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MDM2 Is Essential to Maintain the Homeostasis of Epithelial Cells by Targeting p53

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Keywords
MDM2 · p53 · Apoptosis · Alveolar type 2 cells · Type 2 lung inflammation

Abstract
Introduction: MDM2 is known as the primary negative regulator of p53, and MDM2 promotes lung cancer fibrosis and lung injury through p53-dependent and p53-independent pathways. However, the mechanism by which MDM2 influences the pathogenesis of asthma is unknown. In this study, we investigated the function of MDM2 in lung epithelial cells in type 2 lung inflammation. ***Methods:*** We used type II alveolar epithelial cell-specific heterozygous knockout of Mdm2 mice to validate its function. Then papain-induced asthma model was established, and changes in inflammation were observed by measuring immunohistochemistry and flow cytometry analysis. ***Results:*** In this study, we knockdown the mouse *Mdm2* gene in type 2 alveolar epithelial cells. We demonstrated that heterozygous *Mdm2* gene-deleted mice were highly susceptible to protease allergen papain-induced pulmonary inflammation characterized by increased ILC2 numbers, IL-5 and IL-13 cytokine levels, and lung pathology. A mechanistic study showed that following the decreased expression of *Mdm2* in lung epithelial cells and A549 cell line, p53 was overactivated, and the expression of its downstream genes *p21*, *Puma*, and *Noxa* was elevated, which resulted in apoptosis. After *Mdm2* knockdown, the mRNA expression of inflammation-related gene IL-25, HMGB1, and TNF-α were increased, which further amplified the downstream ILC2 response and lung inflammation. ***Conclusion:*** These results indicate that Mdm2 maintains the homeostasis

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of lung epithelial cells by targeting P53 and regulates the function of lung epithelial cells under type 2 lung inflammation.

Introduction

Asthma is a chronic airway inflammatory disease characterized as airway swelling, increased mucus production, shortness of breath, and cough. Allergic asthma is characterized by high level of type 2 cytokines such as IL-4, IL-5, and IL-13, immunoglobulin IgE, and eosinophils, which are positively correlated with the disease severity [1]. When exposed to various stimuli, pulmonary epithelial cells (ECs) can secrete the innate cytokines such as IL-33, TSLP, and IL-25, as well as the alarmins like HMGB1 [2]. These alarmin cytokines can act on cells that highly express their receptor, such as group 2 innate lymphoid cells (ILC2s), T helper 2 (Th2) cells, mast cells, and basophils, leading to the activation of these immune cells and the development of type 2 lung inflammation [3]. ILC2s, the tissue-resident innate immune cells, can be activated by the alarmins and can express IL-5, IL-13 to stimulate the recruitment of eosinophils, the mucus secretion of goblet cells, as well as the development of Th2 cells, leading to increased type 2 lung inflammation [4]. Many other EC-derived factors also regulate the response of ILC2s. For example, TNF-α, a proinflammatory cytokine elevated in severe asthma, enhances ILC2s survival and function in ILC2-dependent asthma [5, 6]. Therefore, ECs play a very important role in the initiation and amplification of type 2 immunity.

The alveolar ECs consist of alveolar type 1 cells, which are responsible for gas exchange, and alveolar type 2 cells (AT2 cells), which make the surfactant protein to prevent alveoli collapse and function as progenitor cells to maintain alveolar epithelium integrity [7]. AT2 cells are the main ECs that make up the alveolar structure. Surfactant protein C (SPC) is one of the surfactant proteins of the lung [8]. SPC is specifically expressed in AT2 cells [9], so it is often used to identify AT2 cells.

ECs represent the first line to defense and express pattern recognition receptors to recognize type-2 cell-mediated immune insults like allergens or helminths. It also has been reported that ECs play an important role in the development of lung diseases [2]. So the status of lung ECs is very important for maintaining the normal life activities of the lung. The alveolar ECs undergo apoptosis, which can lead to impairment of the epithelial barrier function in acute lung injury or acute respiratory distress syndrome (ARDS). Epithelial apoptosis has been considered to be the pathological characteristic of acute lung injury [10, 11]. It was also reported that apoptosis of ECs and the Fas/Fas ligand system plays an important role in the pathogenesis of ARDS [12].

Our laboratory previously found that MDM2 acts as an E3 ubiquitinase to affect IL-33 stability in vitro [13], and this led us to focus on the role of MDM2 in asthma. MDM2 is an E3 ubiquitin ligase that can interact with more than 100 proteins. Different proteins interact with the positions of MDM2 to form complexes that determine MDM2 activity in response to different signaling pathways [14]. Among these proteins, p53 is the primary partner that interacts with MDM2 [15]. MDM2 interacts with p53 mainly through a negative feedback loop [16]. In unstressed cells, p53 is usually maintained at low levels by ubiquitination of MDM2 [17–19].

P53 primarily functions as a tumor suppressor, suppressing tumor development in various organs. P53 is also a regulatory factor in the cell growth cycle and is related to cell cycle regulation, DNA repair [20], cell differentiation, cell apoptosis, and other pathways. The cell apoptosis is induced predominantly by p53 through the upregulation of p53-regulated genes such as *Puma*, *Noxa*, and *Bax* [21]. P53 also can upregulate expression of p21 to induce cell cycle arrest [22]. Regarding apoptosis mediated by P53 downstream genes, *Noxa*, *Pumaα*, and *Pumaβ* interact with antiapoptotic Bcl-2 family members, such as Bcl-2, resulting in cyto C release from mitochondria to cytosol and the activation of caspase-9 [23]. The cytoplasmic Bax responds to various apoptotic stimuli by translocating to mitochondria, where it is capable of inducing cyto C release, activating caspase-9 through Apaf-1, and initiating the apoptosis cascade [24, 25].

The study of the MDM2-p53 interaction is concerned mainly with cancer research, and how MDM2 regulates p53 function and maintains its tumor suppressor function is well investigated [26]. Amplification and overexpression of MDM2 occurs in many cancers, resulting in a loss of p53-dependent activities, such as apoptosis and cell cycle arrest [27]. Besides, there are some studies about MDM2-p53 in lung development and diseases. Inactivation of MDM2 in lung ECs during development leads to reduced lung lobe number and lung size [28]. The attenuation of MDM2-p53 interaction and of p53 degradation may be involved in the EC apoptosis seen in idiopathic pulmonary fibrosis [29]. In the acute lung injury induced by LPS, p53 can protect LPS-induced endothelial barrier dysfunction through MDM2-p53 axis [30]. However, there is no report about the role of

the MDM2-p53 in regulating the homeostasis of ECs and its potential link to asthma.

In the present study, we generated mutant mice with lung alveolar EC-specific knockout of the *Mdm2* gene. Due to the lethality of homozygous *Mdm2* KO mice, heterozygous (HE) mice were used in all experiments. We found that *Mdm2* knockdown mutant mice showed increased susceptibility to papain-induced lung inflammation, with increased ILC2 numbers, secretion of more IL-5 and IL-13 cytokines, and more severe tissue inflammation than those of the WT mice. Lung ECs isolated from these mice exhibited increased p53 signal, suggesting that the reduction of Mdm2 resulted in ectopic activation of p53 in lung ECs. Consequently, the expression of apoptosis-related genes, such as *Puma* and *Noxa*, which are regulated by p53 were increased. TUNEL staining revealed that the *Mdm2* knockdown mutant mice showed increased apoptotic cells both at homeostasis and after allergen challenge. Consistently, *Mdm2* knockdown cell line showed higher expression of apoptosis-related genes and more apoptotic cells, resembling the phenotype of *Mdm2* mutant mice. At the same time, the expression of proinflammatory factors such as IL-25, HMGB1, and TNF-α in ECs is increased, which can activate ILC2 and aggravate inflammation. Overall, we conclude that reducing *Mdm2* expression in ECs causes activation of p53, which induces cell apoptosis, disrupts the homeostasis of lung epithelium, and increases sensitivity to allergen stimuli which lead to exacerbation of type 2 inflammation.

Methods

Mice

C57BL/6 mice were purchased from the Shanghai Research Center for Model Organization (Shanghai, China) and $Mdm2^{fl/fl}$ and SPC-CreERT2 mice were purchased from Cyagen. For gene knockdown of *Mdm2*, $Mdm2^{fl/+}$ SPC-CreERT2 mice were intraperitoneally injected with tamoxifen (75 mg/kg) once every 24 h for a total of 5 consecutive days to induce Cre activity and *Mdm2* knockdown in alveolar epithelial type II cells. Littermate controls were treated in the same way. The mice were maintained under specific pathogen-free conditions at the Animal Care Facility of the Chinese Academy of Sciences (Shanghai, China) and used at 8–10 weeks old. The animal care and use procedures complied with the guidelines of the CAS Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences.

