

# CTNNAL1 enhances glucocorticoid sensitivity in HDM-induced asthma mouse model through deactivating hsp90 signaling pathway

Di Wu<sup>a,b,1</sup>, Lexin Liu<sup>a,1</sup>, Jiahui Zhu<sup>a</sup>, Caixia Liu<sup>c</sup>, Chunjiao Long<sup>a</sup>, Chi Liu<sup>a</sup>, Xiangping Qu<sup>a</sup>, Huijun Liu<sup>a</sup>, Ming Ji<sup>a</sup>, Xiaoqun Qin<sup>a</sup>, Yang Xiang<sup>a,\*</sup>

<sup>a</sup> Department of Physiology, School of Basic Medical Science, Central South University, Changsha 410008, Hunan, China

<sup>b</sup> School of Medicine, Foshan University, Foshan 528000, Guangdong, China

<sup>c</sup> School of Integrated Chinese and Western Medicine, Hunan University of Chinese Medicine, Changsha 410208, Hunan, China

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## ABSTRACT

**Aims:** Adhesion molecules play vital roles in the induction of airway hyperresponsiveness (AHR) or airway inflammation. The down-regulation of catenin alpha-like 1 (CTNNAL1) in the bronchial epithelial cells of asthma patients and mice models has been noted in our previous study. In this work, we further explore the underlying mechanism of CTNNAL1 in asthma.

**Main methods:** We constructed a house dust mite (HDM)-induced asthma animal model on control mice and applied CTNNAL1-siRNA transfection to create CTNNAL1-deficient mice.

**Key findings:** We documented much more severe airway inflammation and increased leukocyte infiltration in the lungs of the CTNNAL1-deficient mice comparing to control mice, along with elevated expression of inflammatory cytokines. Dexamethasone (DEX) treatment led to less reduced inflammation in CTNNAL1-deficient mice compared with control mice. Immunoprecipitation confirmed the interaction between heat shock protein90 (hsp90) and CTNNAL1. The expression of hsp90 was upregulated after CTNNAL1 silencing. Meanwhile, the use of hsp90 inhibitor geldanamycin significantly decreased the expression of NR3C1, ICAM-1 and the ratio of p-p65/p65 in CTNNAL1-silenced 16HBE14o- cells. Both geldanamycin and DEX could function to suppress the expression of ICAM-1 and the phosphorylation level of p65. Nevertheless, the anti-inflammatory effect of DEX proved less potent than geldanamycin in the CTNNAL1-silenced group. The combined therapy of geldanamycin and DEX significantly decreased the inflammatory responses in CTNNAL1-deficient HBE cells than DEX monotherapy.

**Significance:** Our study corroborates that CTNNAL1 deficiency induced aggravated airway inflammation and rendered insensitivity to glucocorticoids via triggering hsp90 signaling pathway.

## 1. Introduction

Asthma is a common respiratory illness characterized by reversible airflow obstruction with airway inflammation and hyper-responsiveness [1,2]. The mechanism of airway inflammation includes imbalance of lymphocyte subsets, eosinophilic airway infiltration and immunoglobulin overexpression [3]. According to the estimation of 2019 Global Burden of Disease (GBD) Study, asthma inflicts a substantial health and economic burden on up to 358 million people on a global scale. The incidence of adult asthma in Asia ranges from 0.7 % to 11.9 % [4]. The statistics of the China Pulmonary Health Study in 2019 showed that 45.7

million asthmatics over the age of 20 are in China, amounting to a prevalence rate of approximately 4.2 % [5].

Airway epithelium is the first cellular barrier to allergens, therefore the structural damage and loss of function in epithelium cells could trigger airway inflammatory reactions after allergic stress. Epithelium dysfunction is implicated in the pathogenesis of lung inflammation disorders, including asthma and COPD [6,7]. CTNNAL1, a protein homologous to  $\alpha$ -catenin, functions to facilitate the recovery of human bronchial epithelium damage [8,9]. Our previous study reported the markedly down-regulation of structural adhesion molecule catenin alpha-like 1 (CTNNAL1) in the lungs of ovalbumin-treated asthma

\* Corresponding author.

E-mail address: [xiangyang@csu.edu.cn](mailto:xiangyang@csu.edu.cn) (Y. Xiang).

<sup>1</sup> Di Wu and Lexin Liu contributed equally to this work.

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mouse model, highlighting the negative correlation between pulmonary resistance ( $R_L$ ) in asthmatic mice and mRNA levels of CTNNAL1 in the 8-day course of repeated ovalbumin (OVA) challenge [8]. Moreover, a negative correlation between the down-regulation of CTNNAL1 and airway hyperresponsiveness has been observed [10]. CTNNAL1 deficiency led to intensified lung inflammation and mucus hypersecretion through activating YAP-ROCK2 signaling pathway [11].

Inhaled and intranasal glucocorticoids (GCs) are the most common and effective drugs for controlling the symptoms of airway inflammation in respiratory diseases, such as allergic rhinitis, chronic rhinosinusitis [12] and asthma [13,14]. Unfortunately, up to 10 % of patients suffering from difficult-to-control asthma have shown unsatisfactory or even dismal clinical response, even though they are on the prescription of high-dose glucocorticoid. An advanced understanding of the molecular mechanisms of glucocorticoid resistance may pave the way to a more promising therapeutic approach for patients with severe asthma.

The anti-inflammatory effects of GCs are mediated through the glucocorticoid receptors (GRs), which bind to GCs cross the cell membrane and to form glucocorticoid/GR complexes in the cytoplasm. The glucocorticoid/GR complexes are then translocated to the nucleus to interact with glucocorticoid response elements (GREs), thereby elevating the expression of anti-inflammatory (transactivation) or pro-inflammatory (trans-repression) genes [15]. The bronchial epithelium, with abundant glucocorticoid receptors, is a primary target of the anti-inflammatory actions of GC [16].

To delve into the molecular mechanism of CTNNAL1 in the pathogenesis of asthma, we performed immunoprecipitation (IP) assay coupled with mass spectrometry to screen out the potential candidate proteins that interact with CTNNAL1. Our data confirmed the interaction between CTNNAL1 and hsp90, a process essential for the proper folding of the ligand-binding domain of GR. After the maturation and final folding of GR, the continued binding of hsp90 to GR would thwart the transcriptional activity of GR by reducing DNA binding and disrupting its formation of transcriptional complexes [17,18], thereby evoking glucocorticoid resistance. Furthermore, the aberrantly up-regulation of hsp90 could be partially attributable to the progressing severity of asthma. It has been reported that the inhibition of hsp90 diminished the expression of pro-inflammatory mediators via the deactivation of NF- $\kappa$ B signaling pathway [19].

In light of these findings, we hypothesized that CTNNAL1 might play a key role in mediating glucocorticoid sensitivity in the mouse model of asthma. We intend to probe the mechanism that underlies the reduced glucocorticoids sensitivity by carrying out a series of solid experimental assays on CTNNAL1-defective mouse model and airway epithelial cells model.

## 2. Material and methods

### 2.1. Ethics statement

This study was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol of animal study was approved by the Xiangya Animal Protection and Use Committee of Central South University. All surgeries were performed under sodium pentobarbital anesthesia, and every effort has been made to minimize the suffering of the animals.

### 2.2. AAV synthesis and transfection

The effective AAV5-CON305 (sequence: TTCTCCGAACGTGT-CACGT) and AAV5-CTNNAL1-RNAi (sequence: TCAGACAATAACTAA-GACA) were designed and synthesized by Shanghai Genechem (Genechem Inc., Shanghai, China). Mice were anesthetized and intratracheally injected with CTNNAL1-RNAi-AAV (containing  $10^{10}$  vector genome copies per injection) or AAV5-CON305 as control after one

week of adjustable feeding.

### 2.3. Animals and challenge protocols

C57bl/6J mice were obtained from the experimental animal center, Xiangya School of Medicine, Central South University. Twenty-four C57bl/6J mice (female, 8 weeks old, free of murine-specific pathogens) were obtained and housed under barrier conditions in air-filtered, temperature-controlled units under a 12 h light–dark cycle and were granted free access to food and water. Following the transfection, the HDM-stressed asthma model was established according to the protocol of the previous study [20]. At first, the mice were sensitized to purified HDM extract (10  $\mu$ g protein in 50  $\mu$ L saline; Greer Laboratories, Lenoir, NC) via intranasally (i.n.) on days 14, 21, 28 and 34. The allergen challenge was done via an atomization treatment of HDM on four consecutive days (days 35–38) and the mice were sacrificed 24 h after the last challenge (day 39). The control group received sterile PBS via i. n. route and atomization route on days 14, 21, 28, 34, 35, 36, 37 and 38.

### 2.4. Treatment of mice with DEX

DEX (Sigma) was dissolved in endotoxin-free saline and administered at a dose of 1 mg/kg by intraperitoneal injection in a volume of 0.1 mL. The treatment of DEX was given 30 min before the atomization of HDM.

### 2.5. Cell culture, transfection of siRNA, treatment of HDM, DEX and hsp90 inhibitor

The 16HBE14o- cells, which refer to SV40-transformed human central airway epithelial cells, were a generous gift of Dr. Dieter C Gruenert from University of California San Francisco [21]. The cells were cultured in a mixture medium of DMEM: F12 (1:1) (Sigma, St. Louis, MO), supplemented with 100  $\mu$ M streptomycin, 10 % fetal bovine serum (Sigma, St. Louis, MO), and 100  $\mu$ M penicillin, and were incubated at 37 °C in 5 % CO<sub>2</sub>. Human bronchial epithelial cells were seeded into six-well tissue culture plates at an appropriate density and were cultured in a conditioned medium until reaching sub-confluence. The cells were then washed twice with PBS, and the media was replaced with DMEM containing 5 % FBS. After 24 h of co-culturing, the medium was replaced with DMEM containing 10 % FBS. Simultaneously, the cell culture system was treated with HDM (100 pg, Sigma) for 24 h after the transfection. In the group of DEX treatment, the cell culture system was pretreated for 30 min with DEX (0.1  $\mu$ M/L, Sigma) before HDM treatment. The effective CTNNAL1 siRNA and nonsense siRNA were synthesized by Guangzhou Ribobio (Ribobio Inc., Guangzhou, China). Transfections were performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. In the group of inhibitor treatment, cells were pretreated for 24 h with HSP90 inhibitor geldanamycin (20  $\mu$ M) (Selleck Chemicals, USA) before incubation with HDM.

