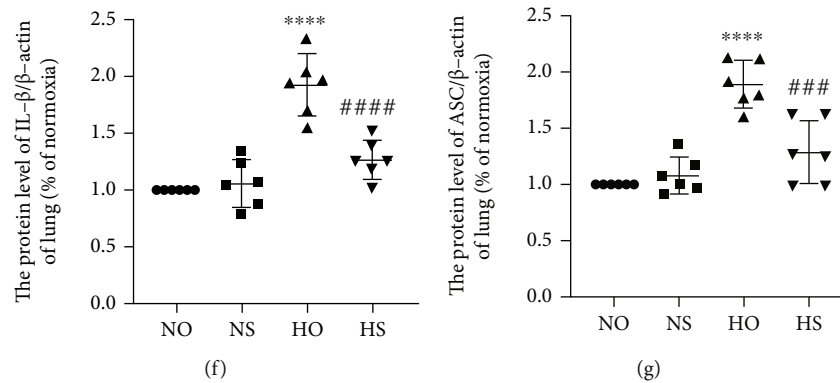


FIGURE 4: Hyperoxia exposure can activate NLRP3 inflammasome. Simvastatin treatment ameliorated this hyperoxia-induced change. (a) IF staining images of NLRP3 in the lungs of each group (microscopy,  $\times 200$ ). Scale bars =  $100 \mu\text{m}$ . (b) Western blotting results of NLRP3, pro-caspase-1, cleaved caspase-1, mature IL- $1\beta$ , and ASC in different lung tissues. (c–g) The quantification of protein levels of NLRP3, pro-caspase-1, cleaved caspase-1, mature IL- $1\beta$ , and ASC (normalized with  $\beta$ -actin). The values are the mean  $\pm$  SD;  $n = 6$ , analyzed by two-way ANOVA with the Bonferroni post hoc test,  $*P < 0.05$  and  $****P < 0.0001$  versus the normoxia group;  $###P < 0.01$ ,  $####P < 0.001$ , and  $#####P < 0.0001$  versus the hyperoxia group.

demonstrated in Figure 3(c), the ratio of wet and dry weight is calculated in each group; the water content was increased in the hyperoxia group but decreased following simvastatin treatment. As presented in Figures 3(d) and 3(e), TNF- $\alpha$  levels in the lung of the hyperoxia group were higher than those of the normoxia group. Conversely, the simvastatin treatment reversed the increase of TNF- $\alpha$  on hyperoxic inducement. Similar changes in IL-6 and IL- $1\beta$  levels were found. Our findings indicated that simvastatin treatment significantly reduced lung edema in acute stages of hyperoxia lung injury, implying that simvastatin ameliorated oxidative stress and hyperoxia-induced inflammation infiltration in the lungs of neonatal rats.

**3.4. Effect of Simvastatin on the NLRP3 Inflammasome Signaling Pathway in Hyperoxia-Induced Bronchopulmonary Dysplasia.** By Western blotting and immunofluorescence, we quantified the expression of NLRP3, pro-caspase-1, cleaved caspase-1, mature IL- $1\beta$ , and ASC in the lungs to evaluate the influence of simvastatin on NLRP3 inflammasome. Compared with those in the normoxia group, NLRP3 levels increased in the hyperoxia group, which could be partly reversed by simvastatin treatment (Figure 4). Similar changes were observed in the protein levels of pro-caspase-1, cleaved caspase-1, mature IL- $1\beta$ , and ASC.

**3.5. KLF2 Plays a Positive Role in Hyperoxia-Induced Lung Injury.** To further verify and explore the effect of simvastatin and its specific mechanism, we conducted *in vitro* experiments using A549 cell lines. Firstly, CCK8 analysis was used to select the optimal timing for hyperoxia. As indicated in Figure 5(a), 4 h after hyperoxia exposure, the viability of A549 cells begins to reduce and cell survival after 48 h of exposure remains more than 50%. As a result, 48 h of exposure was employed in all subsequent cell experiments. Based on the above *in vivo* studies, we hypothesized that simvastatin protects against hyperoxic lung injury via the KLF2 mechanism. To further explore this possibility, we used KLF2 shRNA for the *in vitro* study. To investigate the role

of KLF2 in hyperoxic lung injury, we established two groups: normoxia + KLF2 shRNA group (NO + KLF2 shRNA) and hyperoxia + KLF2 shRNA group (HO + KLF2shRNA). As displayed in Figures 5(b)–5(d), KLF2 shRNA and hyperoxia could decrease KLF2 expression. CCK8 analyses and cellular immunofluorescence staining of Ki67 were used to evaluate the proliferation ability of cells. As demonstrated in Figures 6(a)–6(c), KLF2 plays a positive role in hyperoxia-induced lung injury. Silencing KLF2 with KLF2-specific shRNA aggravates the damage to hyperoxia-caused cell viability. In addition, as illustrated in Figures 6(d)–6(g), KLF2 shRNA and hyperoxia could increase the expression of NLRP3 protein and inflammatory cytokines in A549 cells.

