Research Article

Regulatory Effects of Nur77 on Airway Remodeling and ASMC Proliferation in House Dust Mite-Induced Asthma

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Airway remodeling played a vital role in the development of asthma, and airway smooth muscle (ASM) mass was its hallmark. However, few strategies targeting ASM remodeling were developed in treating asthma. Nur77 was the transcription factor nuclear receptor involved in the pathogenesis of several lung diseases. Nur77 distribution and expression were determined in an HDM-mediated allergic asthma model. Its effect on airway hyperresponsiveness (AHR), chronic inflammation, and ASM remodeling in asthmatic mice was evaluated using a lentivirus-mediated shRNA. Possible mechanisms were explored by examining Nur77 actions and its underlying pathways in primary human AMC cells (ASMCs). In this study, we reported that Nur77 expression was mainly distributed along ASM and increased in lungs of HDM-challenged mice. Nur77 depletion by lentivirus-mediated shRNA ameliorated AHR, chronic inflammation, goblet cell hyperplasia, and airway remodeling in the asthmatic mouse model. By means of primary human ASMC, we discovered that Nur77 upregulation by HDM stimulation promoted cell proliferation and ROS production, as well as reduced antioxidant gene expression. These alterations might associate with MFN2/MAPK/AKT pathways. These findings broadened our understanding of airway remodeling and ASMC proliferation, which might provide a novel therapeutic target for asthma patients.

1. Introduction

Bronchial asthma (or asthma) is a common chronic respiratory disease with features of inflammatory cell activation, reversible obstruction, hyperresponsiveness, and remodeling of the airway. It is sweeping the world, and nearly 25 million people suffer from the disease, which is also responsible for over 5000 deaths per year [1, 2]. Airway remodeling plays a pivotal role in the natural history of asthma [3]. It is characterized by thickened epithelium with goblet cell hyperplasia, more fibrotic airway smooth muscle (ASM) layer with increased cell numbers, and altered extracellular matrix (ECM) deposition [4]. These structural changes subsequently lead to progressive lung function decline and irreversible airway obstruction despite optimal treatments [5]. Airway remodeling also hinders the response to bronchodilator and aggravates airway hyperresponsiveness [6]. In spite of great efforts to alleviate airway remodeling in the last decades, few therapeutic options have proved effective to restrict or reverse this

progressive condition. Accordingly, further research on cellular and molecular mechanisms that modulate airway remodeling may offer novel therapeutic strategies in asthma management.

The increased airway smooth muscle mass is a hallmark of airway remodeling [7]. Hyperplasia (cell proliferation) and hypertrophy (cell volume increase) of airway smooth muscle cells (ASMCs) contribute to the reduced airway lumen. Besides, ASMCs are biologically active in modulating airway remodeling by generating reactive oxygen species (ROS) and producing ECM proteins, including cytokines, matrix metalloproteinases (MMPs), and growth factors as well as angiogenic factors (e.g., vascular endothelial growth factor (VEGF)) in response to extrinsic detrimental factors [4]. As such, researches on asthma therapies targeting ASM followed by controlling bronchoconstriction should be of primary importance. Ablation of excessive ASM has been proved beneficial to refractory asthma in a series of clinical trials [8–11]. Accordingly, it is of great necessary to clarify novel molecular mechanism underlying ASMC states and further optimize their response in the face of allergens. Such understanding may provide opportunity to develop new solutions of the unmet needs in airway remodeling and asthma cure.

The transcription factor nuclear receptor 77 (Nur77) belonged to NR4A subfamily of nuclear hormone receptors and was a key regulator involved in the pathogenesis of several lung diseases including asthma, acute lung injury, and pulmonary fibrosis [12]. Its expression and activation were induced by various physiological and pathological stimuli (e.g., innate immune activation, tissue injury, and hypoxia) and was able to modulate the transcription of target genes involved in inflammation, oxidative stress, cell survival, and metabolism as well as autophagy [13, 14]. Several lines of evidence suggested that Nur77 protected airway epithelium against overwhelming inflammatory injury, therefore exhibiting beneficial effect in inflammation-related lung diseases, such as asthma [15, 16]. Other investigation indicated that Nur77 accelerated airway epithelium injury by inducing cellular apoptosis under ozone exposure [17]. More recently, Nur77 has been validated to inhibit the proliferation of pulmonary artery smooth muscle, thus displaying promising therapeutic efficacy for the treatment of pulmonary arterial hypertension (PAH) [18, 19]. However, researches focusing on Nur77's role in asthma pathogenesis were currently incomplete, since its influence on the disorder varied with the inducing agents and distinct cell type. Besides, the exact effect of Nur77 on airway hyperresponsiveness (AHR), one of the most important *in vivo* procedures for pulmonary mechanics in asthma research, has never been considered as far as we knew.

In the present study, we examined, for the first time, that Nur77 was expressed mainly along smooth muscle and upregulated by HDM in a murine model of asthma. Furthermore, we systematically investigated the role of Nur77 in AHR and airway remodeling *in vivo*. Possible mechanisms were explored by evaluating Nur77 actions and its underlying pathways in primary human ASMC.

2. Materials and Methods

Additional details are provided in the Supplementary Material—Methods.

2.1. Establishment of HDM-Induced Asthma Murine Model.
Specific-pathogen-free female C57BL/6 mice, aged 6-8 weeks, were purchased from Shanghai Laboratory Animal Co. Ltd, China. All mice model experiments were conducted under the protocols of the Shanghai Committee for Accreditation of Laboratory Animal and were approved by Shanghai General Hospital Institutional Review Board (Permit Number: 2018KY201) (Shanghai, China). All the efforts were made to minimize mouse suffering.

The asthmatic murine model was induced by HDM according to the previous article [20]. Specifically, mice were randomly divided into 4 groups: control, HDM extracts (Greer Laboratories, XPB70D3A2.5, Lenoir, NC), Vehicle +HDM, and shRNA+HDM. Equal amounts (10^7 U per mouse) of recombinant lentiviral vectors of shRNA targeting Nur77 or negative control shRNA (vehicles) (Zorin, Shanghai, China) were given intranasally 48 hrs before HDM challenge. The mice were treated with 50 μL of 100 μg HDM extract or equal volume of sterile normal saline to the trachea with a micropipette 3 times per week for consecutive 4 weeks (Figures 1(a) and 2(a)). The target sequence of Nur77 shRNA was CATGTGCCTTTAAGCCTATAG.

2.2. Measurement of Airway Hyperresponsiveness (AHR).
The development of AHR was evaluated by increased airway resistance in response to various concentrations of acetylcholine (Ach) (0, 8, 16, 32, 64, 128, and 256 mg/mL for 10 μL per mouse). After anesthesia with an intraperitoneal injection of 0.2 mL 1% sodium pentobarbital, mice were tracheostomized and airway resistance was recorded using a whole-body plethysmograph chamber (EMMS, Hants, UK).

2.3. Bronchoalveolar Lavage Fluid (BALF) Acquisition and Analysis.
Details were provided in the Supplementary Material—Bronchoalveolar lavage fluid (BALF) acquisition and analysis.

2.4. Histological Analysis of Lung Tissues.
Lung sections were performed with hematoxylin-eosin (H&E), Periodic Acid-Schiff (PAS), and Masson's trichrome staining, as well as immunohistochemical staining for Nur77, α-SMA, and MMP-9. Details were provided in the Supplementary Material—Histological analysis of lung tissues.