### 2.6. Lung histology and immunohistochemical staining

Paraffin-embedded lung tissue sections were stained by using hematoxylin and eosin (H&E) stain (Sigma). The inflammation score was measured independently by three pathologists, who were blinded to the grouping of experiment. The scores were averaged to obtain a final score [22]. The expression of targeted proteins in airway epithelium was detected via immunohistochemical staining on the lung tissue sections using the following primary antibodies: CTNNAL1 (Ab96184, Abcam), HSP90 (AF6126, Affinity) and NR3C1 (AF5004, Affinity).

Mouse lung paraffin sections were incubated with primary antibody and were subsequently incubated along with secondary antibody. The positive cells were brown and the positive particles were located on the part of antigen. The nuclei were stained blue by using hematoxylin. For

microscopy, a Moticam Pro Microscope (BA410E, Xiamen, China) was used. Images were obtained via Motic Images Plus3.0 (×64).

2.7. Bronchoalveolar lavage fluid (BALF) collection and cell counting

The lungs were lavaged twice by using 1 mL sterile PBS to collect 1 mL of BAL (bronchoalveolar lavage) fluid, which was immediately centrifuged at 1500 ×g. The total cell count was measured, and a cytospin preparation was performed. The cells were stained by using a Diff-Quick reagent (Baxter Dade). A differential count of 300 cells was performed by using the standard morphological criteria, and the accurate quantification of cells recovered was achieved by performing BAL lavage.

2.8. ELISA assay

Mouse IL-1 beta Quantikine ELISA Kit (#MLB00C), mouse IL-5 Quantikine ELISA Kit (#M5000), mouse IL-6 Quantikine ELISA Kit (#M6000B), mouse TNF-α Quantikine ELISA Kit (#MTA00B), human IL-1 beta Quantikine ELISA Kit (#DLB50), human IL-5 Quantikine ELISA Kit (#D5000B), human TSLP Quantikine ELISA Kit (#DTSLP0), human IL-8 Quantikine ELISA Kit (#D8000C), human IL-6 Quantikine ELISA Kit (#S6050) and human TNF-α Quantikine ELISA Kit (#DTA00D) were purchased from R&D systems (Minneapolis, Minnesota, USA). The levels of IL-1β, IL-5, IL-8, TSLP, IL-6 and TNF-α were determined, respectively, in accordance with the manufacturer's protocols.

2.9. Quantitative RT-PCR

Total RNA was extracted from whole-lung tissues of mice or human bronchial epithelial cells from different group by using Trizol (Invitrogen), according to the manufacturer's protocol. One milligram RNA was reverse transcribed into cDNA using AMV reverse transcriptase (Qiagen, Vento, Netherlands) with an RNase inhibitor and oligo d(T) primer (Invitrogen) at 40 °C for 50 min followed by heating at 90 °C for 5 min. Subsequently, 1 μL of reverse-transcript was added to a 30 μL PCR mixture for 40 cycles. Each cycle included 93 °C for 30 s, 54 °C for 60 s and Taq polymerase was used. Negative controls consisted of an equal volume of water substituted for the RNA in the RT reaction. The normalization of mRNA expression data for sample-to-sample variability in RNA input, RNA quality, and reverse transcription efficiency was measured by comparing the copy numbers of target gene and GAPDH. The primer sequences are listed in Table 1 and Table 2.

2.10. Western blot

Proteins from the lung tissues or HBE cells were extracted and analyzed using Western blotting as described in our previous study [23]. Western blotting analysis was performed as described previously. Frozen

Table 1  
Oligonucleotide primers for qRT-PCR.

| Mouse gene | Primer sequence                                                              |
|------------|------------------------------------------------------------------------------|
| CTNNAL1    | Forward: 5'-AGATGAGTGACATGGCGACG-3'<br>Reverse: 5'-CAGCCCGAGCTTTGCTATCT-3'   |
| HSP90      | Forward: 5'-GAAAGCCGCCTCAAACCTTC-3'<br>Reverse: 5'-TTCCTTCTTCTGCGGTCTT-3'    |
| ICAM1      | Forward: 5'-GAAAGCCGCCTCAAACCTTC-3'<br>Reverse: 5'-ATCTCGGGGACAGATCCTTGT-3'  |
| IL-5       | Forward: 5'-AAGCAATGAGACGATGAGGCT-3'<br>Reverse: 5'-CCCCACGGACAGTTTGATTCT-3' |
| IL-8       | Forward: 5'-TGGGTGAAGGCTACTGTTGG-3'<br>Reverse: 5'-AGCTTCATTGCCGGTGGA-3'     |
| NR3C1      | Forward: 5'-CAGTGAAGACAGCACAAT-3'<br>Reverse: 5'-TGGTATCGCCTTTGCCATT-3'      |
| GAPDH      | Forward: 5'-TTGCAGCTCCTTCGTGGC-3'<br>Reverse: 5'-GACCCATTCCACCATCACA-3'      |

Table 2  
Oligonucleotide primers for qRT-PCR.

| Human gene | Primer sequence                                                              |
|------------|------------------------------------------------------------------------------|
| CTNNAL1    | Forward: 5'-CTACACCAGCCATGAGCA-3'<br>Reverse: 5'-GCCTGAGTTGACAGTTCCA-3'      |
| HSP90      | Forward: 5'-GGATGGTCTGGCAACATGGA-3'<br>Reverse: 5'-CTCTGATCAGCGGGTGTCTG-3'   |
| ICAM-1     | Forward: 5'-GGGAACACCGGAAGGTGTAT-3'<br>Reverse: 5'-TGAGGTACAGGCCCTCTGAT-3'   |
| IL-5       | Forward: 5'-GGTGAAAGAGACCTTGGCACT-3'<br>Reverse: 5'-CTCCAGTGTGCCTATTCCCTG-3' |
| IL-8       | Forward: 5'-TCCTGATTCTGCAGCTCTGT-3'<br>Reverse: 5'-CCAGACAGAGCTCTCTTCCA-3'   |
| TSLP       | Forward: 5'-TCGTGGTGGGAAGAGTTAG-3'<br>Reverse: 5'-AAAGGGAACATACGTGGACA-3'    |
| NR3C1      | Forward: 5'-TGGTTTGTCAAGCCCCAGT-3'<br>Reverse: 5'-GCTGTCTTCCACTGCTCTT-3'     |
| GILZ       | Forward: 5'-CATGGAGTGGCGGTCTATC-3'<br>Reverse: 5'-CACCTCCTCTCTACAGCGT-3'     |
| MKP-1      | Forward: 5'-GTACATAAGTCCATCTGAC-3'<br>Reverse: 5'-GGTTCCTTAGGAGTAGACA-3'     |
| GAPDH      | Forward: 5'-GACGCTCACCCAGACA-3'<br>Reverse: 5'-TGACACCCACAGCAAGA-3'          |

lung tissue was homogenized and lysed in RIPA buffer (Sigma-Aldrich) containing protease inhibitor (Roche, Mannheim, Germany). 50 μg protein isolated from bronchial epithelial cells or lung tissue was separated by 10 % SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were incubated with the following primary antibodies: CTNNAL1 (Ab96184, Abcam), NF-κB (#48676, SAB), p-NF-κB-Ser276 (#11011, SAB), Hsp90 (#AF6126, Affinity), ICAM1 (#16174-1-AP, Proteintech), NR3C1 (#AF5004, Affinity), and p-NR3C1-Ser211 (#AF2004, Affinity). Subsequently, HRP-conjugated secondary antibody was added for 2-h incubation. Film density was measured using ImageJ densitometry software and was normalized against GAPDH (#60004-1-Ig, Proteintech).

2.11. Immunoprecipitation

Immunoprecipitation was performed according to the protocol of our previous publication [24]. Briefly, the protein was extracted from HBE cells with RIPA lysis buffer (Sigma-Aldrich). An appropriate dilution of anti-CTNNAL1 antibody (1:100; #Ab96184, Abcam) or anti-Hsp90 antibody (1:200; #AF6126, Affinity) was added to the centrifuge tube to conjugate with the suspension of Protein G. The antibody-bead mixture was incubated at 4 °C for 4 h on a tube rotator. Then, 50 μg of cell lysates were added into the mixtures. The lysate-bead/antibody conjugate mixtures were incubated overnight at 4 °C. The mixtures were further resuspended in a 5 × SDS loading buffer. The samples were boiled for 5 min and analyzed by performing Western blot. The immunocomplexes were stained with anti-CTNNAL1 antibody (#ab96184, Abcam) and Hsp90 antibody (#AF6126, Affinity).

2.12. Flow cytometry

Whole-lung cell suspensions were prepared for flow cytometric analysis. Briefly, the lung cell suspensions were prepared by enzymatically digesting the lung tissue using 5000 caseinolytic units of dispase (Discovery Labware Inc., Corning). Then, the single-cell suspensions were stained with the fluorochrome-labeled monoclonal antibodies, CD45 (Biolegend) and PE gating strategy was used. The cells were analyzed by flow cytometry within 1 h.