Mouse Model of Papain-Induced Lung Inflammation

For establishment of a papain-induced acute asthma model, mice were anesthetized by isoflurane and administered papain (3 μg in 40 μL PBS) or phosphate buffered saline (40 μL PBS, as a control) intratracheally for 5 days and sacrificed on day 6.

Preparation of Cell Suspensions from Lung Tissue

Mouse lung tissue was cut into pieces and digested in Roswell Park Memorial Institute (RPMI) medium containing 10% serum and 5% collagenase I at 37°C for 30 min. The digested lung fragments were poured into a strainer and ground with a syringe pushing head, and the filtrate was filtered through the strainer into a 15 mL EP tube. The supernatant was centrifuged, and red blood cells were lysed and then centrifuged. The supernatant was poured out, and 2 mL of 40% Percoll was added. The suspension was transferred to a flow tube containing 80% Percoll. The samples were centrifuged at 600 *g* for 20 min, and the intermediate layer of the fragment was collected for further analysis.

Flow Cytometric Analysis

After preparation of cell suspensions from lung tissue, suspensions were pre-stained with FVS780 (BD, 565388) in PBS for cell viability analysis to exclude dead cells. For lung ILC2s, which was stained with a cocktail of fluorochrome-conjugated antibodies for various lineage markers, including CD3, CD5, CD45R, CD11b, CD11c, NK1.1, Gr-1, TER119, FcεR1, and TCR γ/δ, to identify Lin^- cells. The following antibodies were used for flow cytometry: anti-GR-1 (BioLegend, RB6-8C5), anti-NK1.1 (eBioscience, PK136), anti-TCRγ/δ (Biolegend, clone GL3), anti-CD3 (eBioscience, clone 2C11), anti-Ter-119 (eBioscience, clone 382 Ter119), anti-B220, anti-CD5 (eBioscience, clone GK1.5), anti-CD11b (Bioscience, clone 379 M1/70), anti-CD11c (eBioscience, clone N418), and anti-FcεR1 (eBioscience, clone MAR-1). For intracellular cytokine staining, cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich), 1 μg/mL ionomycin (Sigma-Aldrich), and 1 μg/mL brefeldin A (BFA; eBioscience) for 4 h at 37°C and then stained to determine intracellular IL-5 and IL-13 levels. And the cells were stained with the lineage antibody mixture above, PerCP-Cy5.5-CD90.2, and FITC-ST2 for the surface. The cells were permeabilized and fixed with Foxp3/Transcription Factor Staining Buffer Set (00-5523-00, eBioscience) according to the manufacturer's instructions (eBioscience) for 30 min or overnight. Next, the cells were stained with BV421-IL-5 (BioLegend, 504311), PE-IL-13 (Invitrogen, 12-7133-82), and A700-Ki67 (Invitrogen, 56-5698-82) in 1 × Permeabilization Buffer (Invitrogen, 00-8333-56) for 30 min on ice. Then flow cytometry analyses were performed on a BD LSR Fortessa (BD Biosciences).

Quantitative Real-Time PCR

Total RNA was extracted from *Mdm2* knockdown A549 cells, lung homogenates, and lung ECs from mice using TRIzol (Invitrogen) and was reverse transcribed into cDNA with synthesis kit (Vazyme). Gene expression was analyzed by quantitative real-time PCR with SYBR Green chemistry (Vazyme) using the following primer set: human-*ACTIN*-F, CATGTACGTTGCTATCCAGGC; human-*ACTIN*-R, CTCCTTAATGTCACGCACGAT; human-*BAX*-F, CCCGAGAGGTCTTTTTCCGAG; human-*BAX*-R, CCAGCCCATGATGGTTCTGAT; human-*PUMA*-F, GACCTC AACGCACAGTACGAG; human-*PUMA*-R, AGGAGTCCCATG ATGAGATTGT; human-*NOXA*-F, TGCTACACAATGTGGCGT C; human-*NOXA*-R, ACTTGGACATGGCCTCCCTTA; human-*p21*-F, TGTCCGTCAGAACCCATGC; human-*p21*-R, AAAGTC GAAGTTCCATCGCTC; mouse-*actin*-F, GTGACGTTGACA TCCGTAAAGA; mouse-*actin*-R, GCCGGACTCATCGTACTCC; mouse-*p21*-F, CCTGGTGATGTCCGACCTG; mouse-*p21*-R, CCA

TGAGCGCATCGCAATC; mouse-*Bax*-F, TGAAGACAGGGG CCTTTTTG; mouse-*Bax*-R, AATTCGCCGGAGACACTCG; mouse-*Puma*-F, AGCAGCACTTAGAGTCGCC; mouse-*Puma*-R, CCTGGGTAAGGGGAGGAGT; mouse-*Noxa*-F, GCAGAGCTA CCACCTGAGTTC; mouse-*Noxa*-R, CTTTTGCGACTTCCC AGGCA.

The Construction of MDM2 Knockout Cell Line

SgRNAs for *MDM2* knockout were inserted into a lenti-CRISPRv2 vector with puromycin resistance for screening. The sgRNAs for *MDM2* and lentivirus packaging plasmids psPAX2 and pMD2.G were cotransfected into HEK293T cells grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. The transfection mixture and medium were discarded after 6 h incubation. The medium was changed to DMEM supplemented with 10% FBS, followed by incubation in the presence of 5% CO_2 at 37°C for 48 h. Based on the cell state, supernatant from 293T cells was collected 24 and 48 h after transfection and stored at 4°C. Purified virus and DMEM were used to infect A549 cells in proportion for one night. Then cell status was analyzed and sorted. All plasmids were from our laboratory, and they were confirmed by sequencing. sgMDM2 sequence: GTGGTTACA GCACCATCAGT.

Papain Treatment of MDM2 Knockout Cell Line

Cells were cultured at a density of 2–5 × 10^5 in cell well plates. The next day, when the cell density was 80%, 5 μg of papain was added to the well plate, the cells were collected after 30 min of treatment, and the RNA was extracted.

Cell Apoptosis Detection

For cell apoptosis detection, in vitro, the *Mdm2* knockdown A549 cell line was stained with an Annexin V-FITC/PI Apoptosis Detection Kit (Vazyme, A211-02) according to the manufacturer's protocol. Cell apoptosis was assessed on Beckman flow cytometer. Annexin V–/PI– cells were considered live cells, while Annexin V+/PI– cells were considered early apoptotic cells. Annexin V–/PI+ cells were considered dead cells, while Annexin V+/PI+ cells were considered late apoptotic cells.

Histological Analysis

For histopathological analysis of inflammation, mouse lungs were fixed in paraformaldehyde and embedded in paraffin, and sections were stained with H&E and PAS to evaluate immune cell infiltration and mucus secretion. Sections were stained with TUNEL and P53 and P21 to evaluate the apoptosis level of lung ECs and the expression levels of P53 and P21 in the lung. Inflammation was quantified by semiquantitative scoring: 0, none; 1, mild; 2, moderate; 3, marked; and 4, severe. An increment of 0.5 was used when the inflammation fell between two levels. Three fields were selected randomly for independent scoring by two treatment-blind pathologists.

Isolation of Murine Lung AT2 Cells

To isolate murine lung AT2 cells, lungs were intratracheally perfused with 0.5 mL cocktail enzyme solution containing Collagenase II (225 U/mL, Worthington, LS004176), Dispase (5 U/mL, Corning, 354235), DNase I (200 U/mL, Worthington, LS002139). Then the lungs were cut into small pieces and incubated in cocktail protease solution for 30 min at 37°C. The cell suspension was then filtered through a 70 μm strainer. After centrifugation, cells were resuspended in 1xRBC lysis buffer to remove red blood cells. For further cell purification, the following antibodies were used for flow cytometry and cell sorting: EpCAM-APC (BioLegend, 118214, 1:100), CD31-FITC (BioLegend, 102405, 1:200), and CD45-FITC (BioLegend, 103107, 1:200), CD140-PE (BioLegend, 135905, 1:100). DAPI was used to gate out dead cells.

Statistical Analysis

All data are presented as the mean ± standard error of the mean (SD). A two-tailed Student's *t* test was applied for comparisons of unpaired data between two groups. For multigroup comparisons, we used one-way ANOVA with Tukey's post hoc test. Data were analyzed with Prism Software (GraphPad Prism 8). Error bars represent the SD. And p value <0.05 was considered to denote statistical significance.