**3.6. Simvastatin Elicits Anti-Inflammatory Effects on Hyperoxia-Induced A549 Cell Injury via the KLF2-Mediated Mechanism.** A549 cells were treated with different simvastatin concentrations simultaneously with normoxia or hyperoxia for 48 h to choose the optimal therapeutic concentration. In Figure 7(a), simvastatin doses ranging from 2.5 to  $15 \mu\text{M}$  exerted lung-protective effects and the most obvious effect was observed at a dose of  $5 \mu\text{M}$ . As a result,  $5 \mu\text{M}$  simvastatin was employed in all subsequent cell experiments. Consistently with *in vivo* experiments (Figures 2–4), we found that high oxygen significantly reduced KLF2 expression and increased the expression of NLRP3 protein and inflammatory cytokines in A549 cells and simvastatin could reverse the effect of high oxygen. Interestingly, during the *in vitro* study, we added hyperoxia + simvastatin + KLF2 shRNA group (HS + KLF2shRNA) and found that KLF2 shRNA could significantly reduce these protective effects of simvastatin. As demonstrated in Figures 7(b)–7(d), hyperoxia reduced KLF2 levels in A549 cells, whereas simvastatin increased KLF2 expression in the hyperoxia group but not in KLF2-silenced group. In addition, hyperoxia increased NLRP3 levels in A549 cells, which was restored by simvastatin treatment but not in the KLF2-silenced group (Figures 7(h) and 7(i)). As indicated in Figures 7(j) and 7(k), simvastatin treatment can reduce hyperoxia-induced

