Effects of Blocking NLRP3 Inflammasome on Type II Innate Lymphoid Cell Response in Allergic Rhinitis

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ABSTRACT

Background: Type 2 innate lymphoid cells (ILC2s) and NLRP3 inflammasome are related to allergic and inflammatory responses. NLRP3 inflammasome inhibitor MCC950 was demonstrated to ameliorate allergic rhinitis (AR) in animal models.

Objective: To elucidate the effect of MCC950 on ILC2 responses in AR.

Methods: NLRP3 inflammasome, ILC2s, IL-5$^+$ILC2s, IL-13$^+$ILC2s, and Th2-related factors were examined in 30 AR patients. ILC2s were identified as Lin$^-$CRTH2$^+$CD127$^+$lymphocytes. ILC2s isolated from PBMCs were stimulated with LPS plus ATP. The effect of MCC950, IL-1β, and IL-18 on ILC2 responses was detected by flow cytometry. AR models were established in 60 BALB/c mice. Nasal symptoms and ILC2 responses in the AR models after MCC950 treatment were detected. Human nasal epithelial cells were stimulated with IL-13 (10 ng/mL) and treated with MCC950 (10 µM).

Results: AR patients showed activated NLRP3 inflammasome and increased ILC2 responses compared to controls. NLRP3 inflammasome levels in the AR patients were positively related to the proportion of ILC2s, IL-5$^+$ILC2s, and IL-13$^+$ILC2s in total PBMCs. MCC950 treatment or IL-1β/IL-18 suppression inhibited ILC2 proliferation and Th2-related factors (GATA3, RORα, IL-5, and IL-13). MCC950 administration alleviated frequencies of nasal rubbing and sneezes in the AR models. ILC2s, IL-5$^+$ILC2s, and IL-13$^+$ILC2s in mice were reduced by MCC950. MCC950 inhibited NLRP3 inflammasome in the *in vitro* models of AR.

Conclusion: MCC950 inhibited ILC2 responses in AR and mice models, suggesting that blocking NLRP3 inflammasome may be a promising target for AR clinical treatment.

Keywords: Allergic Rhinitis, MCC950, NLRP3 Inflammasome, Type II Innate Lymphoid Cell

INTRODUCTION

Allergic rhinitis (AR) is an inflammatory and allergic disease induced by Th2 immune response after exposure to allergens (1). The occurrence and development of AR are related to the activation of the immune system. The adaptive immune system plays a key role in AR, while the role of the innate immune system is unclear. The activation of the innate immune system caused by nasal mucosal epithelial barrier dysfunction in AR patients has attracted extensive attention and become an increasingly focal research topic (2, 3). Type 2 innate lymphoid cells (ILC2s) are a newly discovered class of innate lymphocytes able to produce pro-inflammatory factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, IL-5, IL-13, amphotericin, and eotaxin after activation (4). Previous studies have confirmed that elevated ILC2 and IL-5 levels in AR patients and asthma patients can be detected 6 h after nasal stimulation test (5). In the peripheral blood of AR patients, ILC2s are activated to secrete a large amount of IL-5 and IL-13 (6). Additionally, ILC2s can promote the production of pro-inflammatory factors in murine AR models (7).

NLRP3 inflammasome is widely present in various types of cells and can be activated by a variety of substances, and is known as the most extensive receptor in the nucleotide-binding and oligomerization (NOD) family (8). The activation of NLRP3 inflammasome in AR nasal mucosa has been demonstrated. Wan et al. found that NLRP3 inflammasome is activated in the nasal mucosa and lavage fluid of rats, and produces pro-inflammatory factors in rat models of AR (9). Another study also confirmed that the specific NLRP3 inflammasome inhibitor MCC950 inhibits its activation in the nasal mucosa and lavage fluid of AR mice (10). Based on these reports, NLRP3 inflammasome and ILC2, both belonging to the innate immune system, play crucial roles in the initiation and progression of AR. However, whether NLRP3 inflammasome regulates ILC2 responses in AR has not been yet characterized.

There is no antigen-specific receptor on the surface of ILC2, which cannot be activated by allergens but can be activated by a variety of cytokines (11). Evidence shows that thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 are the main activators of ILC2 (12). Some reports have demonstrated that IL-1β and IL-18, belonging to the same IL-1 family as IL-25/-33, can also activate ILC2 (13-15). The classic IL-1β and IL-18 generation pathway is NLRP3-caspase1-IL-1β/IL-18 pathway, which is also an NLRP3 inflammasome activation pathway (16).