Results

Mdm2 *Deficiency in AT2 Cells Increases Papain-Induced Lung Inflammation*

To explore the influence of Mdm2 on type 2 inflammation, we first generated alveolar epithelial type 2 cell-specific *Mdm2* knockout mice by crossing *Mdm2*-floxed mice (*Mdm2*$^{fl/fl}$) and SPC-CreERT2 transgenic mice (Sftpc-CreERT2). After tamoxifen injection, mice with homozygous Mdm2 knockout in AT2 cells did not survive beyond 1 week, so we used *Mdm2*$^{fl/+}$SPC-CreERT2, heterozygous (HE) *Mdm2* knockout mice as knockdown mice in our following experiments, and used *Mdm2*$^{+/+}$ SPC-CreERT2 mice as controls. Mice were injected intraperitoneally with tamoxifen for 5 days, and we waited for 2 weeks to complete the conditional knockdown of *Mdm2* in lung ECs. In order to verify the knockdown efficiency of *Mdm2* knockout in the heterozygous mice, we designed QPCR primers in the vicinity of the *Mdm2* gene knockout region. Two weeks after the last tamoxifen treatment, lung ECs were sorted from mice by flow cytometry. Then the knockdown efficiency was detected by RT-qPCR. In 2 weeks, *Mdm2* gene is knocked down in lung ECs (shown in online suppl. Fig. 1a; for all online suppl. material, see https://doi.org/10.1159/000539824). To test whether inflammation occurs in the resting state after MDM2 knockdown in lung ECs, we examined the proportion and number of eosinophils in bronchoalveolar lavage fluid (BALF) from PBS-treated mice and found no significant ifference, as well as no change in cytokines IL-5 and IL-13 (shown in online suppl. Fig. 1b, c). We performed histological staining for periodic acid-Schiff staining (PAS) and hematoxylin-eosin staining (H&E) in PBS-treated WT and HE mice. No infiltration of immune cells or secretion of mucus was found (shown in online suppl. Fig. 1d).

To examine the influence of *Mdm2* on type 2 inflammation, we administered papain to mice for 5 days through intratracheal injection, and the mice were sacrificed on the sixth day (shown in Fig. 1a). From PAS and H&E staining of lung tissue, we observed an increase in mucus production and infiltrated cells in the *Mdm2* knockdown mice (shown in Fig. 1b). We also detected the eosinophils in BALF, and flow cytometry analysis of eosinophils was gating by live $CD45^+CD11C^-SiglecF^+$ cells in BALF (shown in online suppl. Fig. 2). Compared with control mice, both the proportion and number of eosinophils in BALF were elevated (shown in Fig. 1c and d). We also examined both macrophages and neutrophils, and it was found that the proportion and number of these two types of cells had no difference (shown in online suppl. Fig. 3). These findings demonstrate that *Mdm2* knockdown in the AT2 cells result in increasing lung inflammation after papain challenge.

Mdm2 *Deficiency in AT2 Cells Increases the Papain-Induced ILC2 Response*

We quantified the levels of type 2 immunity-related cytokine and found that papain treatment led to higher concentrations of IL-5 and IL-13 in the BALF of *Mdm2* knockdown mice (shown in Fig. 2a). Further analysis by flow cytometry revealed that there were more ILC2s in the mutant mice lungs than control lungs (shown in Fig. 2b, c, gating strategy shown in online suppl. Fig. 4). There were more ILC2 cells in mutant mice expressing Ki67, indicating that these $ST2^+$ ILC2s in *Mdm2* knockdown mice were more proliferative. Consistently, the number and proportion of functional ILC2s, such as IL-5^+ ILC2s, IL-13^+ ILC2s, and IL-5^+IL-13^+ ILC2s, were significantly higher in the *Mdm2* knockdown mice (shown in Fig. 2d). Dendritic cells and Th2 cells were also examined, and there was no difference in the number and proportion of DCs. However, the number and proportion of Th2 cells were increased in the *Mdm2* knockdown mice (shown in online suppl. Fig. 5). As we know, ILC2 can stimulate the activation of Th2 cells and the further adaptive immunity. Such results suggest that ILC2 can promote Th2 activation and response, and that Th2 response may occur gradually after 5 days of papain immunization [4]. These results suggested that *Mdm2*- specific knockdown increased the papain-induced innate ILC2 and adaptive Th2 responses.

Mdm2 *Knockdown Increases Apoptosis in vivo*

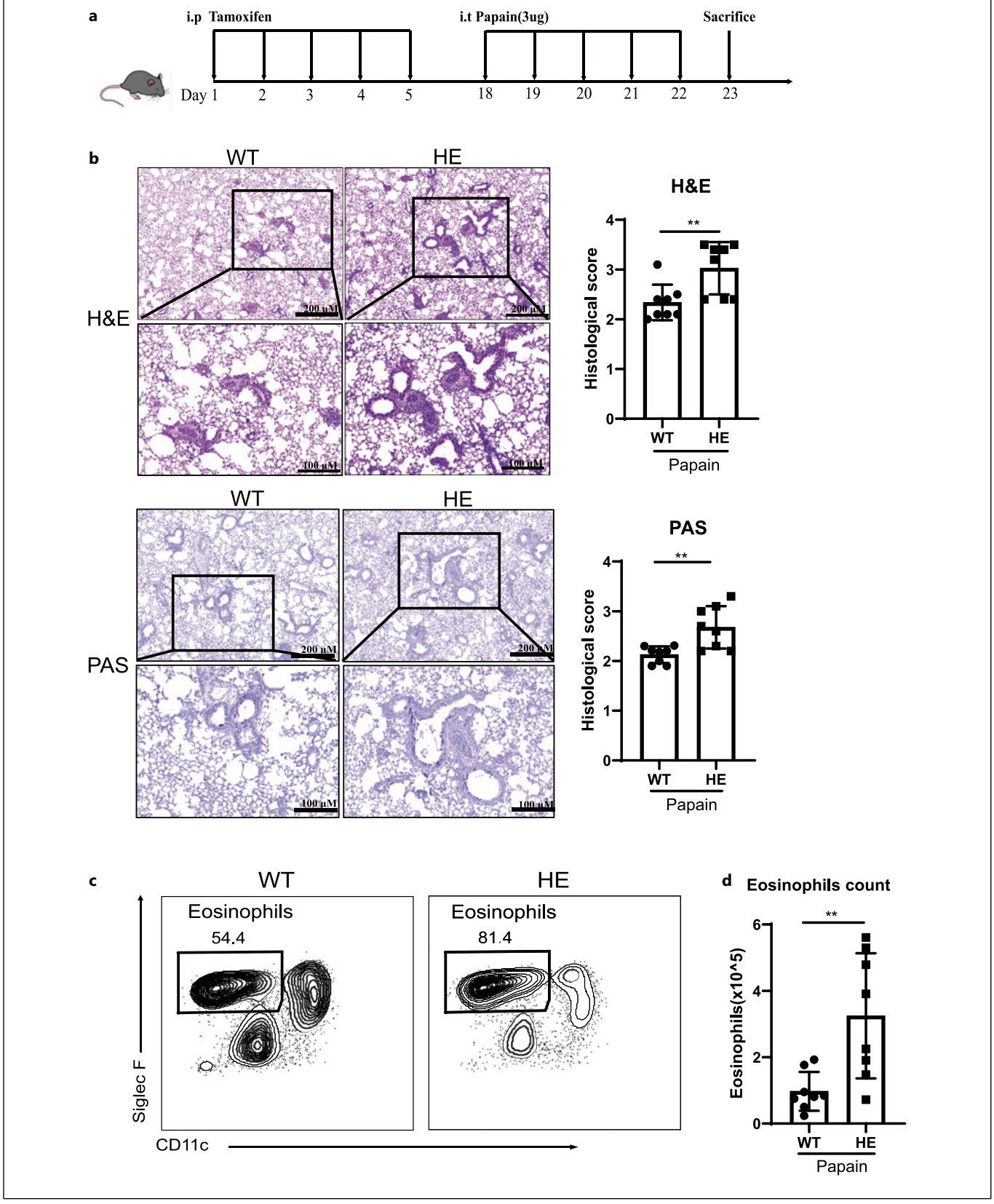
It has been reported that *Mdm2* knockout leads to ectopic accumulation of p53 protein and activation of the p53 signaling pathway, leading to apoptosis [31]. Based on the increased ILC2-induced inflammation in *Mdm2* mutant mice, we then investigated whether the exacerbation of lung inflammation caused by *Mdm2* knockdown is due to activation of the p53 signaling pathway and consequent induction of apoptosis in lung ECs.