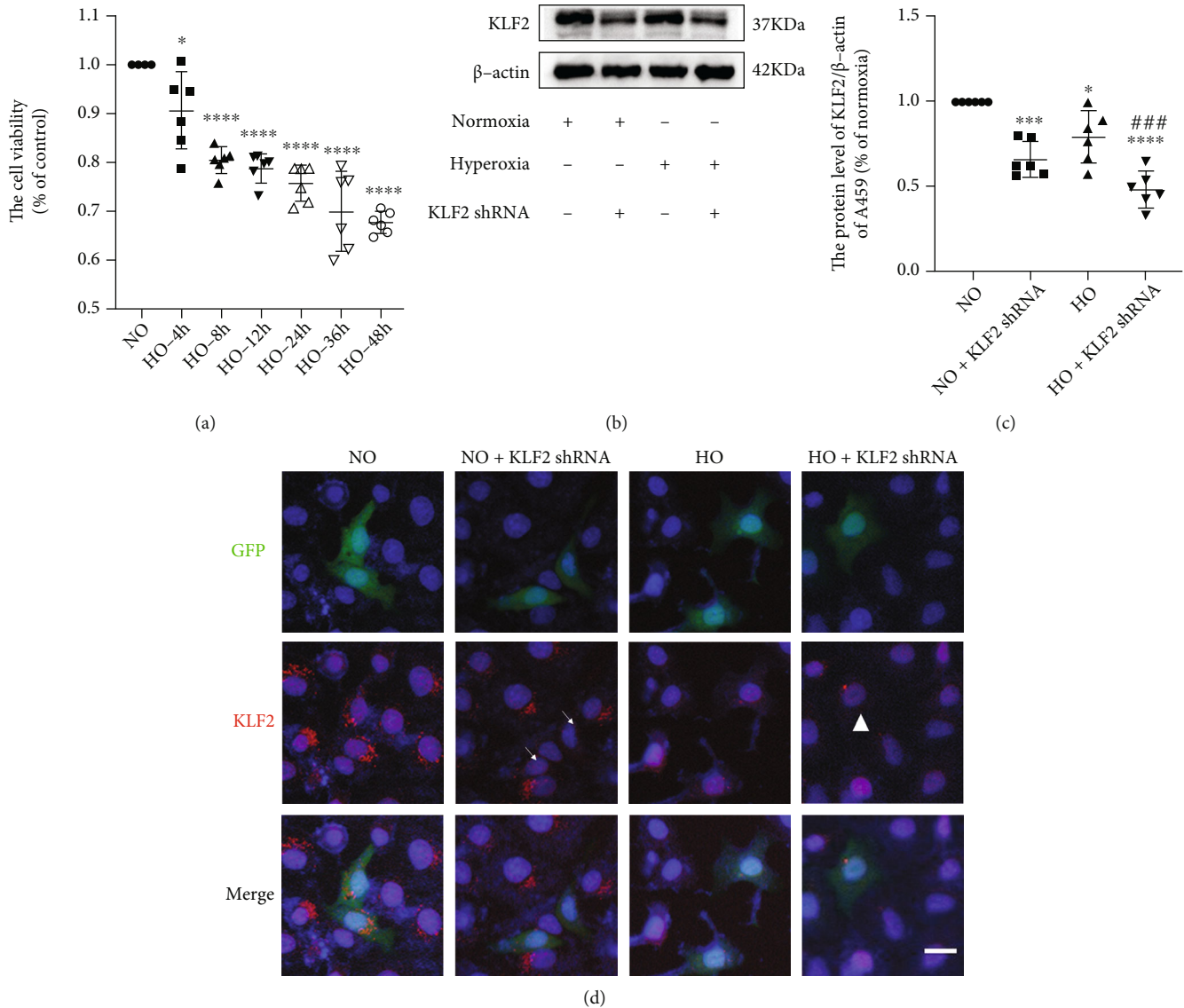


FIGURE 5: Hyperoxia and silencing of KLF2 significantly reduced KLF2 expression. (a) A549 cells were treated from 4 to 48 h, and cell viability was assessed using CCK8,  $n = 6$ , analyzed by one-way ANOVA followed by Tukey's post hoc test,  $*P < 0.05$  and  $****P < 0.0001$  versus the normoxia group. (b) Western blotting results of KLF2 in A549 cells. (c) Analyses of KLF2 expression (normalized to  $\beta$ -actin),  $n = 6$ , analyzed by two-way ANOVA with the Bonferroni post hoc test,  $*P < 0.05$ ,  $***P < 0.001$ , and  $****P < 0.0001$  versus the normoxia group;  $###P < 0.001$  versus the hyperoxia group. (d) IF staining images of KLF2 in each group (microscopy,  $\times 400$ ). Scale bars = 25  $\mu$ m.

elevation of IL-6 and TNF- $\alpha$  but this therapeutic effect of simvastatin was abolished by KLF2 silencing.

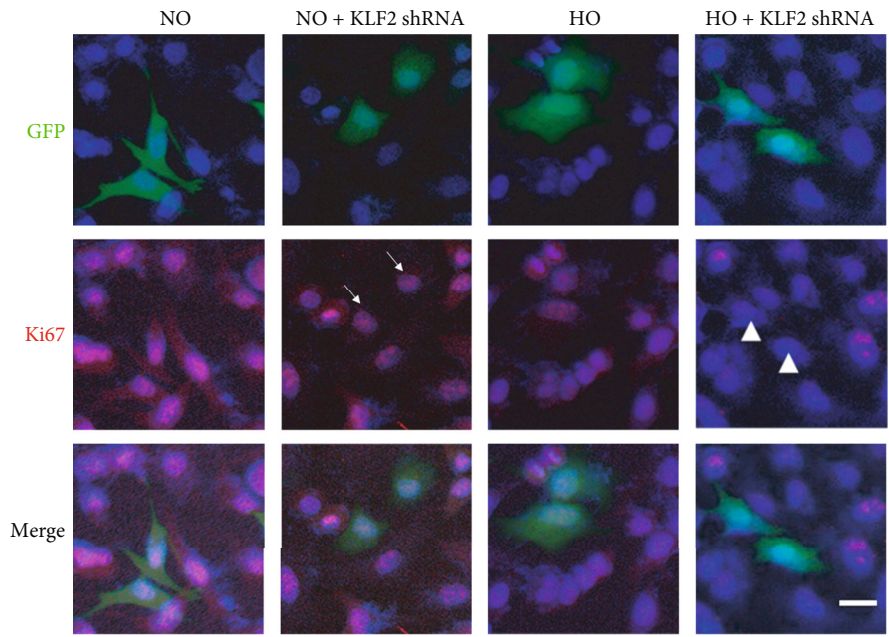
#### 4. Discussion

Bronchopulmonary dysplasia (BPD) is a common complication of premature birth [38]. Exposure to high-oxygen concentrations has been demonstrated to cause simplified lung development, leading to BPD [39]. Modelling BPD induced by high-oxygen levels is the most frequently used model, resulting in arrested lung growth, alveolar simplification, impaired blood vessel development, and abnormal pulmonary function. In this study, we used neonatal SD rats and A549 cells exposed to oxygen for 7 days and 48 hours,