2.5. Cell Culture and Lentivirus Transduction.
Normal human airway smooth muscle cells (ASMCs) were purchased from PromoCell (Heidelberg, Germany). Recombinant lentiviral vector of Nur77 shRNA was designed and constructed by Zorin (Shanghai, China). The target sequence was TGGTGAAGGAAGTTGTCCGAA. ASMCs were infected with 10^7 TU/mL (MOI = 10) lentivirus-mediated shRNA (Nur77i) or negative control shRNA (NEGi), respectively, for 12 hrs. The cells were collected to determine their interference efficiency by reverse transcription-polymerase chain reaction (RT-PCR) and western blotting (WB) assay. Details
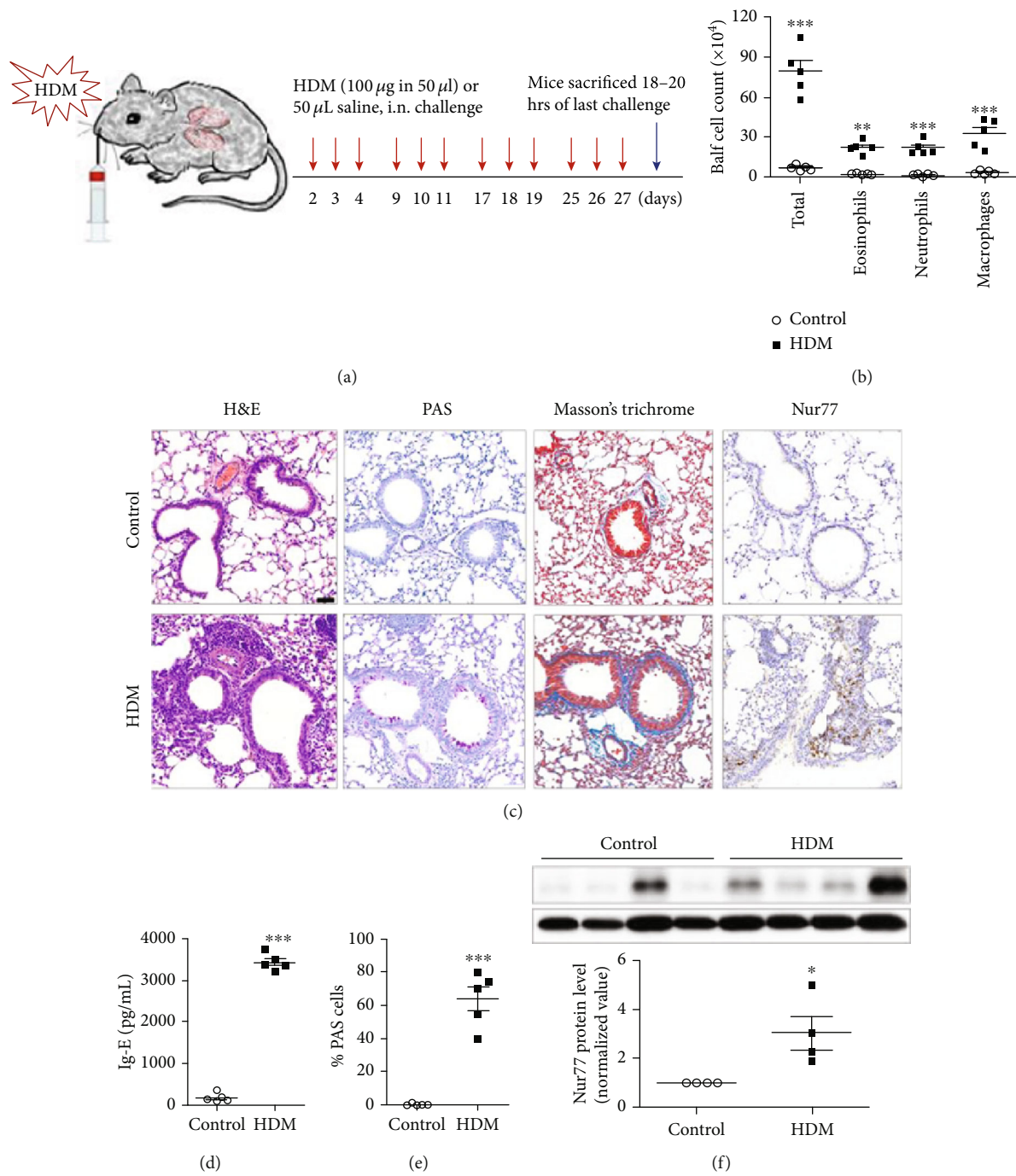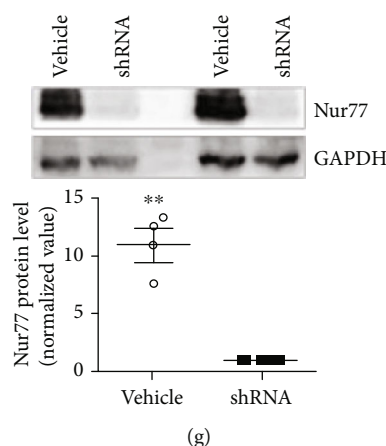
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FIGURE 1: HDM exposure increased lung Nur77 expression in chronic asthma model. (a) The asthmatic mouse model was induced with 50 μL of 100 μg HDM extract to the trachea 3 times per week for consecutive 4 weeks; mice were sacrificed 18-20 hrs after the last HDM exposure for further analysis. (b, d) BALF were collected for detecting the counts of total inflammatory cell, eosinophils, neutrophil, and macrophage (b) as well as Ig-E (d) production. (c) Lung sections were stained with H&E to evaluate leukocyte infiltration (first column), PAS to analyze goblet cell hyperplasia (second column), Masson's trichrome to assess the collagen deposition and fibrosis (third column), and immunohistochemical staining for Nur77 to determine its distribution and expression (fourth column); scale bar for all images: 50 μm. (e) Quantitative analysis of mucus production in lung sections. (f, g) WB assay of Nur77 level in lung tissue affected by HDM challenge and shRNA interfere. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

are shown in the Supplementary Material—Cell culture and lentivirus transduction.

2.6. In Vitro Investigation of ASMC Proliferation. The effect of Nur77 expression on ASMC proliferation was assessed by Cell Counting Kit-8 (CCK-8) and 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay. Details are shown in the Supplementary Material—In vitro investigation of ASMC proliferation.

2.7. Cell Cycle Detection. Cell cycle of ASMC was detected by propidium iodide (PI) staining. Details are shown in the Supplementary Material—Cell cycle detection.

2.8. Western Blotting (WB) Analysis. Details are shown in the Supplementary Material—Western blotting (WB) analysis.

2.9. Detection of Intracellular Reactive Oxygen Species (ROS). Intracellular ROS was detected using DCFH-DA fluorescent probe. Details are shown in the Supplementary Material—Detection of intracellular reactive oxygen species (ROS).

2.10. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Assay. Details are shown in the Supplementary Material—Reverse transcription-polymerase chain reaction (RT-PCR) assay.

2.11. Statistical Analysis. The results were analyzed using the GraphPad Prism software (version7; GraphPad Software, Inc., San Diego, CA). Results were analyzed using one-way ANOVA and Bonferroni's post hoc test (for equal variance) or Dunnett's T3 post hoc test (for unequal variance). Quantitative data was described as mean ± standard error (± SEM). All experiments were performed independently

for at least 3 times. The difference was considered statistically significant when $p < 0.05$.