To detect whether *Mdm2* knockdown influences lung homeostasis, we used lung tissue sections from PBS-treated mice for apoptosis staining. TUNEL staining of the lung tissue revealed that this TUNEL-positive cell had slightly increased in Mdm2 knockdown mice compared to controls (shown in Fig. 3a). Meanwhile, we found that there were still a certain number of lung ECs (shown in online suppl. Fig. 6). As there was no distinct inflammation in the *Mdm2* knockdown at steady state (shown in online suppl. Fig. 1), we suggest that the apoptosis of lung ECs may have little effect on lung homeostasis in the resting state. Then we explored whether *Mdm2* knockdown could lead to apoptosis of lung ECs in papain-induced lung inflammation. As expected, we found more TUNEL-positive cells in the *Mdm2* knockdown mice following papain treatment (shown in Fig. 3b).

Lung Mdm2-*Specific Knockdown Induces the Expression of p53 Downstream Genes*

Previous data indicated that *Mdm2* deletion led to increased cell apoptosis. P53 activation is known to induce apoptosis, cell cycle arrest, and senescence in a variety of tissues. Therefore, we isolated lung ECs from WT mice and *Mdm2* knockdown mice at steady state. Notably, the gene expression levels of *p21*, *Puma*, and *Noxa* were all increased in the *Mdm2* knockdown mice (shown in Fig. 4a), indicating that *Mdm2* knockdown in AT2 cells could induce apoptosis in naive condition. Real-time PCR also demonstrated that expressions of the p53 downstream gene, including *p21*, *Bax*, and *Noxa*, were significantly higher in the knockdown mice than in the control littermates after papain treatment (shown in Fig. 4b), suggesting that *Mdm2* knockdown in AT2 cells leads to upregulated expression of p53 downstream genes.

Immunohistochemical examinations in the lung sections also revealed that the p53 and p21 proteins were highly expressed in the lungs of the $Mdm2^{fl/+}$SPC-CreERT2 mice but were limitedly detected in those of the control littermates treated with papain (shown in Fig. 4c, d). We also found that the expression level of cleaved caspase-3, an active form of caspase-3, increased in the lung of $Mdm2^{fl/+}$SPC-CreERT2 mice treated with papain (shown in Fig. 4e). These findings indicated that lung-specific knockdown of *Mdm2* increases the protein level of p53, p21, and cleaved caspase-3 in the lung, which promotes the cell apoptosis.



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MDM2 *Knockdown Increases Apoptosis and p53 Expression in vitro*

To validate the influence of *MDM2* knockout in lung ECs in vitro, we used the A549 cell line, a human cancer cell line with alveolar basal EC features, for further experiments. We generated *MDM2* knockout A549 cell line using the CRISPR/Cas9 system. We used the mix cells infected with lentivirus carrying sgRNA for further experiments. First, we verified the efficiency of *MDM2* knockout and found that the expression of MDM2 in cells infected with lentivirus carrying sgMDM2 reduced significantly (showed in Fig. 5a), so we used these *MDM2* knockdown cells in the follow-up experiments. We also found that p53 protein level increased in the *MDM2* knockdown cells (shown in Fig. 5a). This result demonstrated that a lack of MDM2 function in lung ECs led to the accumulation of endogenous p53 protein. Next, the protein expression levels of apoptosis-related genes downstream of p53 were detected. The protein expression of Bax and Puma was increased in *MDM2* knockdown cells. Caspase-3, which is a p53 downstream apoptosis-related protein, was activated according to the increasing cleavage form (shown in Fig. 5a). In addition, we examined the transcription levels of p53 downstream genes, such as *p21*, *Noxa*, *Puma*, and *Bax*, and found that the expression of these genes were significantly higher in *MDM2* knockdown cells than in control cells (shown in Fig. 5b). To further confirm the cell apoptosis phenotype, the Annexin V/Propidium iodide apoptosis assay was performed. We found that the proportion of apoptotic cells (Annexin V+) was clearly increased in *MDM2* knockdown cells compared with that in control cells (shown in Fig. 5c). These data suggested that *MDM2* deletion results in the accumulation and activation of p53, which triggers p53 downstream apoptosis-related signaling pathways, leading to cell apoptosis.

MDM2 Was Able to Ubiquitinate p53 in vitro

The negative feedback relationship between MDM2 and p53 has been widely reported. Under stress, p53 is activated after a series of protein translational processes, such as phosphorylation, acetylation, and methylation [32–34]. In non-stressed mammalian cells, p53 is usually maintained at low levels through continuous ubiquitination and by the degradation of the 26S protease system [35]. To verify the interaction of MDM2 and p53, we detected the ubiquitination of p53 catalyzed by MDM2 and the interaction between MDM2 and p53. We overexpressed MDM2, p53, and ubiquitin in 293T cells and then performed immunoprecipitation (shown in Fig. 6). These results showed that in the case of MDM2 and p53 cotransfection, the ubiquitination level of p53 protein increased significantly. In addition, we confirmed the strong interaction between MDM2 and p53. Thus, we confirmed MDM2 was able to ubiquitinate p53 in vitro. We performed ubiquitination of P53 by MDM2 in vitro and verified that MDM2 interacts with P53; therefore, we hypothesized that knockdown of *Mdm2* in mouse lung ECs can reduce P53 degradation and lead to P53 accumulation in the cells, thereby activating downstream apoptosis-related genes.

Mdm2 *Knockdown Increases the Expression of Proinflammatory Factors during Papain Stimulation*

To further investigate the mechanism that papain-induced lung immune inflammation was aggravated after *Mdm2* knockdown in lung ECs, we examined the expression of proinflammatory factors associated with type 2 inflammation. We treated A549 WT cells and A549 *MDM2* knockdown cells with papain, and then collected the cells for RNA extraction. We examined the transcription levels of proinflammatory factors, IL-25, HMGB1, and TNF-α, and found that the expression of these proinflammatory factors were significantly higher in *MDM2* knockdown cells than in control cells (shown in Fig. 7a).

We isolated the lung ECs from $Mdm2^{+/+}$SPC-CreERT2 mice and $Mdm2^{fl/+}$SPC-CreERT2 mice in papain model for RNA extraction to verify the expression of pro-inflammation factors. RNA extraction and RT-qPCR were performed on lung ECs. We found that the expression of IL-25, HMGB1, and TNF-α were all increased in lung ECs of the *Mdm2* knockdown mice (shown in Fig. 7b). These results suggest that papain stimulation of lung ECs can increase the expression of proinflammatory factors in lung ECs and accelerate the activation of ILC2s, thereby aggravating type 2 lung inflammation.

Fig. 1. Histopathology changes and cell phenotype analysis in WT and *Mdm2*-specific knockdown mice following papain challenge. **a** Mice were intratracheally challenged with papain for 5 consecutive days and were sacrificed on day 6. **b** Representative H&E-stained and PAS-stained images of lung sections (scale bars, 200 μm, 100 μm). Each dot represents the ratings on the degree of pathological tissue section. **c**, **d** Quantification and representative flow cytometry analysis of the total eosinophils (live $CD45^+CD11c^-SiglecF^+$) in BALF ($n = 8$ per group). Unpaired Student's *t* test was used to analyze the data. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