Therefore, we hypothesized that NLRP3 inflammasome may promote ILC2 proliferation and cytokine production through the secretion of IL-1β/IL-18. Here, we analyzed NLRP3 inflammasome and ILC2-associated cytokines in the AR patients and the healthy controls. We evaluated the impact of MCC950 (NLRP3 inflammasome inhibitor) on ILC2 responses using cell culture models and the AR mouse models. Our findings might provide a sound reason for NLRP3 inflammasome inhibitor target therapy in AR.

MATERIALS AND METHODS

Participants

Thirty AR patients were recruited in our study. Twenty healthy individuals acted as the controls. Written informed consent was signed by the participants. All the patients were diagnosed according to the results of specific IgE assay, allergen skin prick test, nasal endoscopy, and medical history. Specific immunoglobulin to D farina or Dermatophagoides pteronyssinus, positive skin prick test, and typical symptoms (nasal block, rhinorrhea, sneezing, nose itching) were confirmed as the persistent AR according to the ARIA guidelines. Exclusion criteria for participants were as follows: (1) pregnancy; (2) drug allergies

within the last 14 days; (3) immunotherapy for any allergens; (4) cystic fibrosis diagnosis, immunodeficiency, fungal rhino-sinusitis, posterior nasal polyps, or chronic sinusitis; (5) smoking history; (6) anti-histamines and systemic glucocorticoids within one month before surgery. The study was approved by the Ethics Committee of the General Hospital of Central Theater Command (Wuhan, China) (Approval number: [2023]047-01). Mice protocols used in the present study were approved by the Animal Ethics Committee of General Hospital of Central Theater Command (Wuhan, China).

Clinical Severity

The symptoms of the AR patients, such as runny nose, sneezing, itchy nose, and nasal obstruction, were recorded. According to these symptoms, a total nasal symptom score (TNSS) was calculated (0, none; 1, mild; 2, moderate; and 3, severe). Average scores were taken during the observation period of 8 weeks, as described in the previous studies (17).

ILC2 Detection in Peripheral Blood Mononuclear Cells (PBMCs)

As described in the previous studies (18), 15 mL PBMCs were isolated and collected using density-gradient centrifugation. The PE-Cy7-conjugated CD127 (HIL-7R-M21; BD Biosciences, CA, USA), phycoerythrin-conjugated CRTH2 (BM16, BD Biosciences), allophycocyanin (APC)-conjugated CD161 (BioLegend, Beijing, China), fluorescein isothiocyanate (FITC)-conjugated lineage markers (CD3/14/16/19/20/56 [BD lineage, NJ, USA], TCRγδ, CD4, CD235a, FcεR1α [BioLegend]) were used for staining and examining using flow cytometer (FACSCalibur; BD Biosciences). ILC2s were identified as Lin⁻CRTH2⁺CD127⁺lymphocytes.

ILC2s (1×10^5 cells) were stimulated with 10 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich, MO, USA) for 6 h and were re-stimulated with 500 nM ionomycin (Merck, NJ, USA). Cytofix (BD Biosciences) was used for the cellular staining of IL-5 and IL-13 following the manufacturer's instructions. Briefly, monensin (1:2000 dilution) and 3 mg/mL brefeldin A (eBioscience, CA, USA) were added to the PBMCs and cytokine secretion was blocked. Afterward, fluorochrome-conjugated IL-5/-13 antibodies were used for staining, followed by flow cytometry analysis.

For ILC2 stimulation, ILC2s were incubated with 10 ng/mL IL-25, 50 ng/mL IL-2, 10 ng/mL TSLP, and 10 ng/mL IL-33. MCC950, anti-IL-1β, or anti-IL-18 (all 100 ng/mL) were added. Research and Diagnostic Systems, Inc (Minneapolis, MN, USA) provided these cytokines. Phosphate-buffered saline (PBS) acted as the negative control. After 4-5 days of culture, the cells were centrifugated at 400 ×g for 10 min and the supernatant was stored at -80 °C.