3. Results

3.1. Increased Lung Nur77 Expression in HDM-Induced Chronic Asthma Model. We adopted a classical mice model of chronic asthma induced by HDM, and the diagram of the protocol was summarized in Figure 1(a). After 4 weeks of HDM challenge, the numbers of total inflammatory cell, eosinophil, neutrophil, and macrophage in BALF was significantly elevated (Figure 1(b)). As a hallmark of asthma, the level of airway Ig-E was also enhanced by HDM inhalation (Figure 1(d)). Additionally, the lung tissue histopathology demonstrated that HDM-treated mice developed prominent airway inflammation manifested by leukocytes gathered around terminal bronchioles and capillary vessels (Figure 1(c)). As shown in Figures 1(c) and 1(e), remarkable goblet cell hyperplasia and mucus hypersecretion were viewed in bronchioles of asthmatic mice. Meanwhile, collagen deposition and fibrosis were also enhanced at the interstitium of airways and vessels in asthmatic mice (Figure 1(c)). These alterations suggested chronic pulmonary inflammation and airway remodeling in the experimental asthma model upon HDM exposure.

Immunohistochemistry results showed that Nur77 was mainly distributed in smooth muscle cells around airway and vessels. Its expression in lung tissue was significantly increased in 4-week HDM-inhaled mice compared with the control group (Figures 1(c) and 1(f)). To determine the biological function of Nur77 in chronic asthma, a lentiviral vector of shRNA targeting Nur77 or vehicle was constructed and transfected into C57BL/6 mice through nasal instillation to knock down the lung Nur77 expression. None of the mice died during the lentivirus transfection experiment.
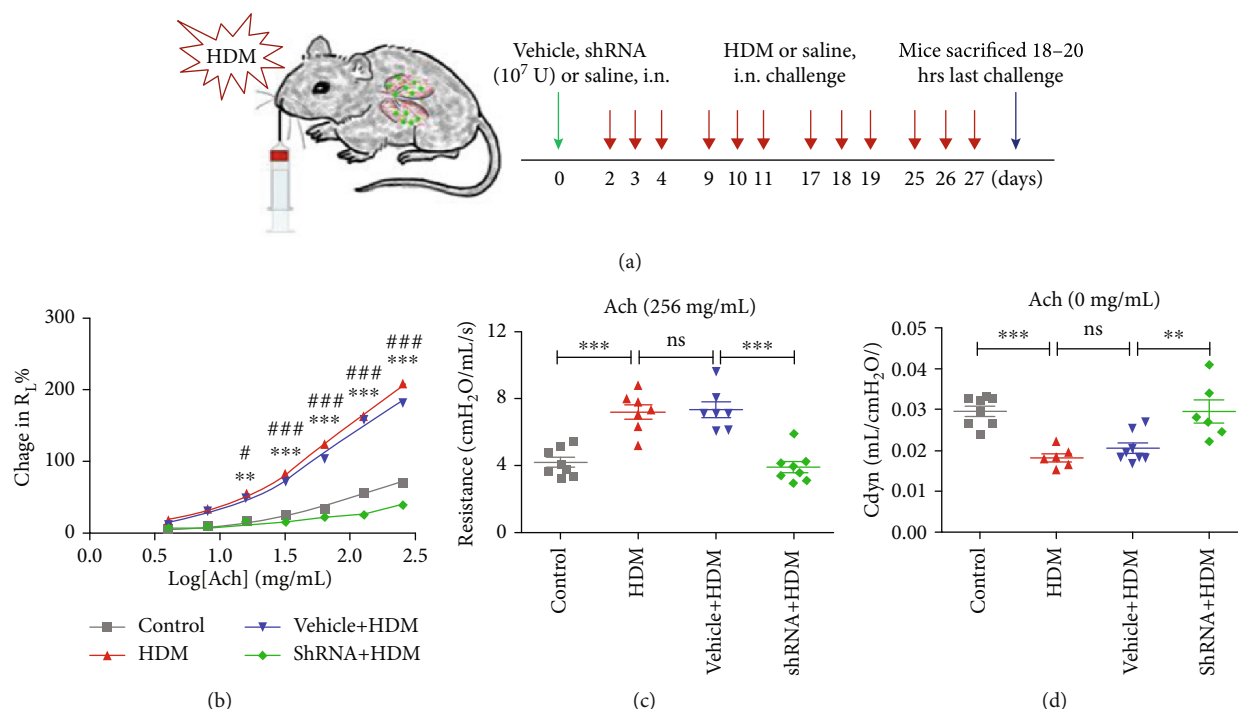
FIGURE 2: Analysis of airway obstruction in HDM-induced experimental asthma. (a) The asthmatic mouse model with lentiviral vector of shRNA or vehicle pretreatments (10^7 U per mouse) intranasally (i.n.) 48 h before HDM challenge; further experiments were conducted 18-20 hrs after the last HDM exposure. (b–d) AHR (b), airway resistance under high concentration of acetylcholine (Ach) (c), and baseline compliance (Cdyn) (d) in response to Ach measured in saline-challenged, HDM-challenged, vehicle-pretreated HDM-challenged, and shRNA-pretreated HDM-challenged mice. (b) $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$: control group compared with HDM-challenged group; $^{**}p < 0.01$, $^{***}p < 0.001$: vehicle-pretreated HDM-challenged group compared with shRNA-pretreated HDM-challenged group. (c, d) $^{**}p < 0.01$ and $^{***}p < 0.001$. ns: not significant.

Moreover, WB analysis demonstrated that Nur77 level in lung tissue was dramatically decreased at 48 hrs after lentiviral vector administration (Figure 1(g)).

3.2. Nur77 Deficiency Protected Mice from AHR and Airway Resistance in HDM-Induced Experimental Asthma.

Since Nur77 expression was increased by chronic HDM exposure, we anticipated that it might be important in the formation and development of asthma. To test this hypothesis, mice were treated with lentiviral vector of shRNA or vehicle intranasally, followed by the first challenge with HDM. The analyses were performed 18-20 h after the last HDM administration (Figure 2(a)).

Airway resistance and dynamic pulmonary compliance (Cdyn) were vital physiological indicators for asthma and airway remodeling. HDM-challenged mice revealed AHR in comparison with saline-challenged ones. Acetylcholine (Ach) reactivity was presented as the percentage increase in airway resistance over baseline reactivity in the absence of cholinergic stimuli. Our experiments showed that Ach reactivity was increased (Figures 2(b) and 2(c)) while baseline Cdyn was decreased (Figure 2(d)) in HDM-challenged mice compared to normal saline-treated mice. Furthermore, loss of Nur77 with lentiviral vector of shRNA improved AHR (Figures 2(b) and 2(c)) and baseline Cdyn (Figure 2(d)), which bring them back to the values measured in control mice. The results suggested that pulmonary Nur77 upregula-

tion contributed to allergen-induced AHR and deteriorated lung compliance in the experimental asthma model.

3.3. Nur77 Depletion Diminished Chronic Inflammation and Airway Remodeling in HDM-Induced Asthma Model.

We next assessed the effect of Nur77 expression on airway inflammation, goblet cell hyperplasia, and collagen deposition/fibrosis in asthma model. As shown in Figure 3(a), Nur77 depletion decreased the counts of BALF total cells and leukocytes (including eosinophil, macrophage, and neutrophil) compared to the vehicle in HDM-challenged mice. Meanwhile, BALF supernatant was used for cytokine detection by Luminex-based Procarta custom 10-plex assay. As key factors in asthma pathology, the level of Ig-E and IL-17a was significantly elevated both in HDM and vehicle +HDM groups compared with the control group. However, this elevation could be abolished by Nur77 shRNA administration (Figures 3(b)–3(d)). Other cytokines were little affected by Nur77 in the asthmatic model (Fig. S1).