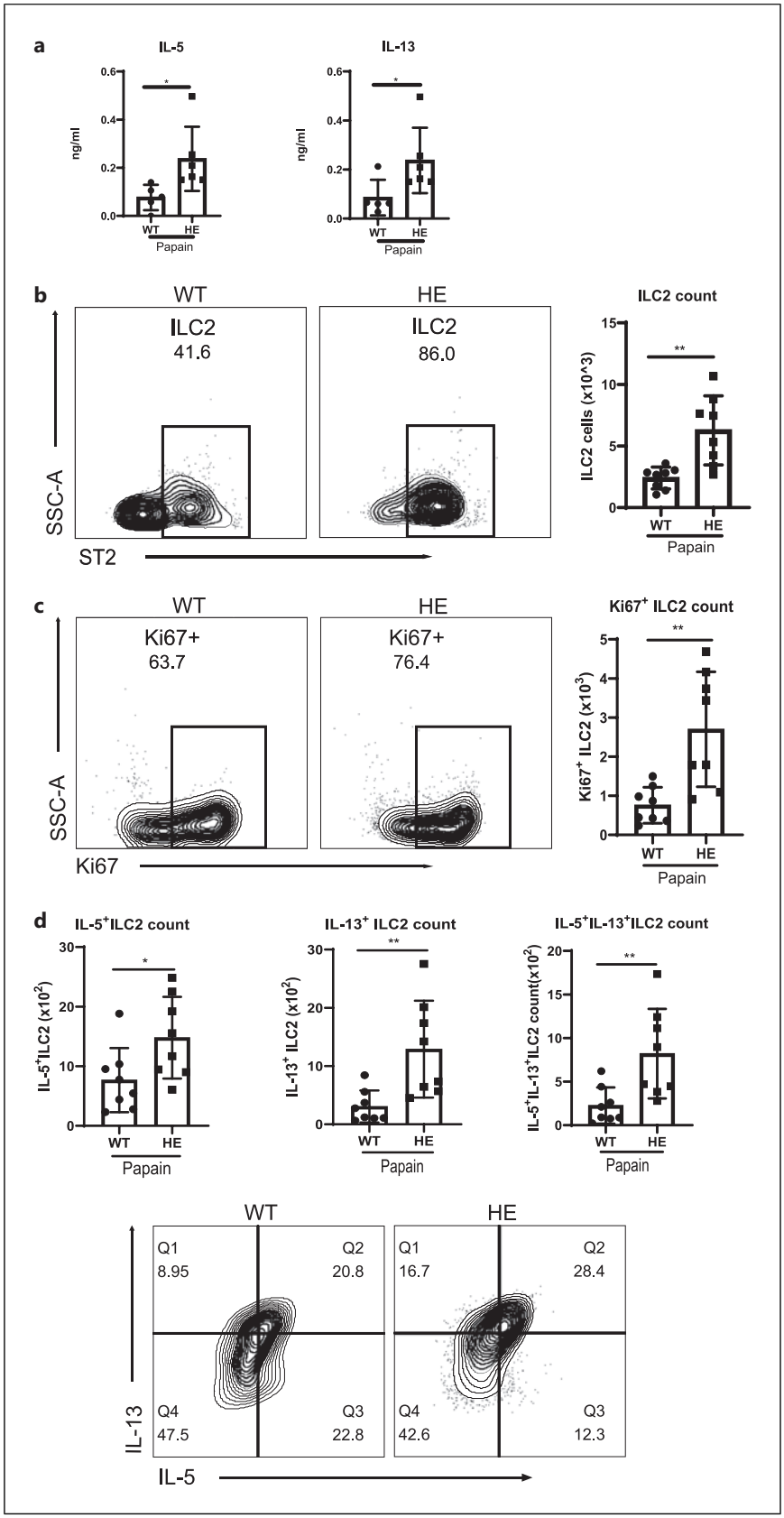


Fig. 2. *Mdm2*-specific knockdown enhances ILC2 response in papain-induced lung inflammation. **a** The levels of IL-5 and IL-13 in BALF were measured by ELISA (n = 8). **b** ST2$^+$ ILC2s were analyzed as live CD45$^+$Lin$^-$CD90.2$^+$ST2$^+$ cells by flow cytometry (n = 8). **c** Ki67 analysis by flow cytometry (n = 8). **d** Quantification and representative flow cytometry analysis of IL-5 and IL-13 production in lung ILC2s after stimulation with phorbol 12-myristate 13-acetate, ionomycin, and BFA for 4 h (n = 8). Unpaired Student's t test was used to analyze the data. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

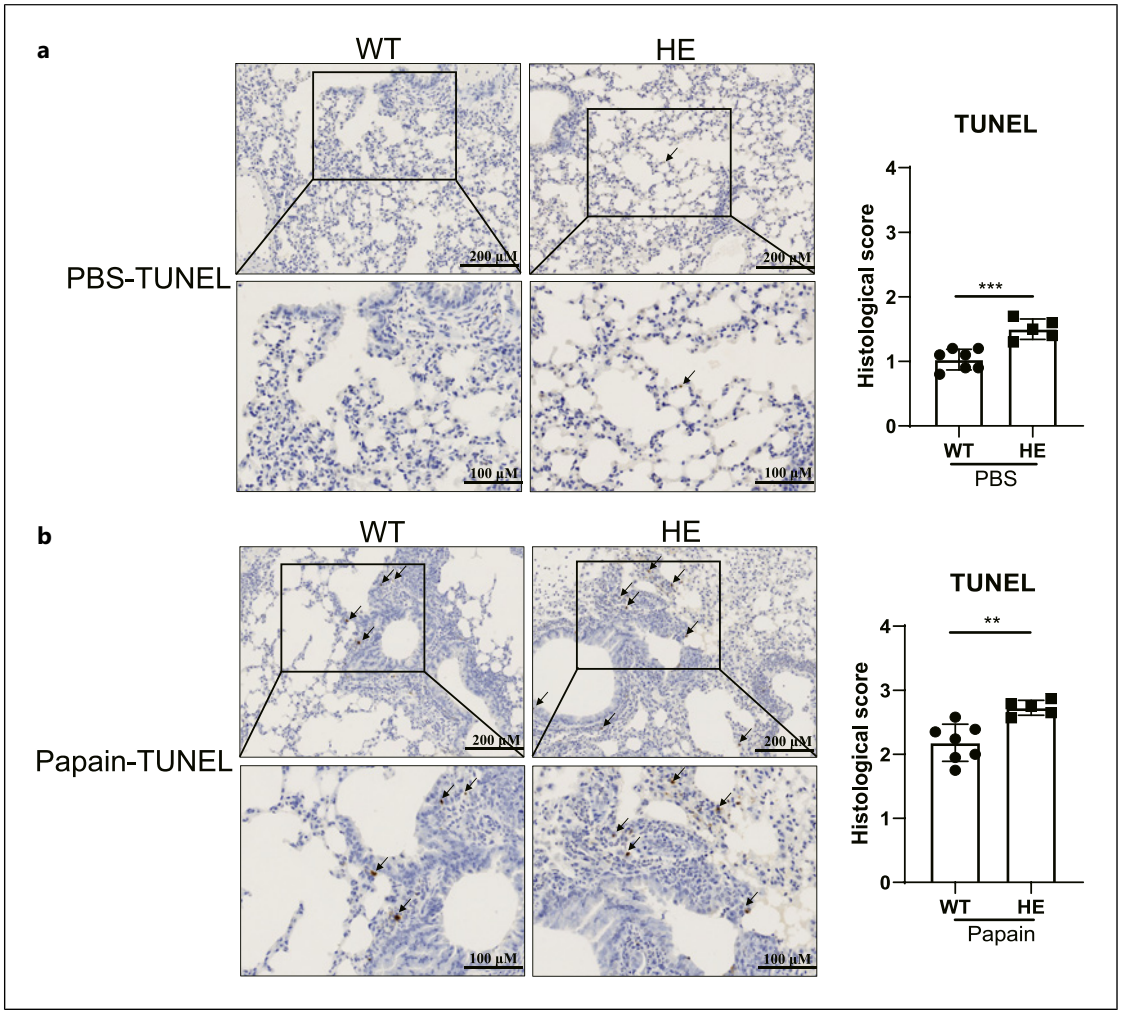
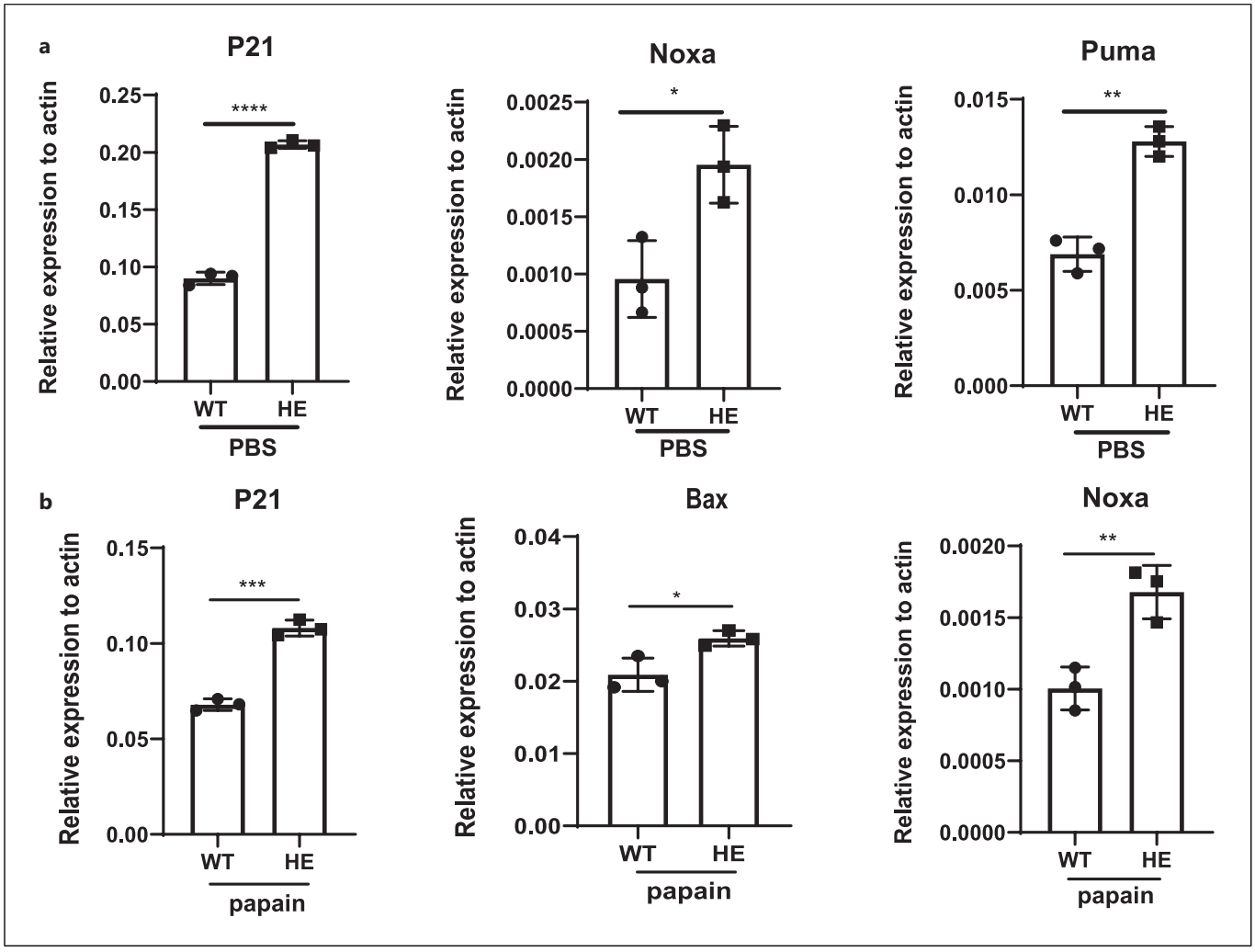