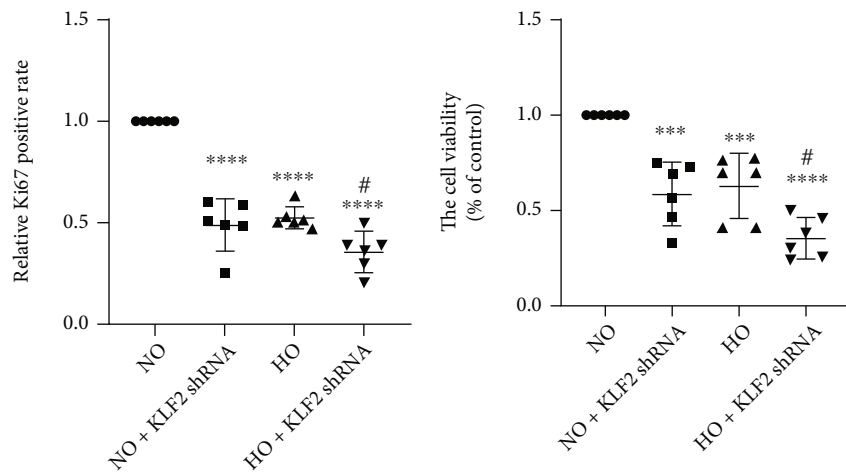
respectively, to establish a model of hyperoxic lung injury *in vivo* and *in vitro*.

Simvastatin is an HMG-CoA reductase inhibitor and is currently used as an anticholesterol drug. In addition, numerous recent studies have revealed that simvastatin has anti-inflammatory, antioxidant, and vascular protective effects [40, 41]. Tulbah et al. believed that anti-inflammatory, immunomodulatory, fibrinolytic, and antithrombotic activities and improvement of endothelial cell function of statins might make them a class of drugs for alternative treatments of chronic lung diseases [42]. Studies have indicated that simvastatin and atorvastatin can improve the health of patients with COPD and asthma by reducing pulmonary artery pressure and inflammatory mediators [43–46]. However, there is no report on whether simvastatin



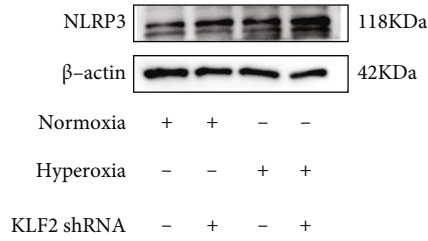


(a)

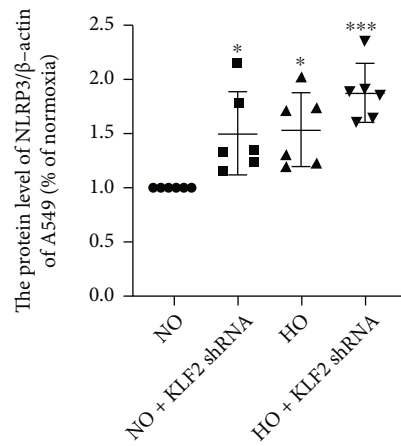


(b)

(c)



(d)



(e)

FIGURE 6: Continued.