2.13. Leukocyte-16HBE14o-cell adherence assay

4 × 10<sup>5</sup> 16HBE14o-cells were added to each well at 37 °C under 5 % CO<sub>2</sub> in humidified air. Human fresh blood was drawn from healthy

donors and treated with erythrocyte lysate. After centrifugation at  $100 \times g$  for 10 min at  $4^\circ\text{C}$ , leukocytes were collected.  $2 \times 10^5$  leukocytes/well were added into 16HBE14o- culture for 12 h at  $37^\circ\text{C}$  under 5 %  $\text{CO}_2$  in humidified air. Non-adherent cells were removed and the remaining cells were collected, which were incubated with CD45-PE-cy7 (Biolegend) for 20 min at  $25^\circ\text{C}$ . After washing, the cells were resuspended in 300  $\mu\text{L}$  of PBS and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) with data stored in list mode files. The percentage of leukocytes binding to 16HBE14o- cells was evaluated first by live gating on 16HBE14o-cells using forward and side scatter, followed then by a combination of anti-CD45-PE-cy7 fluorescence and side scatter. Ten thousand events were measured in the gate and the data were analyzed using CELLQuest Pro software (Becton Dickinson, CA, USA).

#### 2.14. Mass spectrometry

The solubilized protein was centrifuged and the supernatant collected was then used in the assay of protein content according to the Bradford [25] method measured by UV-Visible Spectrophotometer (PerkinElmer, 940 Winter street, Waltham, Massachusetts 02451, USA). Proteins were separated on gel under reducing conditions.

Protein bands from SDS-PAGE were excised and analyzed by MALDI TOF/MS. The MALDI-MS data were acquired on an Ultraflex TOF/TOF spectrometer (Bruker Daltonics, Billerica, MA, USA, and Bremen, Germany), equipped with a 50 Hz pulsed nitrogen laser (337 nm), operated in positive ion reflectron mode using a 90-ns time delay, and a 25 kV accelerating voltage. External calibration was done using the peptide I calibration standard supplied by Bruker (peptides include angiotensin II, angiotensin I, substance P, bombesin, Adrenocorticotrophic hormone (ACTH) clip 1–17, ACTH clip 18–39, somatostatin 28; with masses ranging from 1000 to 3200 Da). The samples were prepared by mixing an equal amount of peptide (0.5 mL) with matrices, dihydroxybenzoic acid and  $\alpha$ -cyano-4-hydroxycinnamic acid saturated with 0.1 % trifluoroacetic acid (TFA) and acetonitrile (1:1).

Monoisotopic peptide masses were assigned and used in the database search. The protein identification was accomplished utilizing the Mascot search engine 31 (Matrix Science, London, UK). Scores  $>66$  were considered to be significant ( $P < 0.05$ ) in the Mascot search. Criteria used for protein identification in the Mascot software were: (1) Significant homology scores achieved in Mascot; (2) significant sequence coverage values; and (3) similarity between the protein molecular mass calculated from the gel and the identified protein.

#### 2.15. Statistical analysis

All experiments were run at a minimum of triplicates, and PRISM (GraphPad, La Jolla, CA, USA) statistical software was used for statistical analysis. Statistical differences between the two groups were determined by performing Mann-Whitney. The Kruskal-Wallis test determined differences among multiple groups. All data are presented in the form of mean values  $\pm$  standard deviation (SD). A significance level of  $P < 0.05$  was set.

### 3. Results

#### 3.1. CTNNAL1 deficiency aggravated HDM-stressed bronchopulmonary inflammation

AAV5-CTNNAL1-RNAi was used to silence CTNNAL1 in the bronchopulmonary tissues of the mice. The silencing efficiency of AAV5-CTNNAL1-RNAi would change with the elapse of time, therefore we detected the mRNA expression of CTNNAL1 at 2, 4, 6 and 8 weeks after AAV5-CTNNAL1-RNAi injection (Supplementary Fig. 1A), respectively. We found that the silencing efficiency of CTNNAL1 at 4 and 6 weeks post-injection was greater than that of 2 and 8 weeks post-injection. To

ensure a low expression of CTNNAL1 in the lung tissues, we constructed the HDM-induced asthma mouse model between 4 weeks to 6 weeks after injection of AAV5-CTNNAL1-RNAi. Our data showed that the protein and mRNA level of CTNNAL1 decreased approximately by 75 % and 70 % respectively in the CTNNAL1-silenced group (Fig. 1A, B, C). We found that the expression of CTNNAL1 was significantly lower than that found in the control group (Fig. 1D, E). Transfection of AAV5-CTNNAL1-RNAi did not cause body weight loss (Supplementary Fig. 1B).

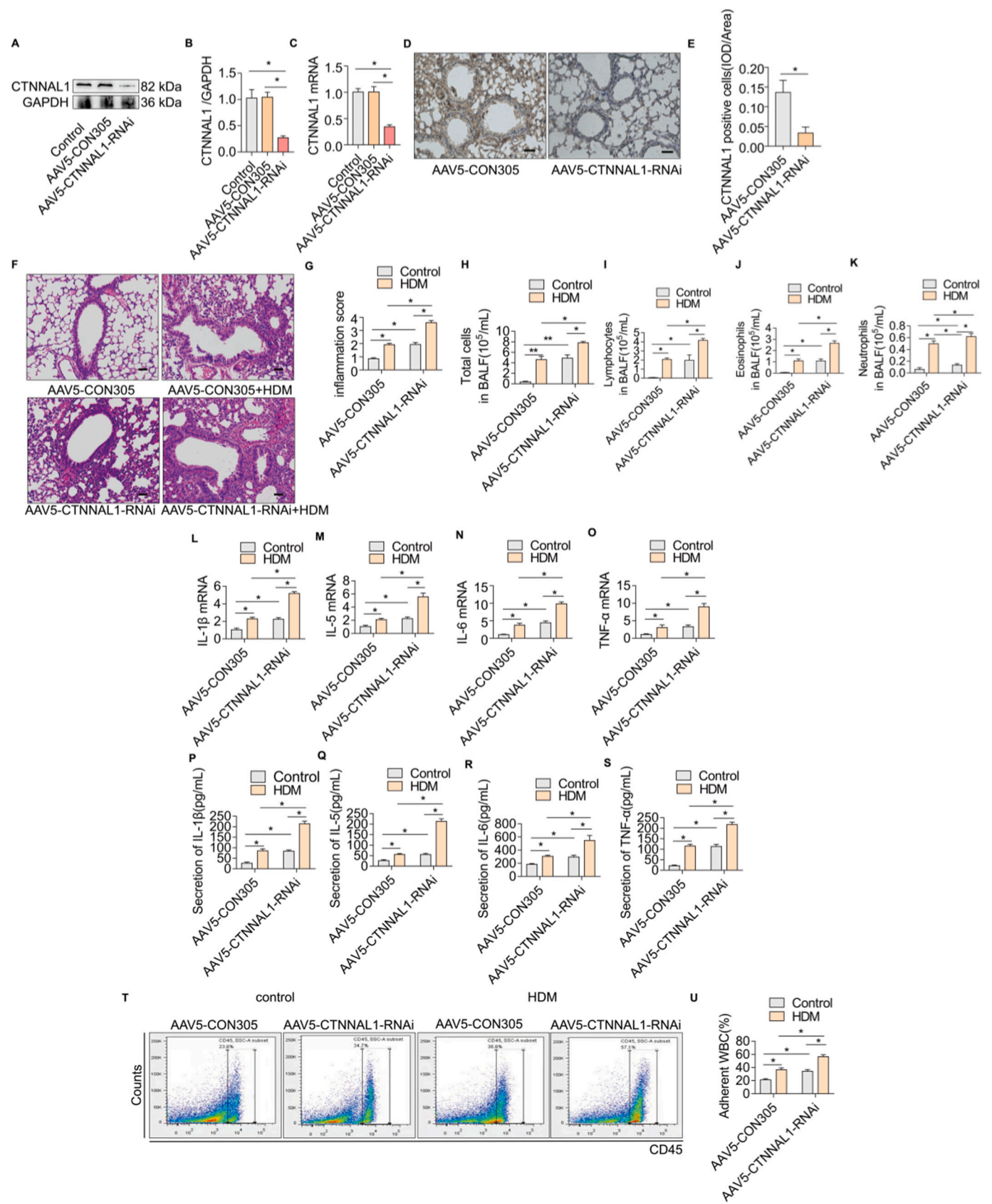
A larger number of inflammatory cells were observed in the perivascular, peribronchial, and parenchymal tissues of HDM-stressed CTNNAL1-silenced mice than in HDM-treated control mice. Both CTNNAL1 deficiency and HDM stimulation could increase the count of inflammatory cells infiltrated into the lungs (Fig. 1F, G). The total number of cells in the BAL fluid of the HDM-treated CTNNAL1-silenced mice group was higher than any other groups. The differential cell count analysis showed a larger number of lymphocytes, neutrophils and eosinophils in the BAL fluid of the HDM-treated CTNNAL1-silenced mice. The number of lymphocytes and eosinophils in the HDM-treated control mice and CTNNAL1-silenced mice were significantly higher than control mice. In addition, the number of neutrophils in HDM-treated control group was larger than control and CTNNAL1-silenced groups (Fig. 1H–K).

Moreover, the levels of pro-inflammatory cytokines, including IL-1 $\beta$ , IL-5, IL-6 and TNF- $\alpha$ , were remarkably increased in CTNNAL1-silenced mice and HDM-stressed control mice, as compared to control mice. Consistently, the secretion of cytokines was increased significantly in HDM-stressed CTNNAL1-silenced group compared with HDM-stressed control group (Fig. 1L–S). Flow cytometry results showed that the count of stained inflammatory cells infiltrating into lungs of CTNNAL1-silenced mice was significantly higher than that in the control mice. Meanwhile, the number of inflammatory cells in HDM-stressed CTNNAL1-silenced group was notably larger than HDM-treated control group (Fig. 1T, U). PAS staining of lung tissues showed goblet cell hyperplasia and mucus overproduction in the airway mucosa of HDM-treated control mice and CTNNAL1-silenced mice compared with control mice. There was a marked increase in mucus over-production of HDM-treated CTNNAL1-silenced mice compared with HDM-treated control mice (Supplementary Fig. 1G).