Fig. 3. *Mdm2* knockdown results in increasing apoptosis in lung tissue of papain-treated mice. **a** TUNEL staining of lung sections from PBS-treated mice (scale bars, 200 μm, 100 μm). **b** TUNEL staining of lung sections from papain-treated mice (scale bars, 200 μm, 100 μm). Each dot represents the ratings on the degree of positive signal in tissue sections. Unpaired Student's *t* test was used to analyze the data. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Discussion

To investigate the influence of *Mdm2* in asthma, we generated AT2 cell-specific *Mdm2* knockdown mice and found that *Mdm2* knockdown exacerbated airway inflammation induced by papain. We found that knockdown of *Mdm2* can lead to the accumulation of p53 in AT2 cells. By isolating lung ECs, we observed that the expression of apoptosis-related genes downstream of p53, such as *Noxa*, *Puma*, and *Bax*, was elevated in *Mdm2* knockdown mice. TUNEL staining showed more apoptotic lung cells in *Mdm2* knockdown mice, and immunohistochemical staining demonstrated that the protein expression levels of p53, p21, and caspase-3 were distinctly increased in *Mdm2* knockdown mice. When we constructed the *MDM2* knockdown A549 cell line, we found that the mRNA and protein expression levels of p53 downstream genes were increased compared with those in WT cells. Annexin V-PI staining indicated that *MDM2* knockdown cells were more likely to undergo apoptosis. In conclusion, the above results showed that a decreased expression level of Mdm2 in AT2 cells can result in the accumulation of p53, and the expression of downstream apoptosis-related genes was consequently increased. The gene expression level of proinflammatory factors, such as IL-25, HMGB1, and TNF-α, were also



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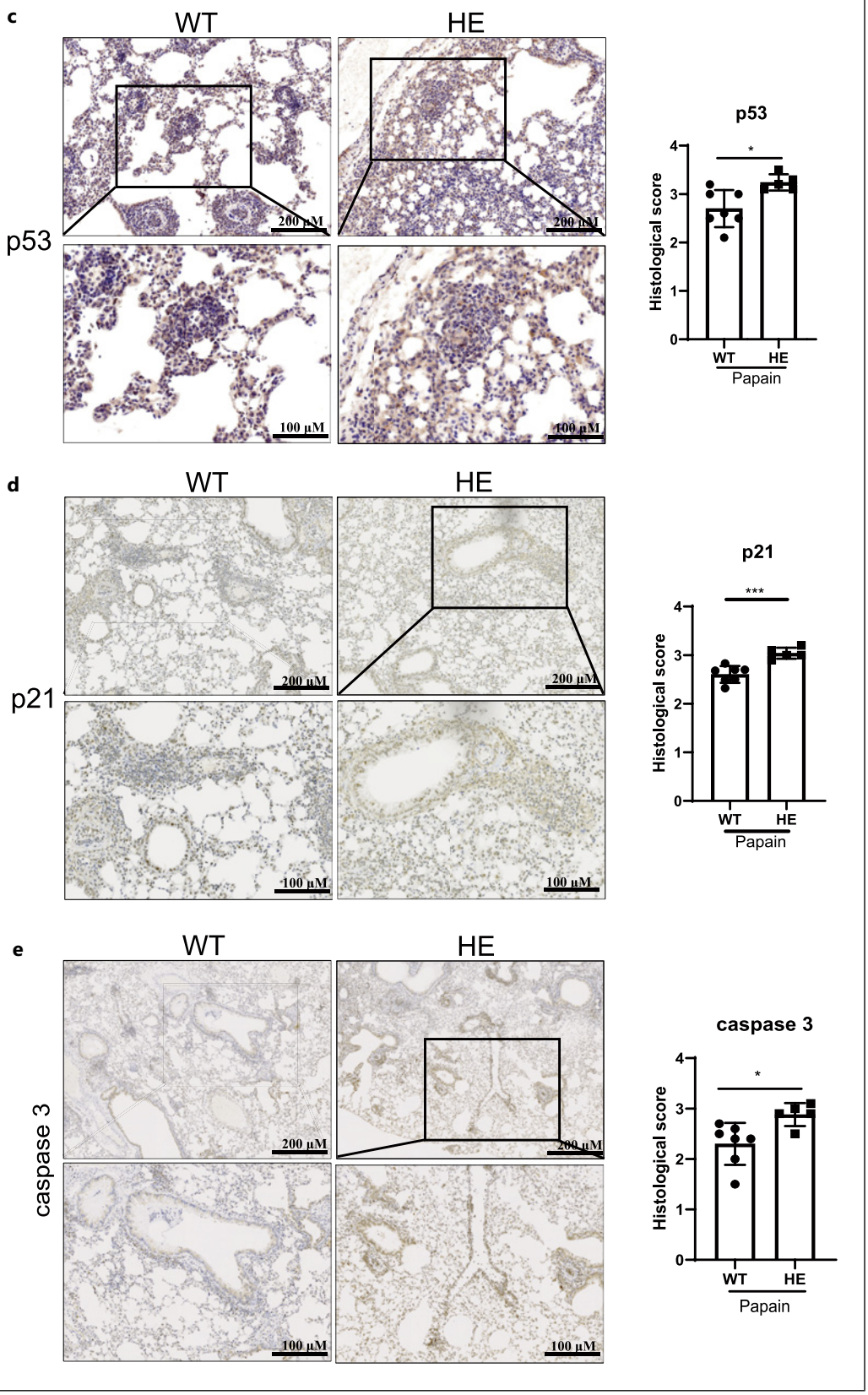
elevated in MDM2 knockdown cells compared with control cells. Therefore, under deletion of *Mdm2*, the pulmonary ECs were easily apoptotic, and the integrity of the lung epithelial barrier was disrupted, leading to the exacerbation of papain-induced lung inflammation.

In our work, we focused on the development of asthma. Allergic asthma is a chronic inflammatory airway disease that is characterized by airway hyperresponsiveness, eosinophilic inflammation, and mucus hyperproduction. The epithelial surfaces of the lungs are in direct contact with the environment and are exposed to various pathogens. AT2 cells are the main ECs that make up the alveolar structure. When lung ECs are stimulated by protease allergens and other external stimuli, they can secrete the alarmins IL-25, IL-33, and TSLP, which can activate immune cells. Recently, more and more researches focus on the role in the asthma development of ILC2s, the tissue-resident innate immune cells, which can be activated by the EC-derived alarmins. Pulmonary ILC2s are capable of producing type 2 cytokines IL-5 and IL-13 to promote the activation of type 2 lung inflammation. IL-5 is able to attract eosinophils into the lung, where they secrete numerous inflammatory cytokines and chemokines. IL-13 affects tracheal ECs and smooth muscle cells, leading to excessive secretion of mucus and airway remodeling. Therefore, lung ECs serve as the significant defense to the various allergen stimulation, regulating the initiation of immune response. When *Mdm2* was knockdown in lung ECs, the homeostasis of lung ECs was damaged, and the response of the AT2 cells to the papain stimulation is extreme. Therefore, MDM2 is important to maintain the homeostasis and the proper response to the allergen stimulation of AT2 cells.