In parallel to BAL analysis, we also estimated the role of Nur77 by means of lung tissue histopathology. It was found that HDM-exposed mice developed obvious inflammatory responses in airways, manifested by excessive leukocytes infiltration over the interstitium of airways and vessels (Figure 3(e)). Pretreatment with Nur77 shRNA markedly suppressed inflammatory cell infiltration, while the vehicle administration did not produce any improvements (Figure 3(e)). Besides, mucus production of goblet cells was
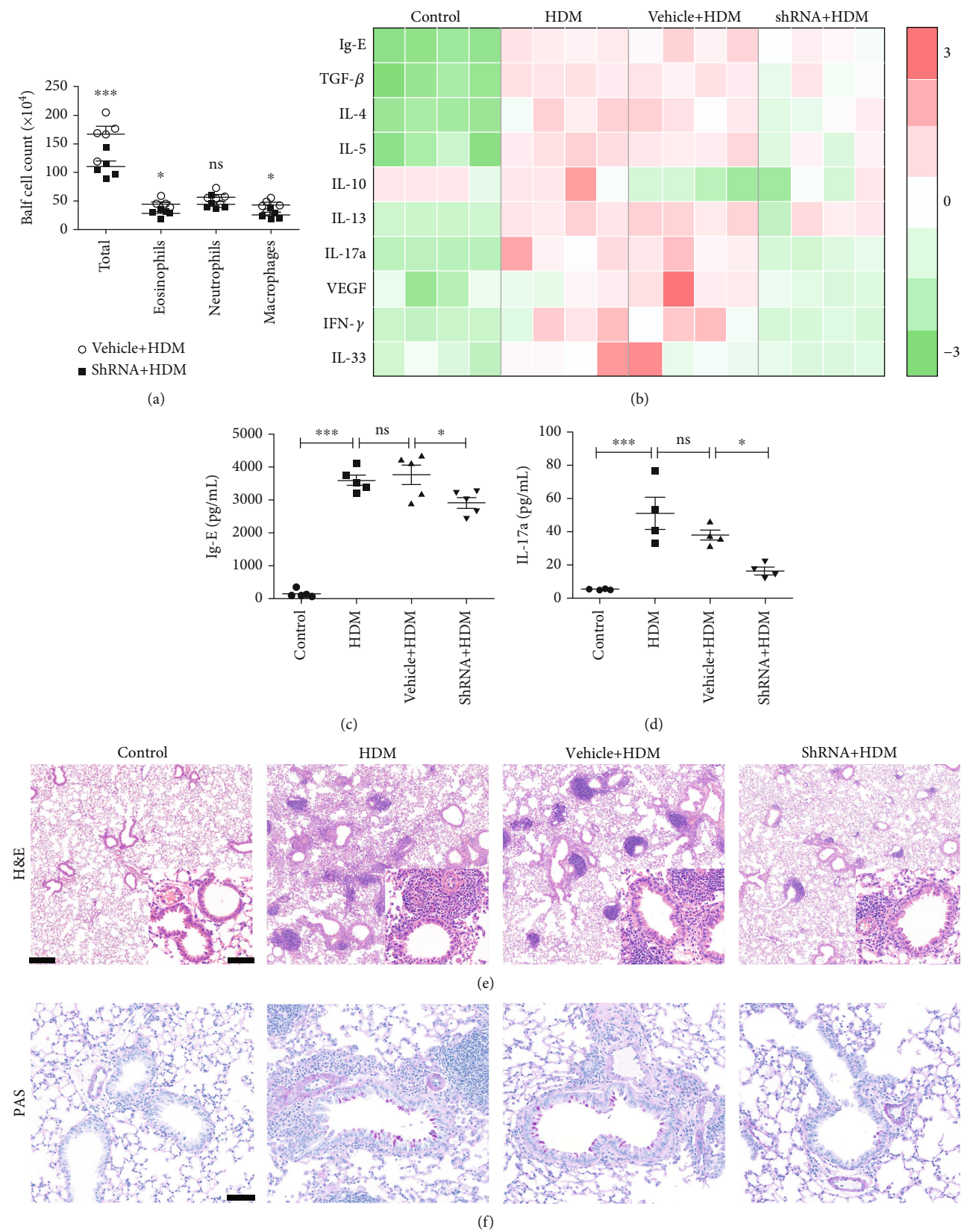
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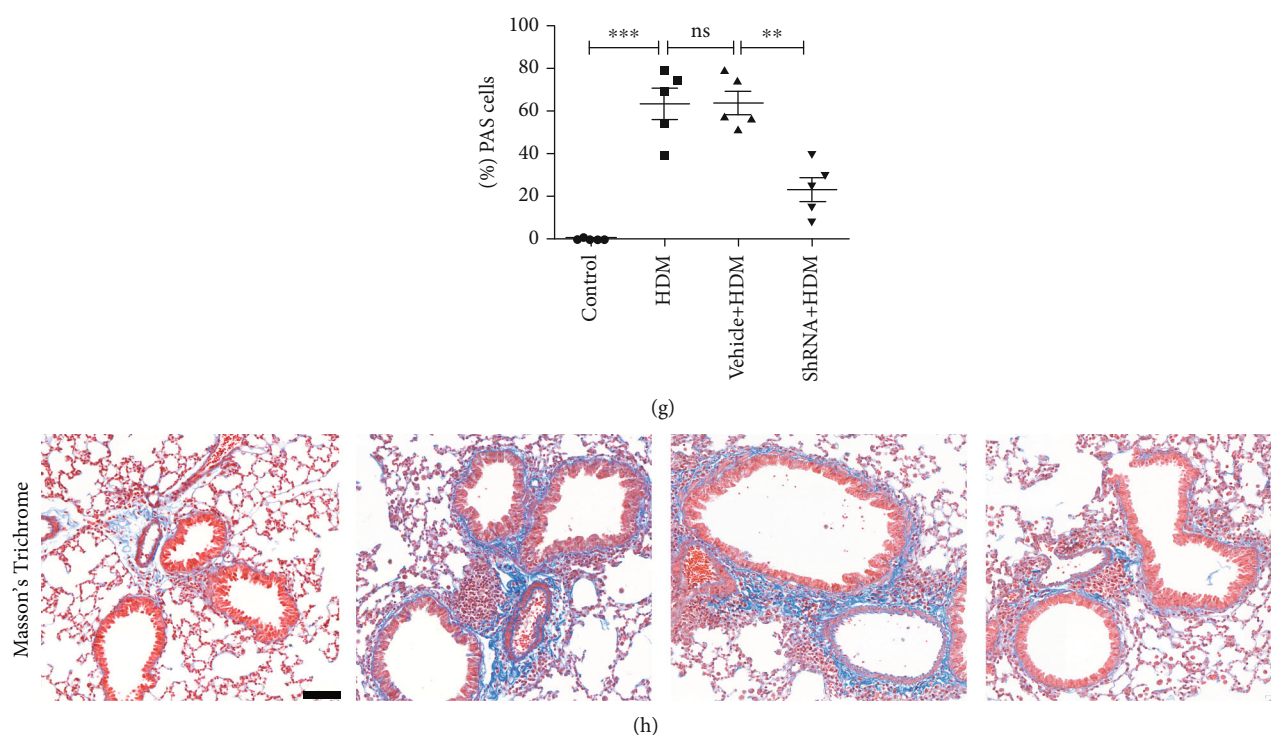