#### 3.2. CTNNAL1 deficiency enhanced inflammatory reactions in HDM stressed airway epithelial cells

As the first barrier to exogenous stimuli, airway epithelial cells need to withstand oxidative stress and chronic inflammatory stimuli [26,27]. Accumulating evidence indicated that epithelium dysfunction is involved in the pathogenesis of lung inflammation disorders, such as asthma [28]. To further verify whether CTNNAL1 deficiency could lead to increased inflammatory response in vitro, we constructed CTNNAL1-silenced 16HBE14o- cells by small interfering RNA (siRNA) transfection. Both mRNA and protein levels of CTNNAL1 expression significantly decreased after CTNNAL1 was silenced (Fig. 2A–D). Both CTNNAL1 deficiency and HDM stimulation could stimulate the secretion of IL-1 $\beta$ , IL-5, IL-8, TSLP, IL-6 and TNF- $\alpha$  in 16HBE14o- cells. Furthermore, the secretion of cytokines was significantly increased in HDM-stressed CTNNAL1 $^{-/-}$  group, compared with HDM-stressed CTNNAL1 $^{+/+}$  group (Fig. 2E–P).

In the progression of allergic airway diseases, bronchial epithelial cells could activate and transmit inflammatory signals by releasing chemokines and recruiting white blood cells (WBC) [29]. To investigate the effect of airway epithelial cells on recruiting leukocytes, we examined the adhesion rate of leukocytes with airway epithelial cells by performing flow cytometry. A higher number of leukocytes were attached to airway epithelial cells in CTNNAL1 $^{-/-}$  group and HDM-treated CTNNAL1 $^{+/+}$  group, compared with CTNNAL1 $^{+/+}$  group. Upon HDM stimulation, the adhesion rate was significantly increased in



(caption on next page)

**Fig. 1.** CTNNAL1 deficiency enhanced HDM-stressed bronchopulmonary inflammation. We named AAV5-CON305 as control group, and named AAV5-CTNNAL1-RNAi group as CTNNAL1-silenced group. (A, B) CTNNAL1 protein expression in the lungs of mice on day 34 after adeno-associated virus infection was detected by Western blot. Significant inhibition of CTNNAL1 was detected in the CTNNAL1-silenced mice ( $n = 5$ ). (C) CTNNAL1 mRNA expression in the lungs of mice on day 34 after adeno-associated virus infection was detected via RT-qPCR ( $n = 5$ ). (D, E) CTNNAL1 expression in the airway epithelium was detected by immunohistochemistry ( $n = 5$ ;  $\times 200$ , Bars = 100  $\mu\text{m}$ ). (F) Lung histopathology in the AAV5-CON305 or AAV5-CTNNAL1-RNAi mice exposed to HDM or PBS as a vehicle control. Lung sections were stained with HE ( $n = 5$ ;  $\times 200$ , Bars = 100  $\mu\text{m}$ ). (G) Inflammation score was measured independently by three pathologists blinded to the experiment ( $n = 5$ ). (H–K) The inflammatory cell profile in the BAL fluid was evaluated. Total cells, neutrophils, lymphocytes and eosinophils were enumerated ( $n = 6$ ). (L–P) mRNA expression of IL-1 $\beta$ , IL-5, IL-6 and TNF- $\alpha$  in the lungs were detected by RT-qPCR ( $n = 5$ ). (Q–U) The secretion of IL-1 $\beta$ , IL-5, IL-6 and TNF- $\alpha$  in the lungs were examined by ELISA kits ( $n = 5$ ). (V, W) After the last HDM challenge, CD45 molecule expression level in the lung tissues was analyzed by flow cytometry ( $n = 5$ ). \* $P < 0.05$ .

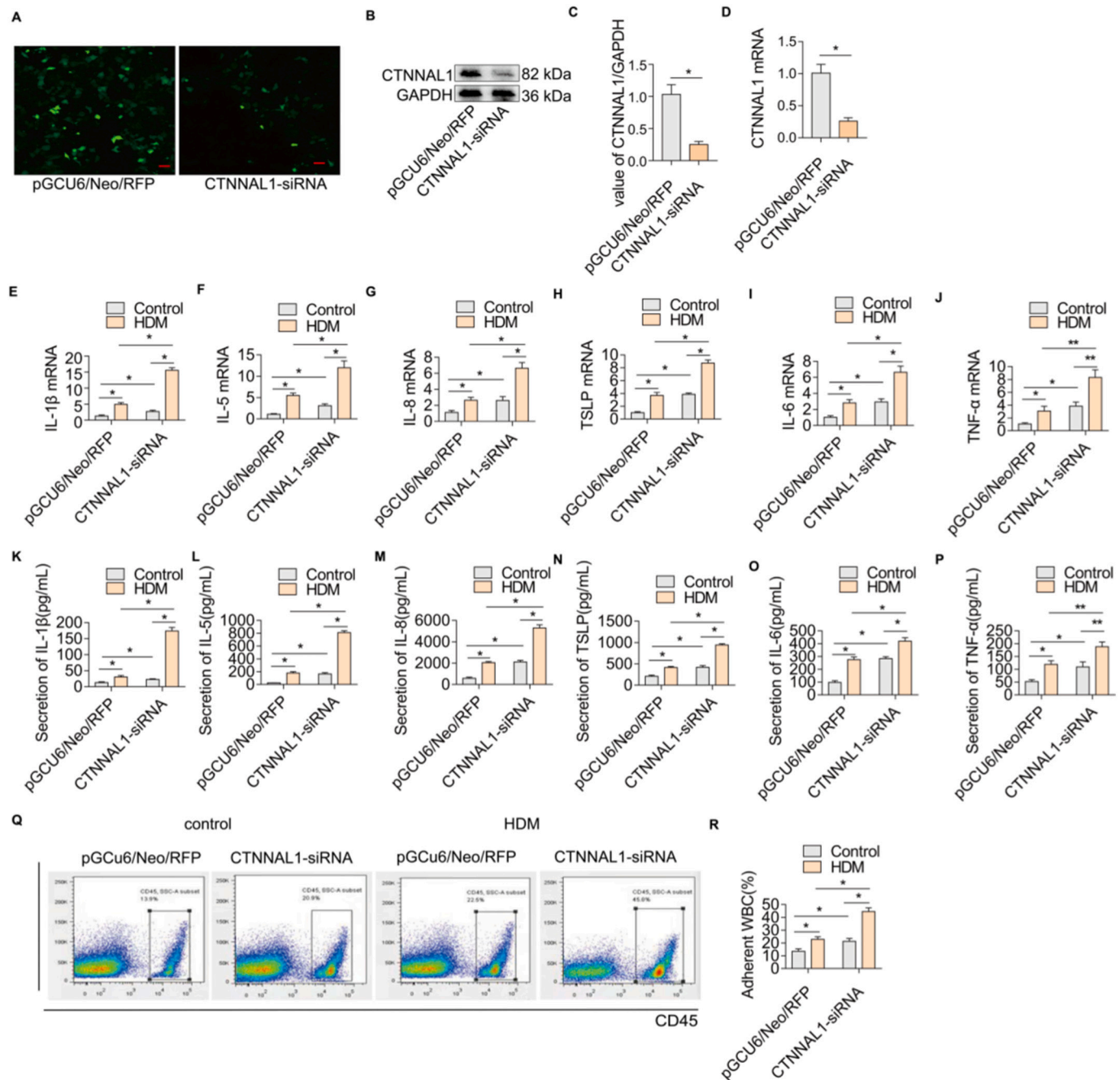


**Fig. 2.** CTNNAL1 silencing enhanced inflammatory reactions in HDM stressed airway epithelial cells. We named pGCU6/Neo/RFP as CTNNAL1<sup>+/+</sup> and named CTNNAL1-siRNA as CTNNAL1<sup>-/-</sup>. (A) Level of CTNNAL1 was assessed by immunofluorescence ( $n = 6$ ;  $\times 200$ , Bars = 100  $\mu\text{m}$ ). (B–C) Expression of CTNNAL1 in 16HBE14o-cells was detected via Western blot ( $n = 6$ ). (D) Expression of CTNNAL1 mRNA in 16HBE14o-cells was detected by RT-qPCR ( $n = 6$ ). (E–J) mRNA expression of IL-1 $\beta$ , IL-5, IL-8, TSLP, IL-6 and TNF- $\alpha$  in 16HBE14o-cells were detected by RT-qPCR ( $n = 6$ ). (K–P) The IL-1 $\beta$ , IL-5, IL-8, TSLP, IL-6 and TNF- $\alpha$  concentrations in the cellular supernatant were assayed by ELISA ( $n = 6$ ). (Q–R) The adhesion rate of white blood cell with airway epithelial cells was examined by flow cytometry ( $n = 6$ ). \* $P < 0.05$ .

CTNNAL1<sup>-/-</sup> group compared to CTNNAL1<sup>+/+</sup> group (Fig. 2Q, R).

### 3.3. CTNNAL1 deficiency increased the expression of ICAM-1 and phosphorylation level of NF- $\kappa$ B

The detection of the expression of inflammatory molecules in the lungs of CTNNAL1-silenced mice revealed that the phosphorylation level of NF- $\kappa$ B was notably elevated, indicating the activation of NF- $\kappa$ B

signaling pathway. A higher phosphorylation level of NF- $\kappa$ B was observed in HDM-stressed CTNNAL1-silenced group compared to other groups (Fig. 3A, B). CTNNAL1 deficiency and HDM stimulation both could increase the ratio of p-p65/p65. Notably, it was significantly higher in HDM-stressed CTNNAL1-silenced group compared with HDM-stressed control group (Fig. 3C). Consistent with these *in vivo* results, the significant activation of NF- $\kappa$ B signaling pathway was noted in CTNNAL1-silenced HBE cells, which were exposed to HDM (Fig. 3F, H).