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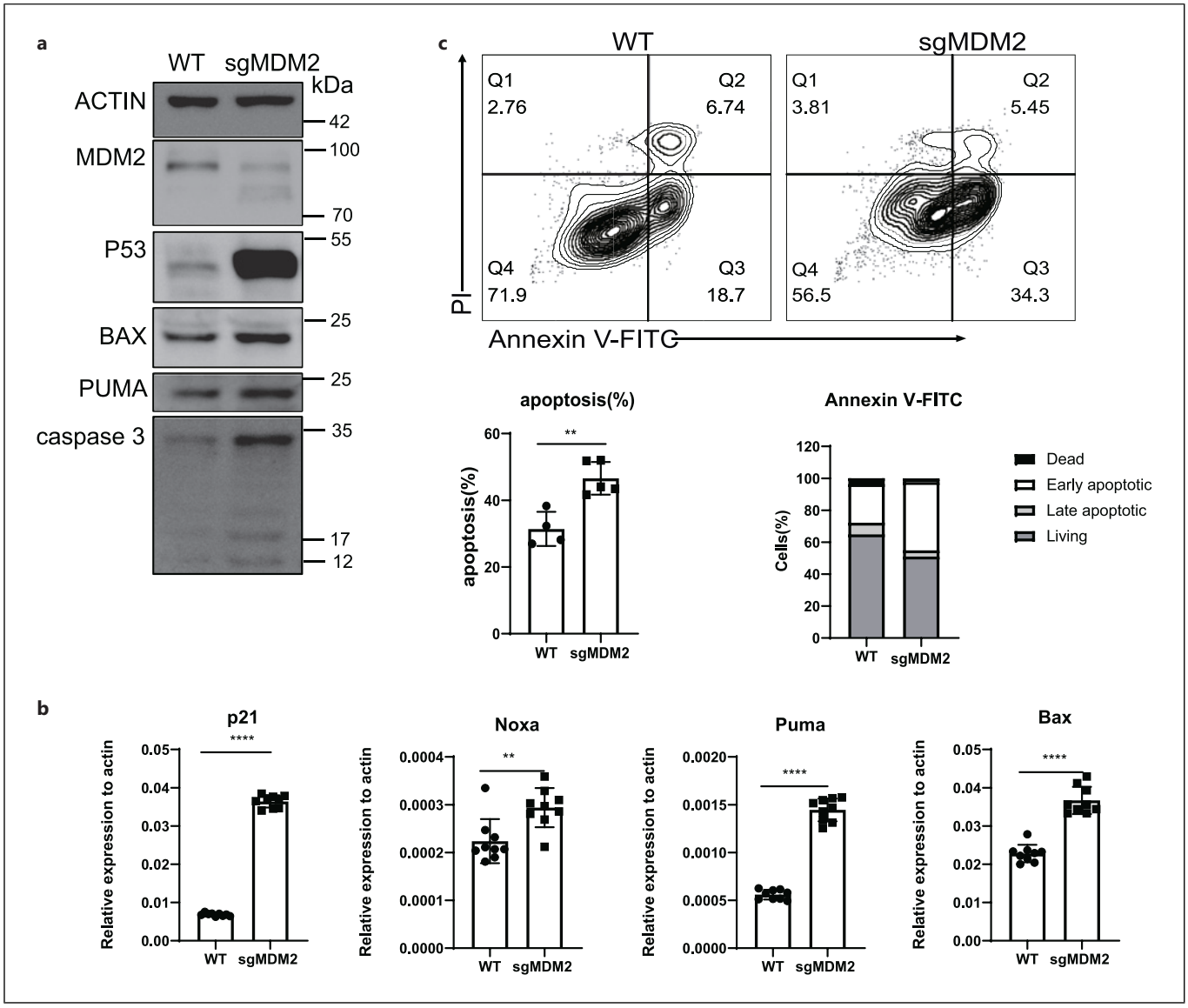

Fig. 5. *MDM2* knockdown enhances apoptosis and expression of p53 downstream genes in vitro. **a** The protein expression of p53, Puma, Noxa, and Bax in Mdm2 knockdown A549 cells was assessed by Western blot analysis. **b** *P21*, *Puma*, *Noxa*, and *Bax* mRNA levels were determined by real-time RT-qPCR (n = 3). Each point indicates a sample from one individual. **c** Apoptosis analysis of *MDM2* knockdown A549 cells (PI/Annexin V-FITC) by flow cytometry and the proportion of apoptosis at different apoptotic stages. Unpaired Student's t test was used to analyze the data. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Fig. 4. Lung *Mdm2*-specific knockdown induces the expression of apoptosis-related proteins. **a** *P21*, *Puma*, and *Noxa* mRNA levels in the lung from PBS-treated mice were determined by real-time RT-qPCR (n = 3). **b** *P21*, *Bax*, and *Noxa* mRNA levels in the lung from papain-treated mice were determined by real-time RT-qPCR (n = 3). **c** Immunohistochemical staining of p53 of lung sections from papain-treated mice (scale bars, 200 μm, 100 μm) (n = 7/5). **d** Immunohistochemical staining of p21 of lung sections from papain-treated mice (scale bars, 200 μm, 100 μm) (n = 7/5). **e** Immunohistochemical staining of caspase-3 of lung sections from papain-treated mice (scale bars, 200 μm,100 μm) (n = 7/5). Each dot represents the ratings on the degree of positive signal in tissue sections. Each point indicates a sample from one individual. Unpaired Student's t test was used to analyze the data. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

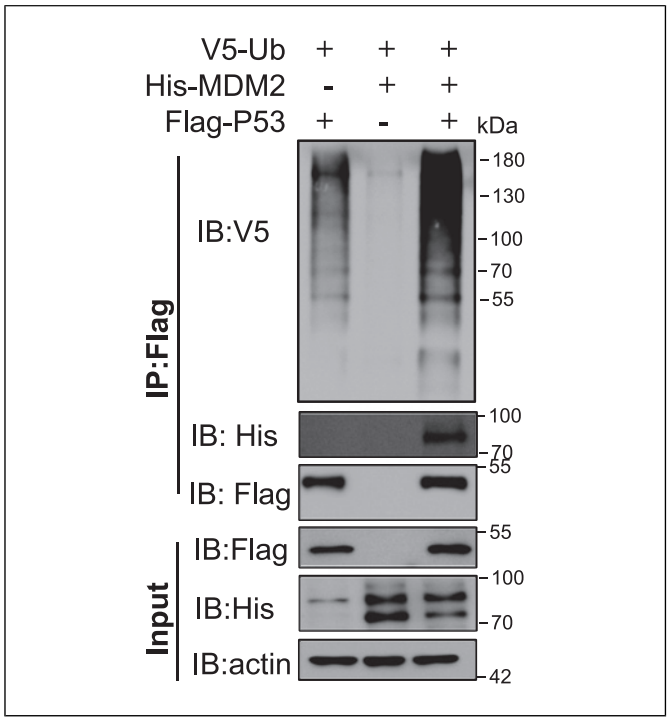

Fig. 6. MDM2 was able to ubiquitinate p53 in vitro. HEK293FT cells were transfected with V5-Ub, His-MDM2, Flag-p53 for 48 h, then with MG132 (10 μM), and analyzed by Western blotting. The result of experiment is shown.

We observed that lung apoptosis and the increased expression of P53 and its downstream apoptosis-related genes like p21, *Noxa*, and *Puma* in AT2 cells occurred in the *Mdm2* knockdown mice, when type 2 inflammation was more intense. When cells face various stress signals, such as DNA damage, hypoxia, cytokines, metabolic changes, and virus infection, the cellular p53 protein level will increase, regulated by posttranslational modifications, such as ubiquitination, phosphorylation, and acetylation [36]. According to the characteristics of apoptosis, apoptotic cells can also form apoptotic bodies, which can maintain membrane completeness. It is possible that lung ECs undergo apoptosis and that the epithelial barrier is damaged, making it easier for external stimuli to trigger inflammation. We found that although the apoptosis of lung ECs was slightly increased at steady state, there were still a certain number of lung ECs; meanwhile, there is no distinct immune response in the lung of *Mdm2* knockdown mice. We suppose that although *Mdm2* knockdown caused apoptosis of lung ECs, but without external stimulation, the cells may be in a relatively stable equilibrium. Under protease allergen stimulation, the apoptosis accounts for exacerbation of response of lung ECs.