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FIGURE 3: Impact of Nur77 on chronic inflammation and airway remodeling in HDM-induced asthma model. (a) BALF cell counts and their differentiation were determined using a haemocytometer in at least 200 cells. (b) The differential expression profiles of the 10 cytokines. (c, d) BALF were collected for detecting Ig-E (c) and IL-17a (d) production. (e) Lung sections were stained with H&E to evaluate leukocyte infiltration; scale bar: 200 μm (inset: 50 μm). (f) Lung sections were stained with PAS to analyze goblet cell hyperplasia; scale bar: 50 μm. (g) Quantitative analysis of mucus production in lung sections. (h) Lung sections were stained with Masson's trichrome to assess the collagen deposition and fibrosis; scale bar: 50 μm. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. ns: not significant.

evaluated by PAS staining, and collagen deposition/fibrosis extent was assessed by Masson's trichrome stain. As shown in Figures 3(f) and 3(g), HDM-challenged mice showed significant goblet cell hyperplasia and mucus hypersecretion with bronchioles, while Nur77 deficiency halted this pathological change in comparison with the vehicle. Simultaneously, collagen deposition and fibrosis were increased around the respiratory tract and vessels in HDM and vehicle+HDM groups, which could be obviously reversed by Nur77 shRNA pretreatment (Figure 3(h)).

3.4. Nur77 Knockdown Relieved ASM Proliferation in Lung Tissue of Asthmatic Mice.

During the pathogenesis of chronic asthma, smooth muscle actin alpha chain (α-SMA) and MMP-9 were crucial factors involved in bronchoconstrictor responsiveness and collagen deposition in airway walls, thus leading to ASM proliferation and remodeling [21, 22]. Our results showed that α-SMA and MMP-9 staining around the bronchioles were enhanced in the HDM-exposed mice compared to the control group. Nur77 shRNA preadministration dramatically reduced their expression in comparison with vehicle-treated HDM-challenged mice (Figures 4(a)–4(d)). These results could be explained by WB assay of VEGF, which was one of the most important angiogenic factors contributing to AHR and airway obstruction. As shown in Figure 4(e), lung VEGF expression was increased in HDM

group, while Nur77 depletion could abolish this upregulation compared to vehicle+HDM group.

When it came to the underlying pathway, phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling were associated with cell proliferation. Dual serine/threonine kinase (AKT) and extracellular signal-regulated kinase (ERK1/2) pathways were able to control ASMC proliferation process. In this study, we also observed enhanced phosphorylation of AKT and ERK1/2 in lung tissues of HDM-induced asthmatic mice. When mice were pretreated with lentiviral shRNA silencing of Nur77, the activation of AKT and ERK1/2 was inhibited compared with vehicle administration (Figures 4(e) and 4(f)). These observations altogether implied that Nur77 might exert proproliferative and proremodeling effects in the development of asthma.

3.5. Nur77 Depletion Inhibited HDM-Induced Proliferation of Primary Human ASMC.

In *in vitro* study, we determined the role of Nur77 expression in ASMC proliferation induced by HDM. We applied primary human ASMC, and only the fourth to eighth passage could be used for the follow-up experiments (Figure 5(a)). We demonstrated, for the first time, that 80 μg/mL HDM treatment (24 hrs) resulted in an increase in Nur77 expression (Figure 5(c)), which was in keeping with the *in vivo* data. Nur77 knockdown in ASMC by selective shRNA led to a suppression of its mRNA and
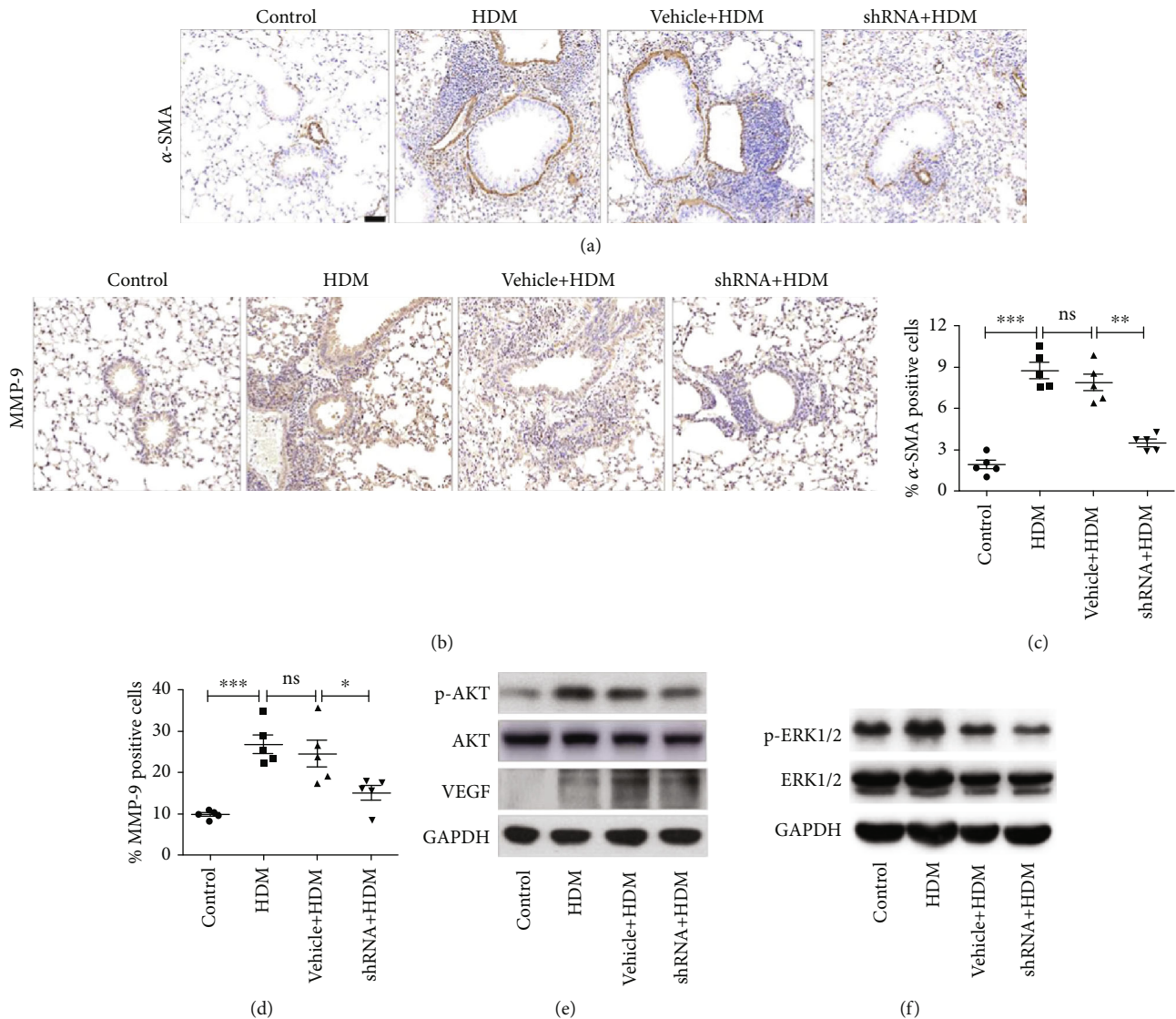
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Figure 4: Effect of Nur77 on ASM proliferation in HDM-induced asthma model. (a, b) Immunohistochemical staining of lung sections was conducted to assess the distribution and expression of α-SMA (a) and MMP-9 (b); scale bar: 50 μm. (c, d) Quantitative analysis of α-SMA (c) and MMP-9 (d) levels in lung tissues. (e, f) WB assay of phosphorylated AKT, total AKT, VEGF (e), phosphorylated ERK1/2, and total ERK1/2 (f) in lung tissues. $^{*}p < 0.05$, $^{**}p < 0.01$, and *** $p < 0.001$. ns: not significant.

protein expression, with shRNA3 (Nur77i) being the most effective (Figures 5(b) and 5(d)).