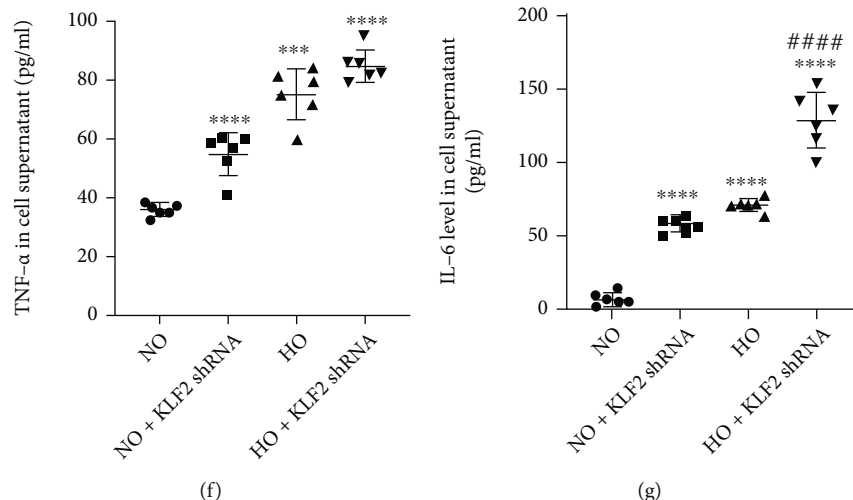


FIGURE 6: KLF2 shRNA reduced the cell viability of A549 and increased the expression of NLRP3 protein and inflammatory cytokines. (a) IF staining images of Ki67 in each group (microscopy,  $\times 400$ ). Scale bars =  $25 \mu\text{m}$ . (b) Quantitative analysis of Ki67<sup>+</sup> cells of A549 cells,  $n = 6$ , analyzed by two-way ANOVA with the Bonferroni post hoc test, \*\*\*\* $P < 0.0001$  versus the normoxia group, # $P < 0.05$  versus the hyperoxia group. (c) Cell viability was determined using CCK8,  $n = 6$ , analyzed by two-way ANOVA with the Bonferroni post hoc test, \*\*\* $P < 0.001$  and \*\*\*\* $P < 0.0001$  versus the normoxia group, # $P < 0.05$  versus the hyperoxia group. (d) Western blotting results of NLRP3 in A549 cells. (e) Analyses of NLRP3 expression (normalized to  $\beta$ -actin).  $n = 6$ , analyzed by two-way ANOVA with the Bonferroni post hoc test, \* $P < 0.05$  and \*\*\* $P < 0.001$  versus the normoxia group. (f–g) TNF- $\alpha$  and IL-6 levels in cells of different groups.  $n = 6$ , analyzed by two-way ANOVA with the Bonferroni post hoc test, \*\*\* $P < 0.001$  and \*\*\*\* $P < 0.0001$  versus the normoxia group. #### $P < 0.0001$  versus the hyperoxia group.

has anti-inflammatory or antioxidant effects on hyperoxia lung injury. Here, we investigated the potential protective mechanism of simvastatin in hyperoxia lung injury through *in vivo* and *in vitro* models and explored its possible mechanism. Simvastatin treatment could improve alveolar simplification caused by hyperoxia in neonatal rats while also decreasing oxidative stress and inflammation. In A549 cells exposed to high oxygen *in vitro*, simvastatin could promote cell survival and reduce inflammation. It is worth mentioning that we found that simvastatin higher than  $20 \mu\text{M}$  could inhibit the proliferation of A549 cells. This may be linked to the antiproliferative effect of simvastatin in lung diseases and its potential to treat lung cancer. Shang et al. found that simvastatin can inhibit the extracellular signal-regulated kinase (ERK) pathway, downregulate the expression of tumor necrosis factor  $\beta$  (TNF- $\beta$ ) receptor II, and inhibit the proliferation of A549 cells [47].

To further explore BPD pathogenesis and the possible mechanism of simvastatin, we examined the role of KLF2 in a hyperoxic lung injury model. Krüppel-like factor 2 (KLF2), alternatively referred to as lung KLF, is implicated in many biological processes, including inflammation [48]. KLF2 gene deletion can cause vascular maturation disorder and abnormal lung development in mice [22, 49]. KLF2 expression has been significantly reduced in many lung diseases, such as influenza virus and lipopolysaccharide-induced ALI, and KLF2 overexpression can significantly improve ALI [22]. Using *in vivo* and *in vitro* models, we investigated the protective effect of KLF2 on hyperoxic lung injury models and its potential mechanisms. Our results indicate that KLF2 expression was significantly decreased in lung tissues and A549 cells following hyperoxia exposure. Furthermore, we discovered that silencing KLF2 gene

expression using KLF2 shRNA plasmid not only reduced cell activity under normal oxygen conditions but also aggravated hyperoxia-caused cell damage. In addition, KLF2 shRNA could increase NLRP3 and inflammatory cytokine expression. Therefore, our results suggest that KLF2 plays an important protective role in hyperoxia-induced lung injury.

Multiple evidences indicate that simvastatin is a strong inducer of KLF2 [30, 50, 51]. Statins can directly induce KLF2 expression by binding to the MEF2 transcription factor in the promoter region of KLF2 [52, 53]. To further confirm whether the protective effect of simvastatin against hyperoxia-induced lung injury is through the KLF2 pathway, we included the HS+KLF2 shRNA group in the *in vitro* experiment. Our study stated that after silencing the KLF2 gene during simvastatin treatment, the anti-inflammatory impact of simvastatin was reversed. The disadvantage is that we have only proved during *in vitro* experiments that simvastatin exerts its anti-inflammatory effect through the KLF2 pathway and we have not investigated whether the antioxidant effect of simvastatin is also correlated with KLF2. Simvastatin has been demonstrated to improve endothelial function by its antioxidant effect in many studies, possibly linked to inhibiting the mevalonic acid (MVA) pathway and isoprenoid synthases and deactivating nicotinamide adenine dinucleotide phosphate (NADPH) [54–56]. *In vivo* and *in vitro* experimental studies have demonstrated that simvastatin could reduce oxidative stress in many lung diseases by inhibiting the GTPases (Rac) pathway and inactivating the NADPH oxidase system at a cellular level, reducing ROS generation [54].