Our previous work found that MDM2 can affect the stability of IL-33. Through expression of MDM2 and IL-33 at different concentrations in 293T cells, it was found that intracellular IL-33 increased with the concentration gradient of MDM2 [13]. Ubiquitination experiments showed that Mdm2 can enhance the ubiquitination of IL-33. Vitamin B6 can regulate MDM2-mediated IL-33 stability and inhibit type 2 immune responses. It is widely known that MDM2 plays an important role in the control of p53. In non-stressed cells, MDM2 promotes p53 degradation via the ubiquitin-proteasome pathway, and p53 is tightly regulated and maintained at low levels [37]. In addition to this, MDM2 also blocks the apoptotic activity of E2F1 [38] and promotes cell proliferation by regulating other important components of the cell cycle. All these phenomena indicate that Mdm2 as an E3 ligase plays different roles in the pathogenesis of asthma, mainly through the ubiquitination of P53 to degradation.

In our work, many questions remain unanswered. To investigate the role of Mdm2 in asthma, we generated type 2 alveolar EC-specific *Mdm2* knockdown mice, in which we observed the consequences of persistent p53 activation in type 2 alveolar ECs and found that the cells underwent apoptosis and aggravated inflammation. Regarding the mechanism that aggravates type 2 inflammation in *Mdm2* knockdown mice, we observed an increase in lung cell apoptosis. Although apoptosis is generally considered to be quiescent cell death that does not cause organ damage, it is unknown whether lung EC apoptosis is directly involved in the development of type 2 inflammation. Some studies have reported that hepatocyte-specific knockout of Bcl-XL, which is a member of the antiapoptotic protein family, can induce massive hepatocyte apoptosis, which may be the cause of spontaneous liver fibrosis [39, 40]. Therefore, apoptosis may also contribute to the development of diseases. The occurrence of inflammation induced by apoptosis has been reported, for example, apoptosis of pulmonary macrophages can induce pulmonary inflammation and fibrosis and that this induction may be associated with increased expression of TNF-α, TGF-β1, MMP2, and MMP9 [41]. There is the evidence indicating that Fas/FasL-mediated lung epithelial apoptosis results in release of proinflammatory cytokines such as TNF-α and TGF-β1, leading to inflammation and progression from ARDS to fibrosis [42]. Induced acute lung injury in mice by intratracheal administration of Fas-activating antibody and demonstrated that apoptosis can lead directly to lung barrier collapse [12]. These studies suggest that apoptosis of lung ECs may disrupt the integrity of the lung epithelial barrier or aggravate inflammation through the secretion

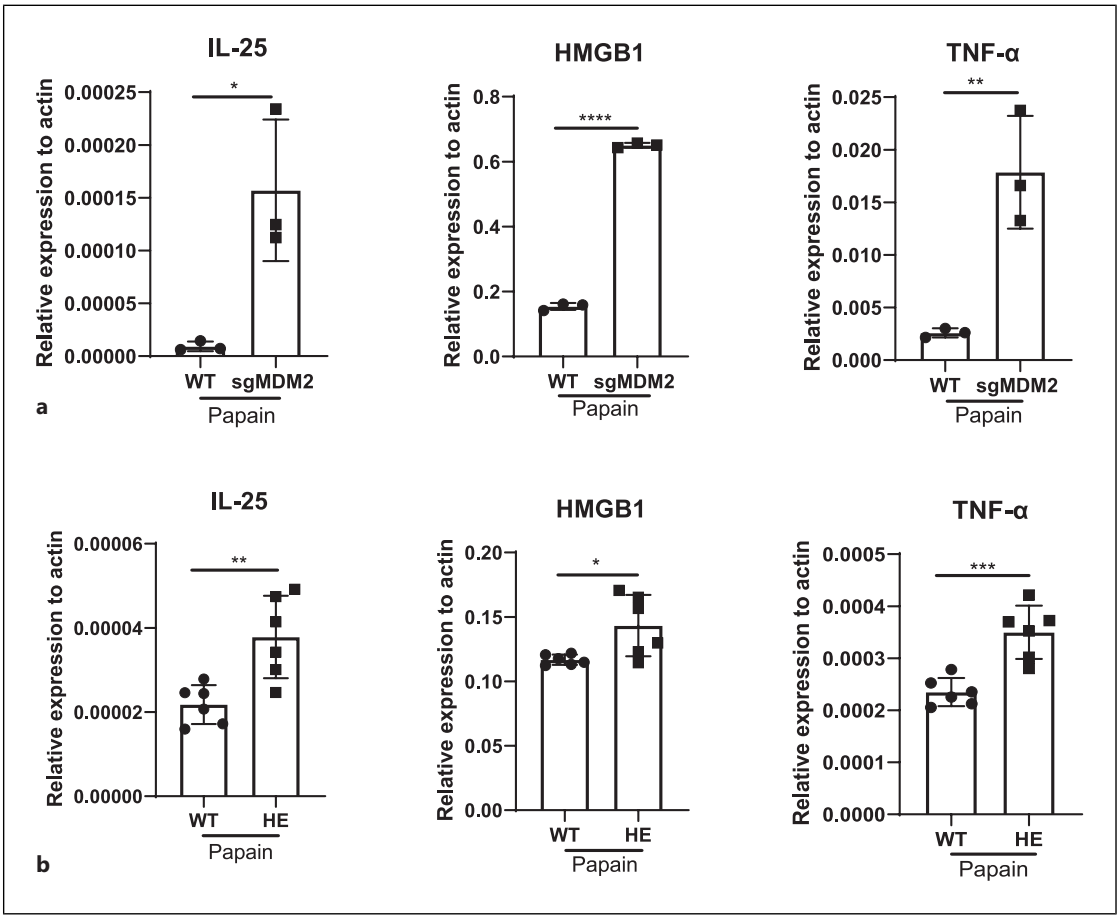

Fig. 7. *Mdm2* knockdown increases the expression of proinflammatory factors during papain stimulation. **a** IL-25, HMGB1, and TNF-α mRNA levels from Papain-treated of MDM2 knockdown A549 cells were determined by real-time RT-qPCR ($n = 9$). Each point indicates a sample from one individual. **b** IL-25, HMGB1, and TNF-α mRNA levels in the lung from papain-treated mice were determined by real-time RT-qPCR ($n = 6$). Each point indicates a sample from one individual. Unpaired Student's *t* test was used to analyze the data. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

of proinflammatory factors. The mechanism by which lung EC apoptosis aggravates type 2 inflammation is still worth exploring.

As we know, IL-25 is one of epithelia-derived alarmins, which can be induced by the stimuli such as allergen proteases and activate ILC2 in type II lung inflammation [2, 43]. HMGB1, another alarmin expressed by ECs, can amplify ILC2-induced type 2 inflammation [44, 45]. TNF-α plays an important role in many pulmonary inflammatory diseases, including asthma [46]. It has been found that TNF-α as an inflammatory factor plays a crucial role in survival, function, and induction of airway hyperresponsiveness through TNFR2 signaling in ILC2 [6]. We found that MDM2 knockdown in lung ECs can induce the high expression of proinflammatory factor IL-25, HMGB1, and TNF-α, leading to the exacerbation of type II lung inflammation. Thus we suggest that the apoptosis of lung ECs regulated by MDM2 can regulate the expression of proinflammation factors to control the development of type 2 lung inflammation.

In the present study, we demonstrated a correlation between the knockdown of *Mdm2* in lung ECs and the aggravation of type 2 immune inflammation, as evidenced by indicators of type 2 inflammation, such as the number and proportion of eosinophil cells and functional ILC2s. In AT2 cells, activation of p53 induces the expression of downstream apoptosis-related genes so that AT2 cells undergo apoptosis. The integrity of the epithelial barrier is destroyed, and homeostasis is damaged. The RNA expression levels of proinflammatory cytokines IL-25, HMGB1, and TNF-α were also increased in lung ECs, resulting in inflammation

being more likely to occur. Therefore, the MDM2-p53 interaction is important for maintaining the homeostasis of lung ECs.

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Statement of Ethics

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Ethics Approval No. SIBCB-S303-1610-030-c2).

Conflict of Interest Statement

The authors declare no competing interests.

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Author Contributions

Su Wang, Shufen Zhong, and Ying Huang performed the experiments and analyzed the data. Songling Zhu, Shuangfeng Chen, Ran Wang, Sonam Wangmo, Bo Peng, Jichao Yang, Houkun Lv, Liyan Ma, and Dr. Zhiyang Ling provided protocols and suggestions. Su Wang, Shufen Zhong, Yaguang Zhang, Bing Sun, and Dr. Pengfei Sui designed the study and wrote the manuscript. Yaguang Zhang and Bing Sun supervised and revised the manuscript.

Data Availability Statement

All data generated and analyzed during this study are included in this article and its online supplementary material files. Further inquiries can be directed to the corresponding author.

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