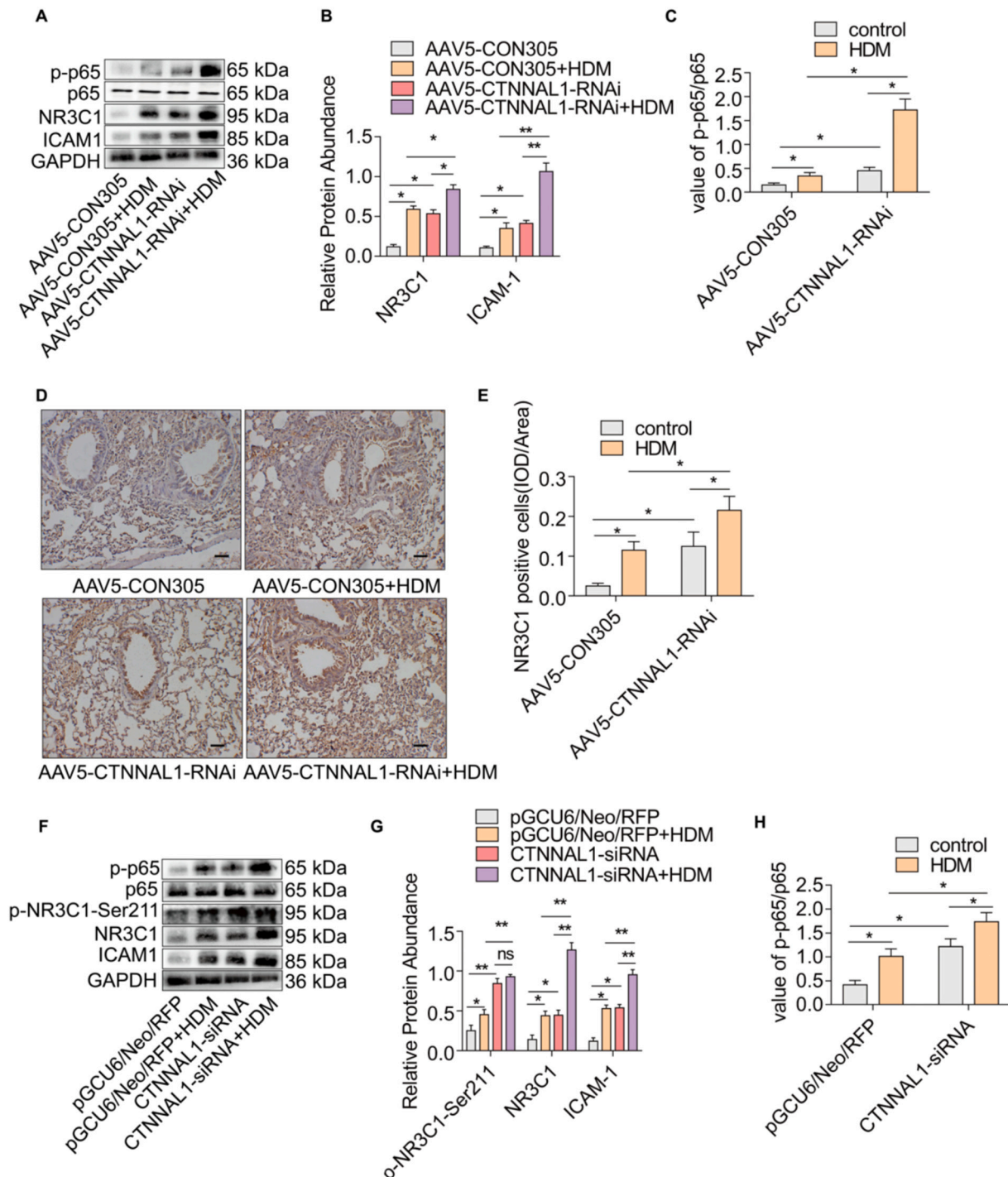


**Fig. 3.** CTNNAL1 deficiency increased p-NF- $\kappa$ B, ICAM1 and NR3C1 expression (A) p65, ICAM-1 and NR3C1 protein expression and phosphorylation of p65 in the lungs were detected by Western blot. (B) The values of ICAM-1/GAPDH and NR3C1/GAPDH were assessed (n = 6). (C) The ratio of p-p65/p65 was assessed (n = 6). (D, E) NR3C1 expression in the airway epithelium was detected by immunohistochemistry (n = 6;  $\times 200$ , Bars = 100  $\mu$ m). (F) p65, NR3C1 and ICAM-1 protein expression, and phosphorylation levels of p65 and NR3C1-Ser211 in the HBE cells were detected by Western blot. (G–H) The values of NR3C1/GAPDH, ICAM-1/GAPDH, p-NR3C1-Ser211/GAPDH and p-p65/p65 were assessed (n = 6). \*P < 0.05 and \*\*P < 0.01.

ICAM-1 is a cell surface glycoprotein and an adhesion receptor known for activating leukocyte recruitment from circulation to the sites of inflammation [30]. There is increasing evidence that NF- $\kappa$ B influences the inflammatory process by regulating the levels of cytokines and chemokines, including ICAM-1, IL-6, IL-8 and TNF- $\alpha$  [31,32]. Our in vivo and in vitro data demonstrated that CTNNAL1 deficiency and HDM stimulation both could upregulate the expression of ICAM-1. Meanwhile, we noted a higher expression of ICAM-1 in HDM-treated CTNNAL-silenced group than in HDM-stressed CTNNAL1<sup>+/+</sup> group

(Fig. 3A, B, F and G).

### 3.4. CTNNAL1 deficiency increased the expression of NR3C1 and phosphorylation of NR3C1

Glucocorticoids participate in a diverse array of physiological processes, including immune-modulatory or anti-inflammatory responses, and play a key role in the pathogenesis of inflammatory diseases [33]. Most of these physiological and pharmaceutical actions are mediated

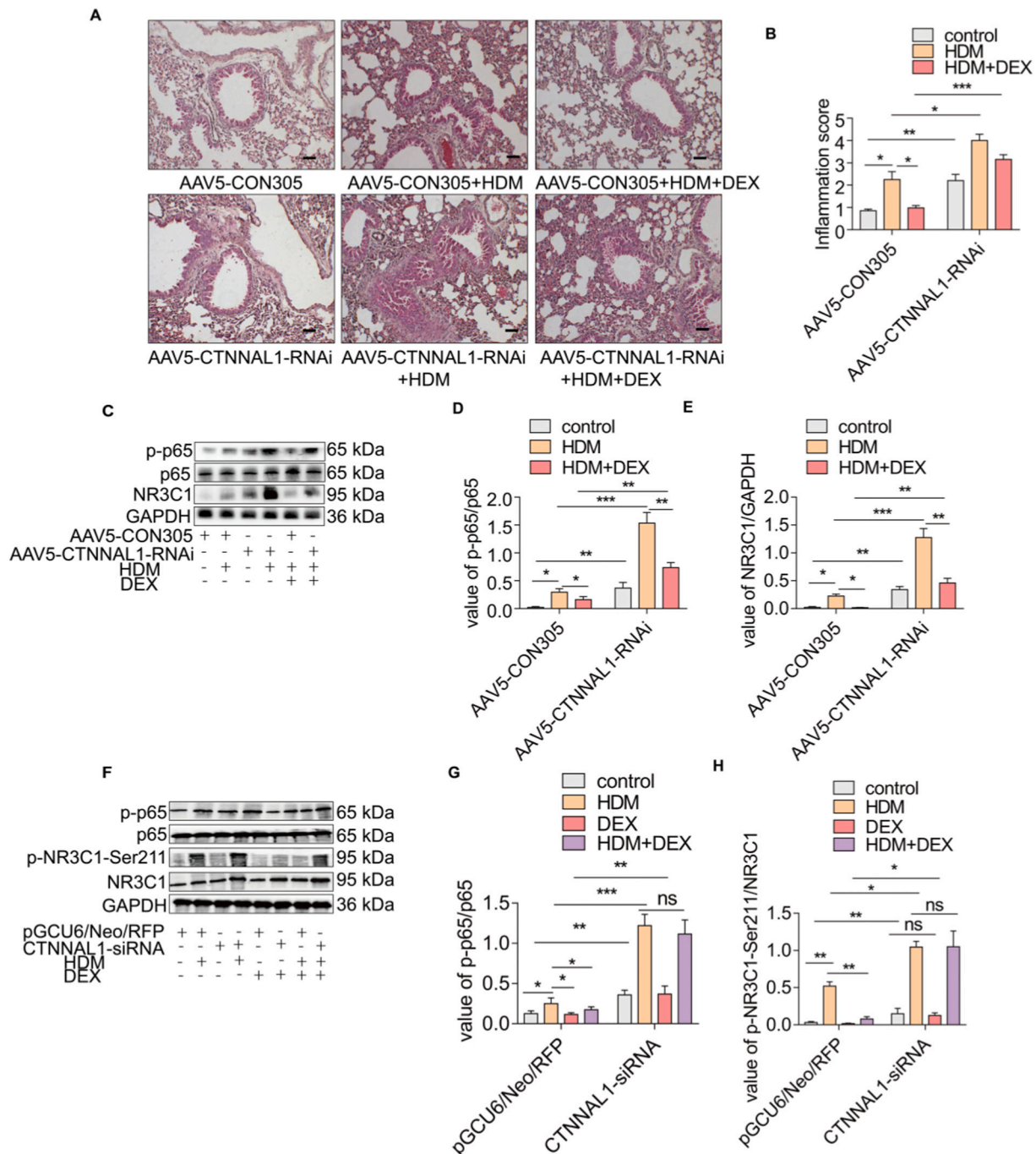


**Fig. 4.** CTNNAL1 deficiency decreased the sensitivity to dexamethasone in vivo and in vitro upon exposure to HDM (A) Lung histopathology in the AAV5-CON305 or AAV5-CTNNAL1-RNAi mice exposed to HDM or PBS as a vehicle control. DEX treated AAV5-CON305 + HDM and AAV5-CTNNAL1-RNAi + HDM mice group. Lung sections were stained with HE (n = 5;  $\times 200$ , Bars = 100  $\mu$ m). (B) Inflammation score was measured independently by three pathologists blinded to the experiment (n = 5). (C-E) Levels of p-p65, p65 and NR3C1 in lungs were detected via Western blot. The values of NR3C1/GAPDH and p-p65/p65 were assessed (n = 5). (F-H) Levels of p-p65, p65, p-NR3C1-Ser211 and NR3C1 in HBE cells were detected via Western blot. The values of p-p65/p65 and p-NR3C1-Ser211/NR3C1 were assessed (n = 5). \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001.

through glucocorticoid receptor (GR) [34,35]. GR is encoded by the nuclear receptor subfamily 3 group C member 1 (NR3C1) gene [36]. Both CTNNAL1 deficiency and HDM stress increased expression of NR3C1. Moreover, NR3C1 expression was significantly increased in HDM-stressed CTNNAL1-silenced group compared with HDM-stressed control group (Fig. 3A, B, D and E). Consistent with these *in vivo* results, both CTNNAL1 deficiency and HDM stress elevated the expression of NR3C1 in HBE cells. Meanwhile, the expression of NR3C1 was significantly up-regulated in HDM-challenged CTNNAL1<sup>-/-</sup> group, as compared with HDM-treated CTNNAL1<sup>+/+</sup> group (Fig. 3F, G).