Although the results of this study indicate that simvastatin protects neonatal rats with hyperoxic lung injury, its clinical application and BPD should be further discussed.

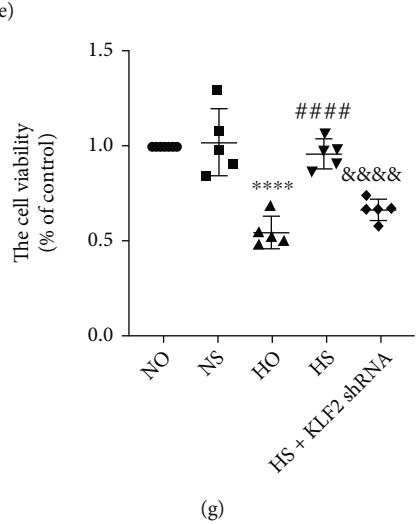
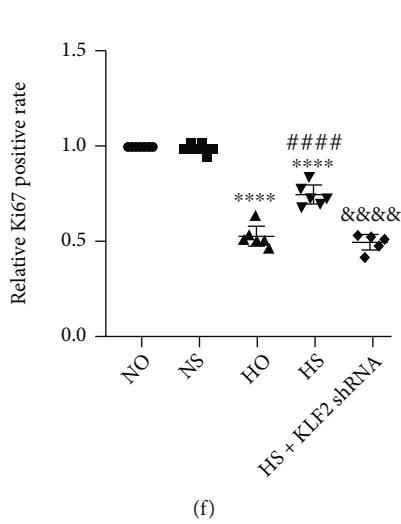
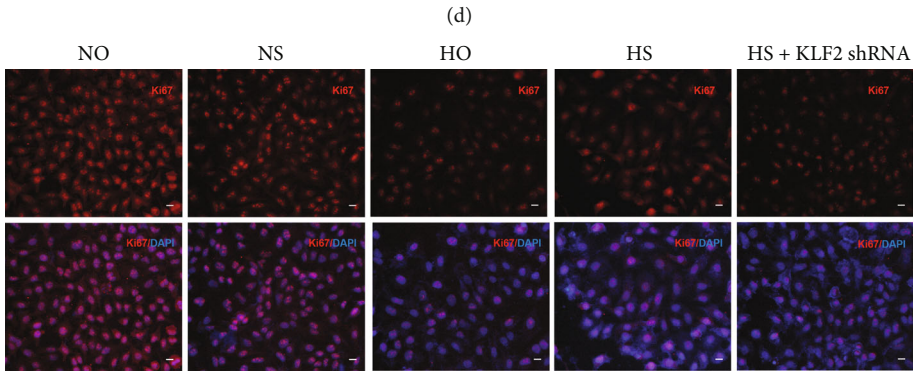
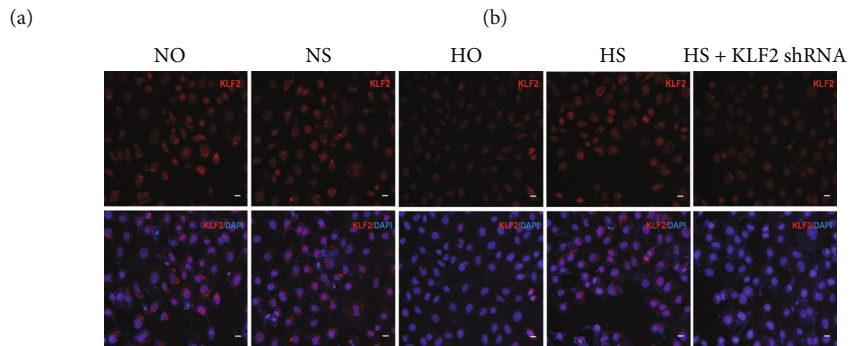
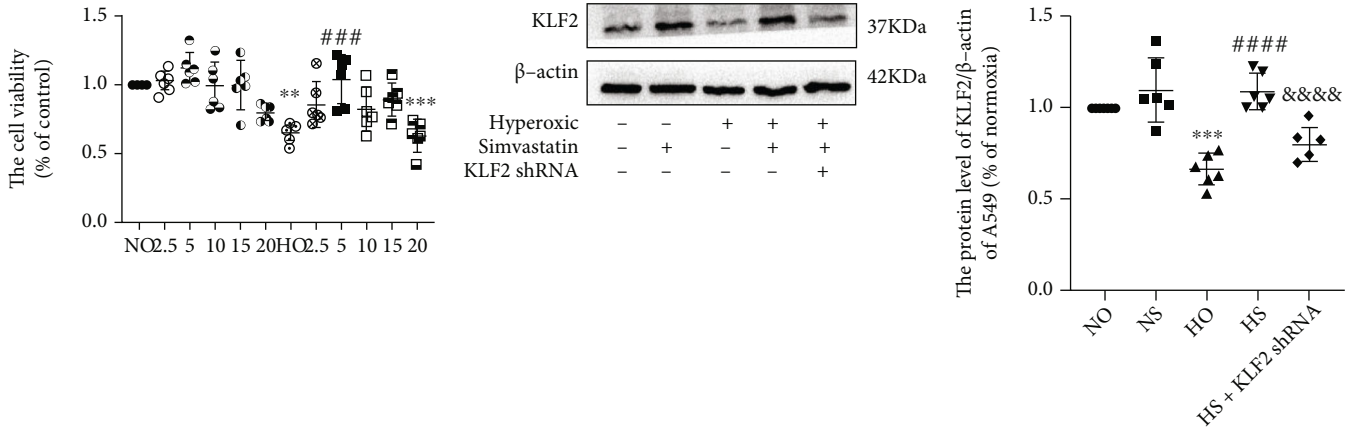