Afterwards, we observed that ASMC proliferation was significantly accelerated by HDM at the concentration of 40 and 80 μg/mL (Figure 5(e)). Nur77 depletion showed an obvious decrease in cell viability compared with the control shRNA (NEGi) (Figure 5(f)). Besides, by using an EdU incorporation assay, we found that cell count incorporating EdU was distinctly elevated after HDM stimulation when compared with the control group, whereas loss of Nur77 notably attenuated HDM-induced ASMC proliferation (Figures 5(g) and 5(h)). We also discovered reduced phosphorylation of AKT in asthmatic ASMC regulated by Nur77 depletion (Figure 5(i)). Furthermore, Nur77 knockdown arrested HDM-stimulated ASMCs in G1 phase indicating by increased G0/G1 and reduced S-phase population compared with the NEGi group (Fig. S2). These findings were highly

consistent with the *in vivo* experiments and suggested that Nur77 participated in controlling ASMC proliferation under HDM challenge.

3.6. Nur77 Deficiency Reduced ROS Production via Antioxidant Gene Expression in HDM-Stimulated Human ASMC. Since ROS played crucial roles in modulating multiple cell functions including proliferation process, we next determined whether Nur77 affected HDM-induced ROS generation in human ASMC. As shown in Figure 6(a), ROS production was significantly promoted by 80 μg/mL HDM. Cells depleted of Nur77 using Nur77i produced less ROS in response to HDM than those pretreated with NEGi (Figure 6(b)), indicating a prooxidant effect of Nur77 in ASMC.

Underlying ROS generation, the oxidant/antioxidant imbalance was regarded to be a key point in asthma pathogenesis. In the HDM- (80 μg/mL) exposed human ASMC,
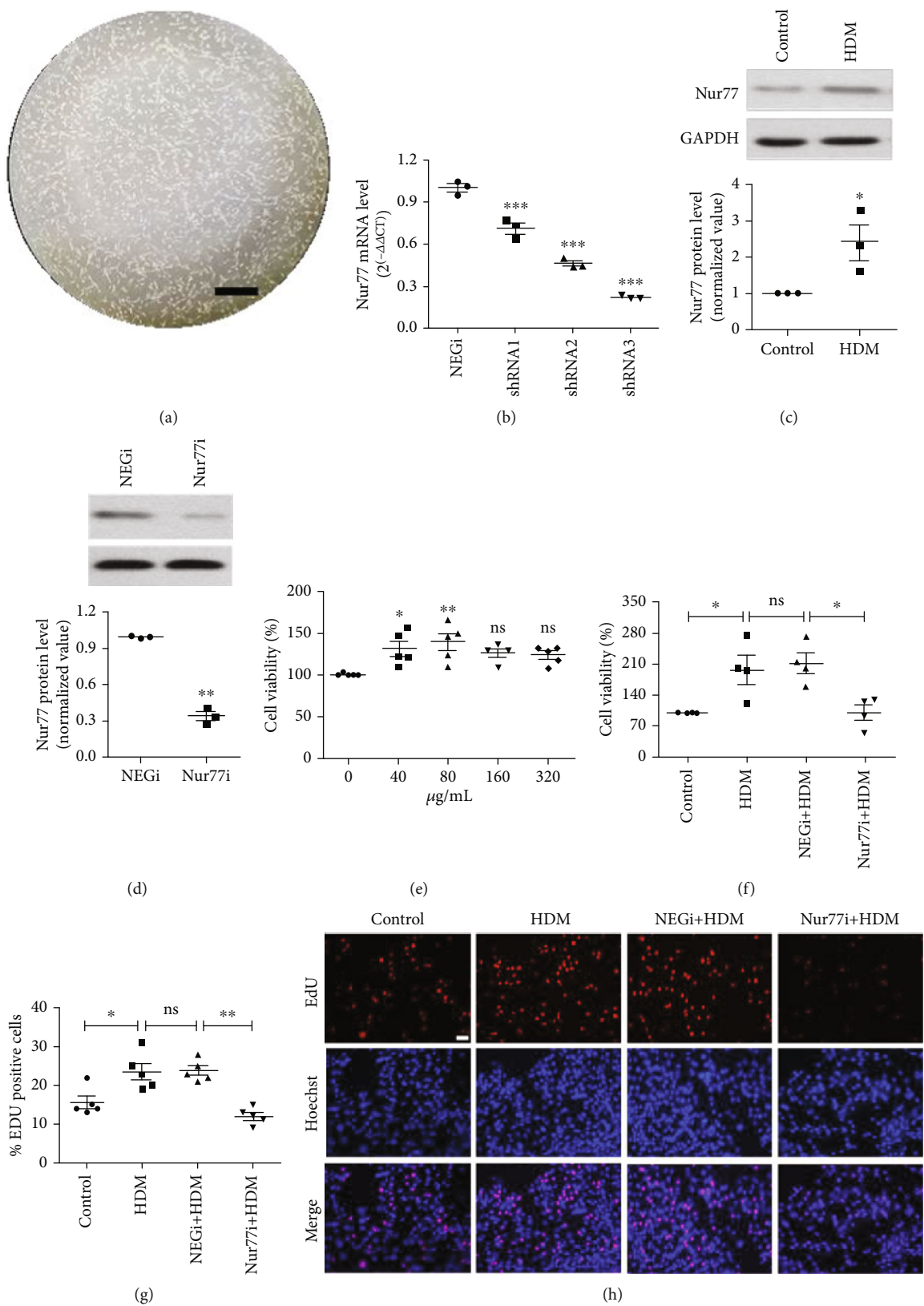
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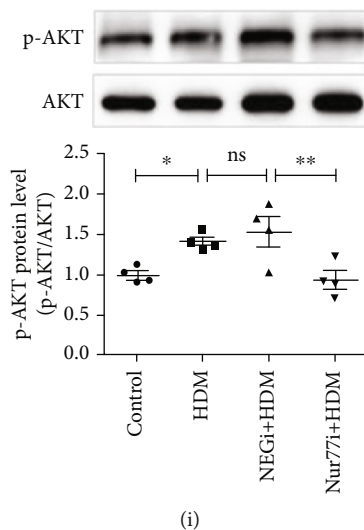
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FIGURE 5: Nur77 modulated proliferation of the primary human ASMC under HDM exposure. (a) One representative microscopic field of primary human ASMC was shown; scale bar: 200 μm. (b, d) Transfection of ASMC with Nur77 shRNA downregulated its mRNA (b) and protein (d) expression at 12 h compared with control shRNA (NEGi) treatment. (c) WB assay of Nur77 in ASMC with or without HDM (80 μg/mL) challenge. (e) Effect of different concentrations of HDM stimulation on ASMC proliferation via the CCK-8 assay. (f–h) Effect of Nur77i on HDM-stimulated ASMC by the methods of CCK-8 and EdU assays. Scale bar: 200 μm. (i) WB assay of AKT phosphorylation in HDM-challenged human ASMC. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. ns: not significant.

we found that Nur77i dramatically upregulated the expression of HO-1 and SOD2, which were important antioxidants responsible for scavenging excessive ROS and maintaining intracellular dynamical redox balance (Figures 6(c) and 6(d)). These results implied that Nur77 might boost ASMC proliferation by inhibiting antioxidant gene expression and facilitating ROS generation.