The phosphorylation of GR induced by several kinases could reduce its binding capacity, stability and translocation to the nucleus, thereby contributing to glucocorticoid resistance [37]. Notably, the phosphorylation of NR3C1 on Ser211 correlates with transcriptional activity of glucocorticoid receptor [38]. We tested the phosphorylation ratio of NR3C1 in airway epithelial cells receiving 30-min HDM stimulation. Our results showed that the phosphorylation level of NR3C1-Ser211 in airway epithelial cells was increased upon HDM stimulation and CTNNAL1 deficiency. Moreover, the phosphorylation rate of NR3C1-Ser211 was significantly increased in HDM-stressed CTNNAL1<sup>-/-</sup> group. However, no significant disparate phosphorylation level of NR3C1-Ser211 was detected between the CTNNAL1<sup>-/-</sup> group and HDM-stressed CTNNAL1<sup>-/-</sup> group (Fig. 3F, G).

To clarify the effect of CTNNAL1 silencing on GR function, the transcription of GR target genes, like glucocorticoid-induced leucine zipper (GILZ) and map kinase phosphatase-1 (MKP-1), were measured. Both HDM stimulation and CTNNAL1 deficiency could decrease mRNA expression of GILZ and MKP-1. Separately, the mRNA expression levels of GILZ and MKP-1 significantly decreased in CTNNAL1-silenced HBE cells upon HDM stimulation (Supplementary Fig. 1E, F).

### 3.5. CTNNAL1 deficiency decreased the sensitivity to dexamethasone *in vivo* and *in vitro* upon exposure to HDM

The therapeutic effect of DEX on airway inflammation of CTNNAL1-silenced mice was assessed by performing H&E staining. As shown in Fig. 4A, a significantly increased number of inflammatory cell infiltration was observed around the airway in HDM-treated control mice, yet it was significantly assuaged by DEX treatment. The count of inflammatory cells infiltrated into lung tissues of HDM-stressed CTNNAL1-silenced mice was increased significantly but was slightly reduced by DEX treatment. That is to say, DEX exhibited a weakened effect on airway inflammation in HDM-challenged CTNNAL1-silenced mice (Fig. 4A, B). Moreover, DEX treatment significantly decreased p-NF-κB/NF-κB ratio in control + HDM group. However, after DEX treatment and HDM exposure, the level of p-NF-κB/NF-κB in CTNNAL1-silenced group was higher than control group (Fig. 4C, D).

Meanwhile, *in vitro* results found that DEX treatment significantly decreased the level of p-NF-κB/NF-κB in CTNNAL1<sup>+/+</sup>+HDM group. After DEX treatment, the ratio of p-NF-κB/NF-κB in the CTNNAL1<sup>-/-</sup> group was notably higher than CTNNAL1<sup>+/+</sup> group. Moreover, after DEX treatment, the ratio of p-NF-κB/NF-κB did not significantly decrease in CTNNAL1<sup>-/-</sup> + HDM group (Fig. 4F, G).

We also found that the ratio of p-NR3C1-Ser211/NR3C1 in CTNNAL1<sup>+/+</sup>+HDM group was significantly decreased after DEX treatment. The level of p-NR3C1-Ser211/NR3C1 in the CTNNAL1<sup>-/-</sup> group was higher than CTNNAL1<sup>+/+</sup> group after DEX treatment. DEX treatment exhibited negligible influence on the level of p-NR3C1-Ser211/NR3C1 in CTNNAL1<sup>-/-</sup> + HDM group (Fig. 4F, H). These results demonstrated that CTNNAL1 deficiency diminished the responses to glucocorticoids both *in vivo* and *in vitro*.

### 3.6. CTNNAL1 deficiency elevated hsp90 expression

To ascertain the mechanism that CTNNAL1 deficiency decreased the sensitivity to glucocorticoid, we screened the molecules that directly

interacted with CTNNAL1. The Mass Spectrometry Sequencing Diagram illustrated that a protein score >66 exhibits a significant difference (outside of the green shadow, Supplementary Fig. 1C). The top-10 list of Protein Score was shown in Supplementary Fig. 1D. Hsp90, which was found to interact directly with CTNNAL1, was selected from the sequencing results. Furthermore, immunoprecipitation assay was carried out to validate the possible interaction between CTNNAL1 and hsp90 in the airway epithelial cells (Fig. 5A, B). The results showed that the expression of hsp90 was increased in CTNNAL1-silenced mice and HDM-stressed control mice, and it was significantly increased in HDM-stressed CTNNAL1-silenced mice compared with HDM-treated control mice (Fig. 5C, D), and a consistent result was observed by immunohistochemistry (Fig. 5E, F). CTNNAL1 deficiency and HDM exposure elevated the hsp90 expression in HBE cells. Moreover, hsp90 expression in HDM-stressed CTNNAL1<sup>-/-</sup> group was significantly increased compared with HDM-stressed CTNNAL1<sup>+/+</sup> group (Fig. 5G, H).

### 3.7. Geldanamycin decreased the expression of ICAM-1 and NR3C1, and the phosphorylation level of p65 in HBE cells when CTNNAL1 was silenced

To verify whether CTNNAL1 deficiency triggers airway inflammation through hsp90 signaling pathway, CTNNAL1-defective HBE cells were treated with hsp90 inhibitor (geldanamycin). We observed that the expression of ICAM-1 and NR3C1, and the phosphorylation level of p65 in CTNNAL1-silenced HBE cells were decreased after geldanamycin treatment. Furthermore, geldanamycin treatment reduced the levels of p-p65, ICAM-1 and NR3C1 in the CTNNAL1-silenced HBE cells after HDM-stimulation (Fig. 6A–D).

### 3.8. CTNNAL1 deficiency decreased responses to glucocorticoids through hsp90 signaling pathway

Both geldanamycin and DEX treatments inhibited the secretion of IL-1β, IL-5, IL-8, IL-6 and TNF-α in CTNNAL1<sup>+/+</sup>+HDM group. The combined treatment of geldanamycin and DEX significantly dwindled the secretion of inflammatory cytokines, compared with monotherapy of geldanamycin or DEX. The secretion of IL-1β, IL-5, IL-8, IL-6 and TNF-α in CTNNAL1<sup>-/-</sup> + HDM group was partially inhibited by geldanamycin or DEX treatment. Notably, the suppressive effect of DEX treatment on the secretion of inflammatory cytokines in CTNNAL1<sup>-/-</sup> + HDM group was inferior to that of geldanamycin. Compared with DEX monotherapy, the combined treatment of geldanamycin plus DEX could more potently suppress the expression of inflammatory cytokines. Interestingly, DEX treatment displayed a negligible effect on the secretion of IL-5 in CTNNAL1<sup>-/-</sup> + HDM group (Fig. 6E–I).

Subsequently, we examined the effect of geldanamycin monotherapy or DEX monotherapy on the production of inflammatory molecules. We noted that the phosphorylation of p65 and the expression of ICAM-1 were partially inhibited by geldanamycin treatment in CTNNAL1<sup>+/+</sup> + HDM group and CTNNAL1<sup>-/-</sup> + HDM group. However, the use of DEX alone displayed a slight effect on the phosphorylation of p65 and the expression of ICAM-1 in CTNNAL1-silenced HBE cells post-HDM stimulation. In addition, the combined treatment using DEX and geldanamycin significantly down-regulated the levels of p-p65 and ICAM-1 in CTNNAL1-silenced HBE cells upon HDM stimulation (Fig. 6J–L). All these results indicated that CTNNAL1 deficiency decreased glucocorticoids sensitivity through triggering hsp90 signaling pathway. A schematic model of CTNNAL1 deficiency decreased glucocorticoid sensitivity in HBE cells through up-regulating hsp90 signaling pathway as shown in Fig. 7.