FIGURE 7: Continued.

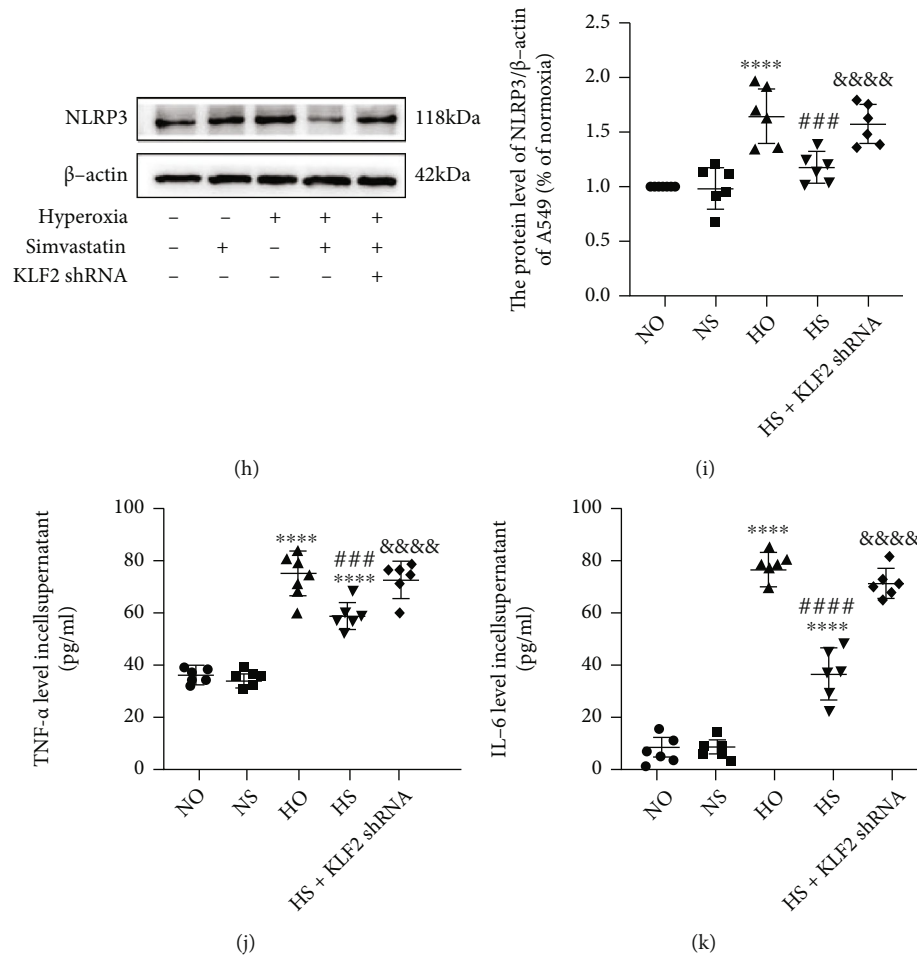


FIGURE 7: KLF2 shRNA could significantly block the protective effect of simvastatin. (a) The cell viability of different simvastatin concentrations,  $n = 6$ , analyzed by two-way ANOVA with the Bonferroni post hoc test,  $**P < 0.01$  and  $***P < 0.001$  versus the normoxia group,  $###P < 0.001$  versus the hyperoxia group. (b) The protein level of KLF2 was evaluated by Western blotting in A549 cells. (c) Analyses of KLF2 expression (normalized to  $\beta$ -actin),  $n = 6$ , analyzed by two-way ANOVA with the Bonferroni post hoc test,  $***P < 0.001$  versus the normoxia group,  $####P < 0.0001$  versus the hyperoxia group,  $#####P < 0.0001$  versus the hyperoxia + simvastatin (HS) group. (d, e) IF staining images of Ki67 and KLF2 in each group (microscopy,  $\times 400$ ). Scale bars =  $25 \mu\text{m}$ . (f) Quantitative analysis of Ki67 $^{+}$  cells of A549 cells,  $n = 6$ , analyzed by two-way ANOVA with the Bonferroni post hoc test,  $****P < 0.0001$  versus the normoxia group,  $#####P < 0.0001$  versus the hyperoxia group,  $#####P < 0.0001$  versus the hyperoxia + simvastatin group (HS). (g) The cell viability of different groups,  $n = 5$ , analyzed by two-way ANOVA with the Bonferroni post hoc test,  $****P < 0.0001$  versus the normoxia group,  $#####P < 0.0001$  versus the hyperoxia group,  $#####P < 0.0001$  versus the hyperoxia + simvastatin group (HS). (h) The protein level of NLRP3 was evaluated using Western blotting in A549 cells. (i) Analyses of NLRP3 expression (normalized to  $\beta$ -actin),  $n = 6$ , analyzed by two-way ANOVA with the Bonferroni post hoc test,  $****P < 0.0001$  versus the normoxia group,  $###P < 0.001$  versus the hyperoxia group,  $#####P < 0.0001$  versus the hyperoxia + simvastatin (HS) group. (j, k) TNF- $\alpha$  and IL-6 levels in cells of different groups,  $n = 6$ , analyzed by two-way ANOVA with the Bonferroni post hoc test,  $****P < 0.0001$  versus the normoxia group;  $###P < 0.001$  and  $####P < 0.0001$  versus the hyperoxia group;  $#####P < 0.0001$  versus the hyperoxia + simvastatin group.

For most people, statins are safe and well tolerated. However, 30% of statins have been associated with intolerance, including the most prevalent muscle and liver toxicities [57]. The strengthening of childhood hypercholesterolemia screening has increased the use of statins in children [58]. In 2010, Cochrane systematic review evaluated the safety of statins in children. Compared with the control group, simvastatin was not found to have significant liver toxicity [58]. However, its long-term safety at this age has not been effectively confirmed. In addition, because statins have not been used clinically in neonates and the use of simvastatin in BPD may inhibit cholesterol synthesis in premature infants