3.7. Nur77 Deficiency Restrained HDM-Increased ASMC Proliferation Partly through MFN2/MAPK Pathways. To further elucidate the underlying mechanisms involved in the effect of Nur77 on airway remodeling, ASMC proliferation, and ROS production, we detected the possible transduction pathways including PI3K/AKT, MAPK, and mitofusin 2 (MFN2). As we knew, MAPK and PI3K/AKT were key components in the signaling associated with ASMC proliferation. Besides, MFN2 was reported to affect cell proliferation via inactivating several proproliferative kinases such as ERK1/2 MAPK [23]. As shown in Figures 5(i), 7(a), and 7(b), HDM (80 μg/mL) induced AKT and MAPK (ERK1/2, P38, and JNK kinases) phosphorylation in ASMC within 30 min while downregulated MFN2 expression after 12 hrs of stimulation. These alterations could be reversed by Nur77i pretreatment compared to NEGi preadministration (Figures 5(i), 7(a), and 7(b)). Based on the results, we supposed that Nur77 promoted airway remodeling and ASMC proliferation by modulating MFN2/MAPK/AKT pathways.

4. Discussion

Asthma is a chronic airway disease induced by persistent exposure of allergens or some environmental hazards [24]. It is commonly characterized by reversible airflow obstruc-

tion, chronic inflammation, and airway remodeling. Among them, airway remodeling has received large amounts of attention since it not only contributed to asthma pathogenesis but also indicated poor prognosis of the disease. Typical structural change of airway remodeling includes ASM thickening, subepithelial fibrosis, mucus cell hyperplasia, and airway neovascularization [25]. It causes lung function decline and fixed airway obstruction. More importantly, airway remodeling decreased bronchodilator response, thus leading to the ineffectiveness of current primary regimen [5]. Although it has been identified as a key therapeutic target of asthma, existing drugs exhibited limited efficacy. Therefore, it is of urgent need to further elucidate the molecular mechanisms of airway remodeling and seek for novel therapeutic alterations. In the present study, we reported that Nur77 expression was mainly distributed along the ASM and increased in lungs of HDM-challenged mice. Nur77 depletion by lentivirus-mediated shRNA ameliorated AHR, chronic inflammation, goblet cell hyperplasia, and airway remodeling in the asthmatic mouse model. By means of primary human ASMC, we discovered that intercellular Nur77 was upregulated in response to HDM exposure. Conversely, Nur77 knockdown by shRNA inhibited cell proliferation and ROS production, as well as elevated antioxidant gene expression in HDM-stimulated ASMC. These alterations might associate with MFN2/MAPK/AKT pathways.

As mentioned above, increased ASM mass is the hallmark of airway remodeling in asthma. The underlying mechanisms were investigated to enhance ASMC proliferation and migration as well as diminished ASMC apoptosis in response to various stimuli [26, 27]. Current therapeutic strategies of asthma concentrated to attenuate ASM contraction or suppress airway inflammation, with few treatment options to
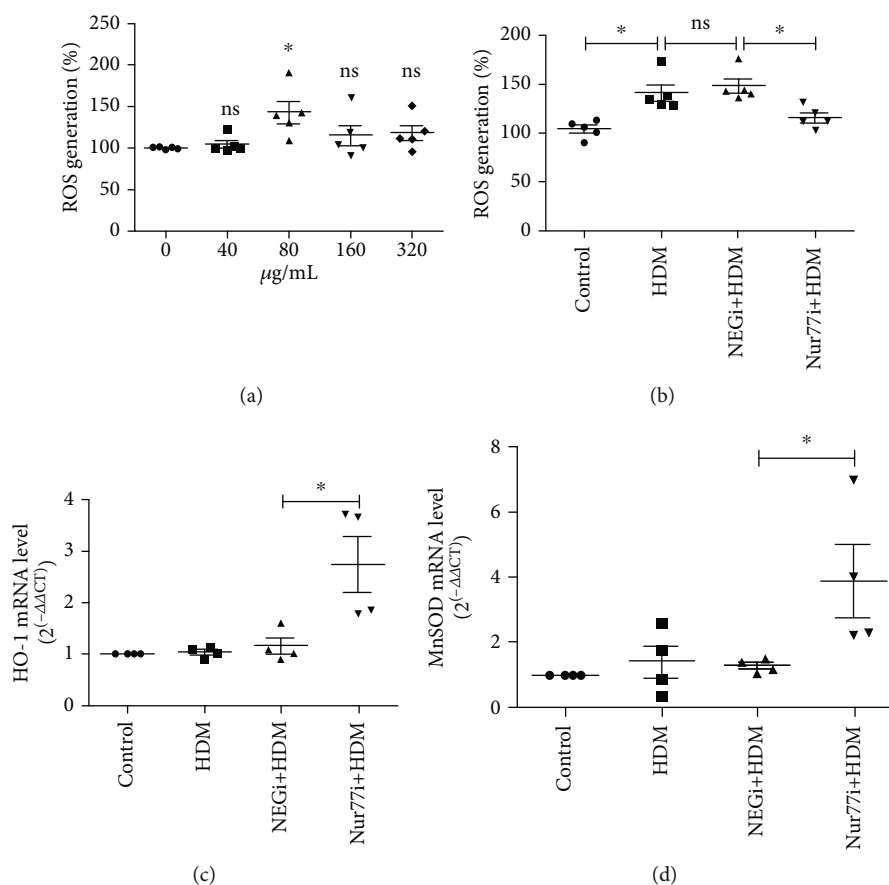
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FIGURE 6: Effect of Nur77 expression on oxidative stress in human ASMC under HDM stimulation. (a) Influence of different concentrations of HDM exposure on ASMC ROS production by DCFH-DA fluorescence assay. (b) Effect of Nur77i on HDM-stimulated ASMC through DCFH-DA fluorescence assay. (c, d) RT-PCR assay of HO-1 (c) and SOD2/MnSOD (d) mRNA expression in HDM-exposed human ASMC. $^{*}p < 0.05$ and $^{**}p < 0.01$.

prevent, blunt, or reverse ASM remodeling. Several inhalation preparations, such as leukotriene receptor antagonists and anticholinergics were proved to regulate ASMC hyperplasia in animal models [28]. Novel monoclonal antibodies (e.g., mepolizumab, lebrikizumab, and omalizumab) might play some roles in airway remodeling but still needed more evidence to elucidate their clinical impact and magnitude [29–31]. Besides, peroxisome proliferator-activated receptor- (PPAR-) γ ligands and vitamin D were also found to inhibit ASMC proliferation and had a certain impact on airway remodeling in asthma development [32, 33]. Nevertheless, due to the lack of therapeutic targeting, over- or long-term usage of these preparations might exhibit poor effect on ASM remodeling and even influence physiological functions of other cells. To date, bronchial thermoplasty (BT) was the only approved therapy that could decrease existing ASM mass and lastingly modify airway remodeling in severe asthma patients [34]. However, this technology has not been widely applied because of its relatively high cost and limited suitable crowd. Herein, we put forward a new idea that pulmonary Nur77 expression contributed to airway inflammation, AHR, and ASM remodeling in HDM-challenged mice, while Nur77 shRNA interference improved these pathogenic changes in asthmatic mice. Since the specific expression of

Nur77 in ASMC, we speculated that genetic regulation of Nur77 could effectively modulate ASMC hyperplasia, thus providing novel therapeutic target in asthma.