## 4. Discussion

The current study intends to explore the biological effect of CTNNAL1 deficiency on airway inflammation and the mediation of glucocorticoid sensitivity. We applied immunoprecipitation and mass

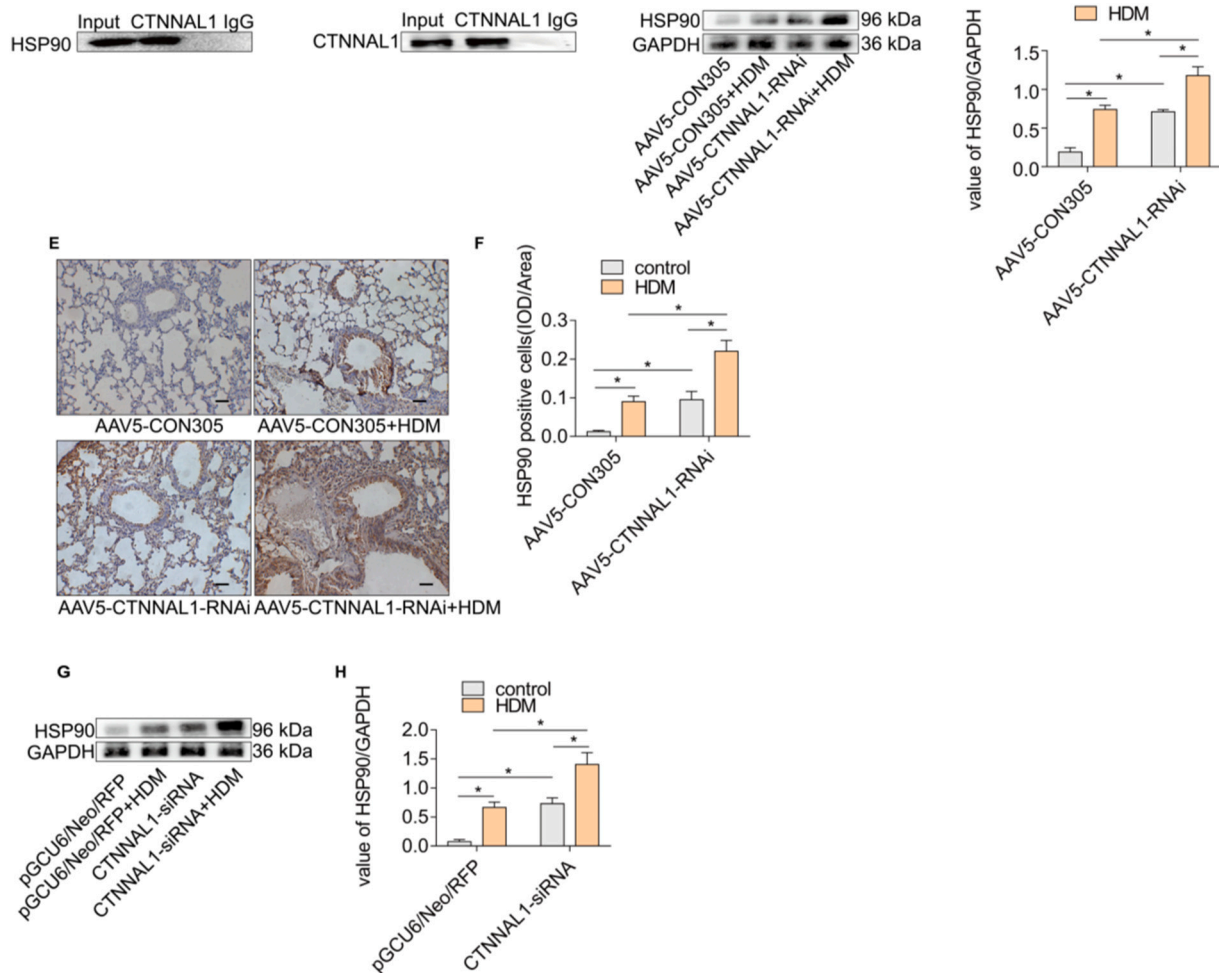


**Fig. 5.** CTNNAL1 deficiency elevated hsp90 expression. (A, B) Interaction between CTNNAL1 and hsp90 was assessed by immunoprecipitation on airway epithelial cells ( $n = 5$ ). (C, D) Hsp90 protein expression in the lungs was detected by Western blot ( $n = 6$ ). (E, F) Hsp90 expression in the airway epithelium was detected by immunohistochemistry ( $n = 5$ ;  $\times 200$ , Bars = 100  $\mu\text{m}$ ). (G, H) Hsp90 protein expression in the HBE cells was detected by Western blot ( $n = 6$ ).  $*P < 0.05$ .

spectrometry to screen the molecules which interact with CTNNAL1 (Fig. 7). We confirmed that hsp90 is the most promising candidate.

Hsp90 belongs to the heat shock family of molecular chaperones that regulate protein conformation and activity [39,40]. Hsp90 regulates a variety of cell signaling pathways by mediating the abundance and activity of essential protein kinases and cell cycle-related proteins [41]. The inhibition of hsp90 could induce the activation of Rho GTPase, leading to a dramatic formation of actin stress fiber in human tumor cell lines [42]. Conversely, the deactivation of Rho could prevent geldanamycin-induced actin reorganization. Hsp90 plays an important and specific role in regulating Rho activity and Rho-dependent actin cytoskeleton remodeling [42]. Notably, CTNNAL1 was identified as a part of the Rho signaling pathway, a member of the dbl. family of the Rho guanine nucleotide exchange factors (GEFs) [43]. When CTNNAL1 was silenced, the expression of hsp90 increased both in vivo and in vitro. In addition, CTNNAL1 deficiency significantly increased the expressions of hsp90, ICAM-1, NR3C1 and the ratio of p-p65/p65, upon HDM exposure.

The secretion of several pro-inflammatory chemokines, cytokines, adhesion molecules, respiratory mucins, growth and angiogenic factors could be induced via the Rel/Nuclear Factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) family of transcription factors [44]. NF- $\kappa\text{B}$  is a redox-sensitive transcription factor participating in the extensive inflammatory network to regulate cytokine activities [45]. It has been reported that the inhibition of hsp90 could diminish NF- $\kappa\text{B}$  activation in human myeloid leukemia THP-1 cells [46], and human lung endothelial cells [47]. In this study, the

phosphorylation level of NF- $\kappa\text{B}$  was significantly increased in the lungs of HDM-stressed CTNNAL1-silenced mice. The inhibition of hsp90 has been shown to inhibit NF- $\kappa\text{B}$  phosphorylation in CTNNAL1-silenced HBE cells.

In endothelial cells, NF- $\kappa\text{B}$  induces ICAM-1 expression, in the presence of stimulation by TNF- $\alpha$  or IL-1 $\beta$  [48]. The induction of ICAM-1 gene transcription has previously been shown to be dependent on NF- $\kappa\text{B}$  activation and its binding to ICAM-1 promoter [49]. NF- $\kappa\text{B}$  is responsible for the transcriptional activation of a set of genes that mediate inflammation, including ICAM-1, IL-6, TNF- $\alpha$  and IL-8. @@@@Our data showed that HDM stimulation or CTNNAL1 deficiency both could increase the expression of NF- $\kappa\text{B}$  target genes, like ICAM-1, IL-6, IL-8 and TNF- $\alpha$ .

ICAM-1 is a key regulatory player of cellular responses in inflammation, functioning to mediate the recruitment of leukocytes from circulation to the sites of inflammation [50,51]. The up-regulation of ICAM-1 is often induced in epithelial and immune cells to cope with inflammatory stimulation [52]. More importantly, ICAM-1 serves as a biosensor to transduce outside-in-signaling via the actin cytoskeleton, following ligand engagement of the extracellular domain [53]. Our results demonstrated that CTNNAL1 deficiency and HDM stimulation both functioned to elevate ICAM-1 expression. Intriguingly, CTNNAL1 deficiency in 16HBE14o-cells induced leukocytes to attach to airway epithelial cells. Consistently, bronchial epithelial cells play an immunomodulatory role by expressing ICAM-1 on cell surface in the progression of allergic airway diseases [54]. Using hsp90 inhibitor

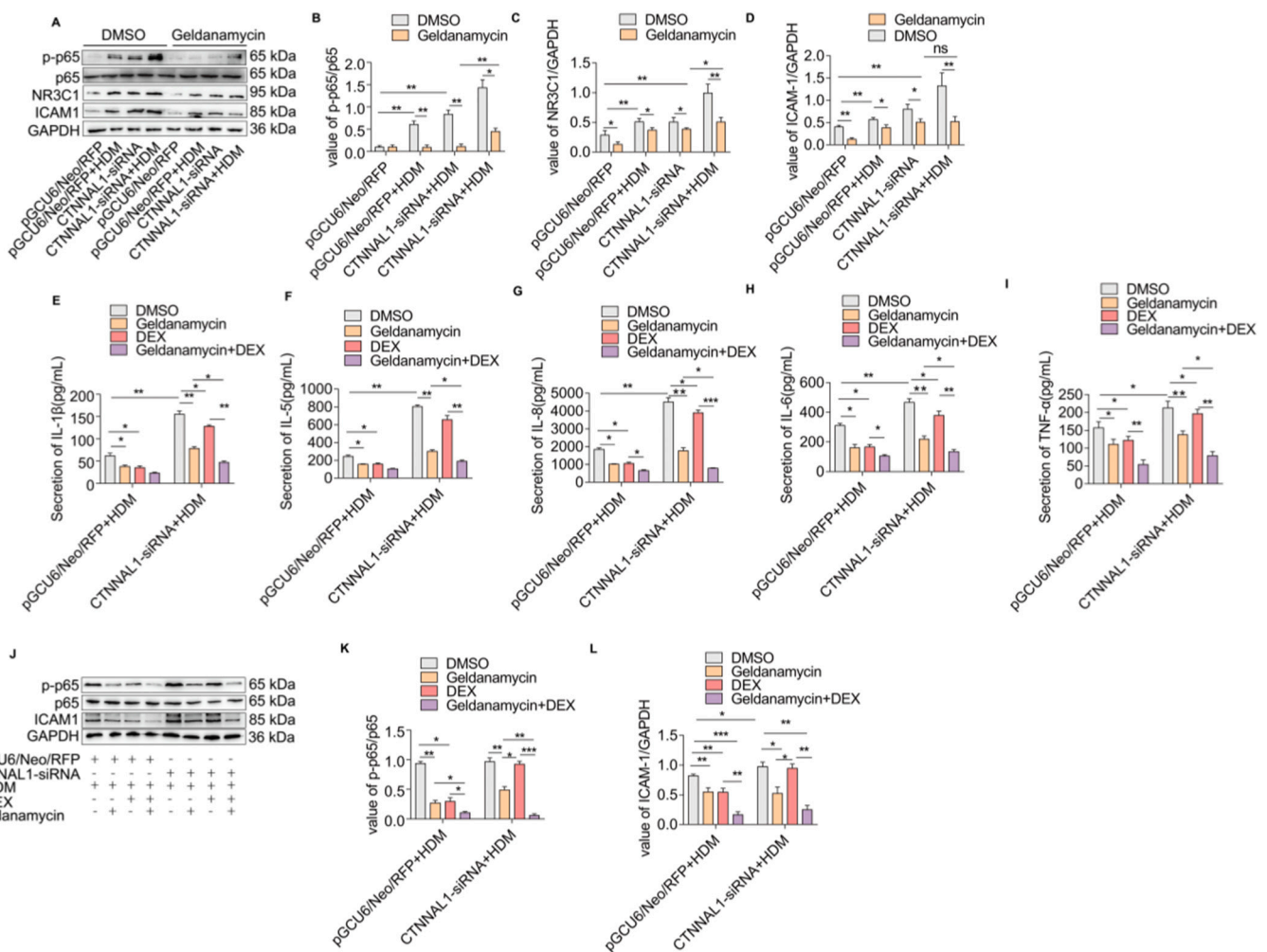


**Fig. 6.** Geldanamycin decreased the levels of p-NF- $\kappa$ B, NR3C1 and ICAM1. (A–D) Levels of p-p65, p65, NR3C1 and ICAM-1 in HBE cells were detected via Western blot. The values of p-p65/p65, NR3C1/GAPDH and ICAM-1/GAPDH were assessed ( $n = 6$ ). (E–I) The IL-1 $\beta$ , IL-5, IL-8, IL-6 and TNF- $\alpha$  concentrations in the cellular supernatant were assayed by ELISA kits ( $n = 6$ ). (J–L) Levels of p-p65, p65 and ICAM-1 in HBE cells were detected by Western blot. The values of p-p65/p65 and ICAM-1/GAPDH were assessed ( $n = 6$ ). ns, no significance, \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