[59, 60], so, the application of simvastatin in neonatal bronchopulmonary dysplasia warrants additional investigation.

Our study has some limitations. (i) Because we only examined the effect of silencing KLF2 on hyperoxic lung injury and the therapeutic impact of simvastatin *in vitro*, *in vivo* inhibition of KLF2 should be included in future studies. (ii) Although this study found that knocking down KLF2 at the cellular level decreased alveolar epithelial cell activity and elevated inflammatory cytokines, we did not investigate whether KLF2 overexpression was protective. (iii) We have only employed *in vitro* experiments to confirm that simvastatin exerts its anti-inflammatory effect through the KLF2



pathway, but we did not investigate whether the antioxidant effect of simvastatin is also linked to KLF2. (iv) We selected the A549 cell line for *in vitro* experiments. Although it is a human-derived cell, the A549 cell line is derived from a 58-year-old male lung cancer cell. It may be different from neonatal lung epithelial cells, and additional research is required to rule out the effect of simvastatin on lung cancer.

## 5. Conclusion

In summary, this study demonstrated that simvastatin could ameliorate lung injury following hyperoxia exposure by stimulating KLF2 expression and suppressing NLRP3 inflammasome formation. While future clinical applications of simvastatin require additional research, it may be a promising treatment for BPD.

## Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

## Ethical Approval

All Sprague-Dawley rats used in animal experiments were obtained from the Animal Center of the Chinese Academy of Sciences (Shanghai, China), following the Guidelines for the Use of Experimental Animal Care issued by the National Institutes of Health and approved by the Experimental Ethics Committee of Wenzhou Medical University.

## Conflicts of Interest

The authors declare that they have no conflict of interest.

## Authors' Contributions

X.W., S.C., and X.F. conceived and designed the experiments; X.W., R.H., and Z.L. performed the experiments and wrote the paper; C.X., R.H., and T.C. analyzed the data; J.L., L.L., W.L., B.P., Q.W., and L.D. contributed materials and analysis tools; S.C. and X.F. edited and approved final draft. All authors contributed to the article and approved the submitted version.

## Acknowledgments

This study was funded by the Natural Science Foundation of Zhejiang (LY21H040007) and Wenzhou Science and Technology Project (Y20180092 and Y20180006).

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