Nur77 is the transcription factor nuclear receptor involved in a wide range of physiological and pathological process, among which cell proliferation and apoptosis are the primary regulatory targets [12]. Interestingly, Nur77 played completely distinct roles in cell survival differed with cell types and irritant agents [35]. Within some tumor cells and cardiomyocytes, Nur77 translocation towards mitochondria triggered cytochrome c release and subsequent cell apoptosis [36–39]. On the contrary, Nur77 was reported to intensify the aggressive proliferation and migration of colorectal cancer cells [40], as well as inhibit cardiomyocyte apoptosis induced by isoproterenol [41]. Besides, Nur77 could promote the proliferation of vascular endothelial cells under histamine and serotonin stimulation [42]. In the research field of asthma, Nur77 also exhibited dual functions. On one hand, Nur77 was suggested to protect airway epithelium against excessive inflammatory injury [15, 16]; on the other hand, it accelerated airway epithelium damage by aggravating cellular apoptosis under ozone exposure [17]. Because of the important roles but uncertain effects of Nur77 in asthma pathogenesis, we systematically examined its
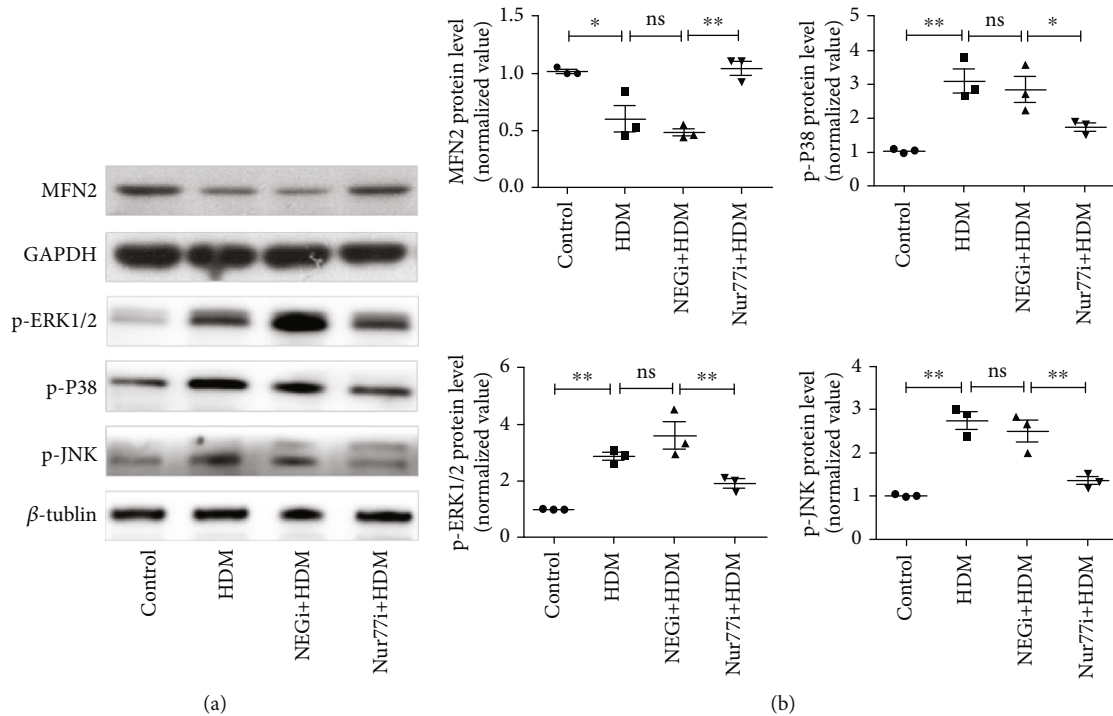
Figure 7: Effect of Nur77 on MFN2 expression and MAPK activation in human ASMC under HDM challenge. (a, b) The levels of MFN2 and phosphorylated ERK1/2, P38, and JNK were measured by WB assay. $^{*}p < 0.05$ and $^{**}p < 0.01$. ns: not significant.

biological functions using an HDM-mediated allergic asthma model. In this study, we demonstrated that HDM exposure significantly upregulated Nur77 expression in ASM of asthmatic mice. The increased Nur77 contributed to airway remodeling and ASMC proliferation, which might through induction of α-SMA, MMP-9, and excessive ROS. To our knowledge, we were the first to demonstrate that ASMC Nur77 upregulation by HDM increased the susceptibility of lung to develop asthma. Since experimental asthma could be induced by various allergens except for HDM, such as ovalbumin (OVA), cockroach, and Aspergillus species extracts, our results might have some limitations. The distribution, expression, and function of Nur77 might be different in other asthmatic models, and these needed further exploration in order to elucidate the association between Nur77 and asthma pathology.

One of the major molecular mechanisms underlying airway remodeling and ASMC proliferation was ROS generation [43]. Excessive intracellular ROS severed as the second messenger or directly regulated the phosphorylation and ubiquitination of cell cycle-related enzymes, followed by activating growth factor receptors and promoting cell proliferation [44, 45]. In the development of asthma, ROS overproduction was proved to be responsible for the increased vascular endothelial permeability, mucus hypersecretion, airway hyperresponsiveness, and epithelial injury [46]. N-Acetylcysteine (NAC), a classical free radical scavenger, has been reported to alleviate oxidant-induced AHR and ASMC hyperplasia [47, 48]. In this study, ASMC ROS level rose dramatically under HDM stimulation, while Nur77 deficiency reversed this increase as well as inhibited cell proliferation.

Besides, Nur77 depletion also upregulated antioxidant gene (HO-1 and SOD2) expression, suggesting that Nur77 might disrupt the oxidant/antioxidant balance within ASMC, therefore leading to cell hyperplasia. We also demonstrated that loss of Nur77 reduced HDM-induced phosphorylation of AKT and MAPKs in human ASMC, which were key components in signaling associated with oxidative stress and cell survival. MFN2 was the mitochondrial fusion-related protein and also regarded as an antiproliferative factor in recent years [23]. It was reported to inactivate several proproliferative kinases, including ERK1/2 MAPK and PI3K/AKT [49]. Our results showed that HDM-decreased MFN2 expression could be reversed by Nur77 depletion in human ASMC. This might be explained by the translocation of Nur77 from nuclear to mitochondria and its regulation on mitochondrial fragmentation (decreased mitochondrial fusion and increased mitochondrial fission) under certain stress [50].

5. Conclusions

Building on the HDM-challenged asthmatic murine model, we demonstrated that Nur77 was mainly distributed along ASM and its upregulation might be a key factor to enhance the susceptibility of lung to develop AHR, goblet cell hyperplasia, and airway remodeling. Moreover, with the help of primary human ASMC, Nur77 depletion notably attenuated HDM-induced cell proliferation through modulating ROS production and MFN2/MAPK/AKT pathways. These findings broadened our understanding of airway remodeling and ASMC proliferation, which might provide a novel therapeutic target for asthma patients.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Kun Wang and Muyun Wang contributed equally to the work.

Acknowledgments

Supplementary Materials

Additional details of Methods and Results section are provided in the Supplementary Materials. (*Supplementary Materials*)

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