geldanamycin decreased ICAM-1 expression in CTNNAL1-silenced HBE cells. These findings verified that CTNNAL1 deficiency increased ICAM-1 expression through hsp90 signaling pathway.

Glucocorticoids are widely used to treat inflammatory lung diseases, such as asthma and COPD [55]. GCs, by binding to the glucocorticoid receptor (GR) upon activation, are translocated to the nucleus to mediate gene expression [56]. Moreover, GCs could be used to inhibit airway inflammation through regulating the activities of human bronchial epithelial cells [57].

Glucocorticoid receptor (GR), a ligand-activated transcription factor involved in critical biological processes, is a well-known obligate hsp90 client [58]. In vivo, hsp90 is required for GR to bind ligand and to become active [59]. Hsp90 promoted the nuclear translocation of GR upon the formation of GR-hsp90 complex. After maturation and final folding of GR, the continued binding of hsp90 to GR would thwart the transcriptional activity of GR by reducing DNA binding and disrupting its formation of transcriptional complexes [17,18], thereby evoking glucocorticoid resistance. Our results demonstrated that GR (NR3C1) expression was elevated after CTNNAL1 was silenced in vitro and in vivo, and that CTNNAL1 deficiency significantly increased NR3C1 expression after HDM stimulation. To further demonstrate the effect of CTNNAL1 silencing on GR function, we examined the phosphorylation level of NR3C1 in CTNNAL1-silenced HBE cells to investigate the activation of NR3C1. Notably, our results demonstrated that the

phosphorylation of NR3C1-Ser211 was also significantly increased in CTNNAL1<sup>-/-</sup> group and HDM-stressed CTNNAL1<sup>-/-</sup> group, which could mostly be attributable to the phosphorylation of GR by p38 mitogen-activated kinase (MAPK) and JNK, which could be activated by IL-13 and IL-4 [60]. These Th-2 cytokines are considered to increase the phosphorylation rate of NR3C1-Ser211, as the subsequent effect of the inflammation caused by HDM. Notably, the phosphorylation level of NR3C1-Ser211 in CTNNAL1<sup>+/+</sup> + HDM group was lower than CTNNAL1<sup>-/-</sup> + HDM group after DEX treatment. After being phosphorylated by kinases, the binding capacity and stability of GR could decline [60]. We next examined the transcription of GR target genes, including GILZ and MKP-1, to further investigate the activation of glucocorticoid receptor. Both HDM stimulation and CTNNAL1 deficiency could decrease mRNA expression of GILZ and MKP-1. Separately, the mRNA levels of GILZ and MKP-1 were significantly decreased in CTNNAL1-silenced HBE cells upon HDM stimulation.

Moreover, after HDM exposure, CTNNAL1 deficiency further provoked the secretion of IL-1 $\beta$ , IL-5, IL-8, IL-6 and TNF- $\alpha$ . In addition, a remarkably higher number of inflammatory cells could be observed in the perivascular, peribronchial, and parenchymal tissues of HDM-induced CTNNAL1-silenced mice. However, such an upward trend was somewhat reversed by mono-treatment of DEX, although to a slight extent. The ratio of p-p65/p65 was significantly increased in HDM-challenged CTNNAL1-silenced mice. By contrast, a lower ratio of p-

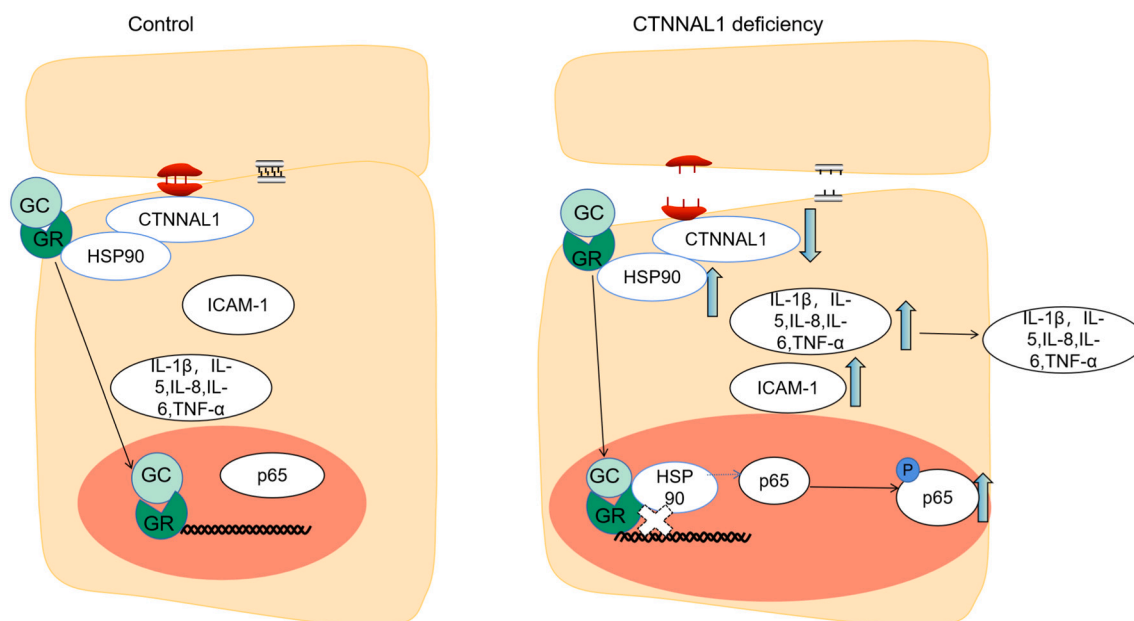


**Fig. 7.** Schematic model of CTNNAL1 deficiency decreased glucocorticoid sensitivity in HBE cells through up-regulating hsp90 signaling pathway. GC, glucocorticoid; GR, glucocorticoid receptor; HDM, house dust mite; HSP90, heat shock protein 90; p-p65, phosphorylated-nuclear factor kappa B; ICAM-1, intercellular cell adhesion molecule-1.

p65/p65 was observed in control + HDM group after being treated with DEX, as compared with CTNNAL1-silenced + HDM group. The converging results in vitro and in vivo jointly showed the lower sensitivity to DEX when CTNNAL1 was silenced.

The treatment of geldanamycin significantly decreased the levels of p-p65, ICAM-1 and NR3C1 and inflammatory cytokines (including IL-1 $\beta$ , IL-5, IL-8, IL-6 and TNF- $\alpha$ ) in CTNNAL1-silenced HBE cells after HDM stimulation. Furthermore, the anti-inflammatory effect of DEX is similar to that of geldanamycin in CTNNAL1<sup>+/+</sup> HBE cells, yet it was significantly weaker in CTNNAL1-silence cells. Moreover, the use of geldanamycin in combination with DEX significantly reduced the expression of ICAM-1 and the phosphorylation level of p65 in CTNNAL1-silenced HBE cells compared with monotreatment of DEX.

## 5. Conclusion

Taken together, this study demonstrates that CTNNAL1 deficiency could deteriorate airway inflammation and render resistance to glucocorticoids therapy via activating hsp90 signaling pathway.

## Abbreviations

|                |                                               |
|----------------|-----------------------------------------------|
| CTNNAL1        | catenin alpha-like 1                          |
| HDM            | house dust mite                               |
| hsp90          | heat shock protein90                          |
| NF- $\kappa$ B | nuclear factor kappa B                        |
| ICAM-1         | intercellular cell adhesion molecule-1        |
| NR3C1          | nuclear receptor subfamily 3 group C member 1 |
| GR             | glucocorticoid receptor                       |
| AAV            | adeno-associated virus                        |
| DEX            | dexamethasone                                 |

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lfs.2022.121304>.

## CRedit authorship contribution statement

Conceptualization: X.Q. (Xiaoqun Qin) and Y.X. (Yang Xiang); methodology: D.W. (Di Wu); software: L.L. (Lexin Liu); validation, Y.X.; formal analysis, C.L. (Caixia Liu) and C.L. (Chunjiao Long);

investigation, D.W. and L.L.; resources, Y.X.; data curation, D.W.; writing—original draft preparation, D.W.; visualization, J.Z. (Jiahui Zhu) and H.L. (Huijun Liu); supervision, X.Q. (Xiangping Qu); project administration, D.W.; funding acquisition, X.Q. (Xiaoqun Qin), C.L. (Chi Liu) and Y.X. All authors have read the manuscript and agreed to its publication and dissemination.

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## Declaration of competing interest

The authors have declared that no competing interest regarding the publication of this manuscript.

## Data availability

Data will be made available on request.

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