Isolation of ILC2s

EasySep FITC selection kit was used to enrich the lineage-negative (Lin) cells in PBMCs. After PE-Cy7-labeled CD127 and PE-labeled CRTH2 were used to stain the Lin⁻ cells, a MoFlo XDP cell sorter (Beckman Coulter, CA, USA) was prepared to sort out ILC2s (Lin⁻CRTH2⁺CD127⁺). The fluorescence minus one (1 antibody against CD127 or CRTH2 was individually omitted) and the isotype control was prepared. ILC2s (1.5×10^5 cells/mL) were cultured in DMEM containing 10% FBS, 1% penicillin/streptomycin, IL-2 (50 ng/mL), TSLP (10 ng/mL), IL-33 (10 ng/mL), and IL-25 (10 ng/mL) for 72 h. Tritiated thymidine incorporation was conducted to measure the ILC2 proliferation.

Cell Transfection

IL-1β/IL-18 short hairpin RNAs (sh-IL-1β/IL-18) generated by GenePharma were used to knockdown IL-1β/IL-18. Lipofectamine 3000 (Takara) was used following the manufacturer's instructions.

Cell Treatment

Human nasal epithelial cells (hNECs) purchased from PromoCell GmbH were

cultured in DMEM containing 1% penicillin/streptomycin and 10% FBS at 37 °C with 5% CO_2. The hNECs were treated with 10 ng/mL IL-13 (PeproTech, Inc., NJ, USA) for 24 h to establish cell models of AR. The hNECs were treated with MCC950 (10 μM) for 24 h prior to IL-13 stimulation.

Enzyme-linked Immunosorbent Assay (ELISA)

The concentration of cytokines (NLRP3, caspase-1, IL-1β, IL-18, IL-5, IL-13, GATA3, RORα, IgE, IL-4, IL-5, and IL-13) were examined using ELISA kits (R&D Systems, USA) based on the supplier's specifications.

Western Blotting

The lysates from nasal mucosa and ILC2s were obtained using a total protein extraction reagent. The protein was loaded on 10% SDS-PAGE gels and transferred onto PVDF membranes. Specific signals on the membranes were blocked using 5% fat-free milk. The membranes were probed with primary antibodies: NLRP3 (1:1000, ab263899, Abcam, USA), caspase-1 (1:1000, ab207802), IL-1β (1:1000, ab254360), IL-18 (1:1000, ab243091), and GAPDH (1:10000, ab8226) at 4 °C overnight, and further with (1:1000, ab102248) for 1 h after washing. Immunocomplexes were visualized with an ECL detection kit (Millipore) and quantified by Image Lab software.

Quantitative Reverse Transcription PCR

Total RNA isolated from nasal mucosa and ILC2s by TRIzol (Invitrogen) was reverse transcribed to cDNA using the PrimeScrip Reagent kit (Invitrogen). The qPCR was performed using SYBR GreenPCR reagent (Bio-Rad Laboratories, Inc.) and a Bio-Rad C1000 Thermal Cycler according to the manufacturer's protocol. The primers are presented in Table 1. GAPDH acted as an internal reference. The alternation in relative mRNA expression was examined using the $2^{-\Delta\Delta Ct}$ method (19).

Experimental Design

Sixty female BALB/c mice (7-week-old) were used and separated into three groups: the normal, ovalbumin (OVA), OVA+MCC950 (n=10). According to previous studies (20), OVA was utilized to establish the murine AR model. First, 0.2 mL suspension containing 20 mg/mL aluminum hydroxide and 0.5 mg/mL OVA (Sigma-Aldrich) were intraperitoneally injected into mice on days 1, 8, and 15 for sensitization. Next, the animals were administrated with OVA (20 μL, 40 mg/mL) by intranasal instillation daily on days 22 to 29. As for MCC950 treatment, the mice in the OVA+MCC950 group received MCC950 (400 μg, S81447; Yuanye, Shanghai, China) dissolved in 30 μL PBS by intranasal installation. The mice in the normal and OVA groups were injected with the same amount of PBS.

Table 1. Sequences of primers used for RT-qPCR

Gene name	Forward sequence (5'-3')	Reverse sequence (5'-3')
NLRP3	ACTCTGTGAGGGACTCTTGC	GGTCGCCCAGGTCATTGTT
Caspase-1	GCACACGTCTTGCTCTCATTA	TTCACATCTACGCTGTACCCC
IL-1β	CGAATCTCCGACCACCACTAC	GCACATAAGCCTCGTTATCCC
IL-18	TCTTCATTGACCAAGGAAATCGG	TCCGGGGTGCATTATCTCTAC
IL-5	ACCTTGGCA CTG CTT TCT ACT CA	ACTCTCCGTCTTTCTTCTCCACAC
IL-13	CTCCTCAATCCTCTCCTGTT	GTTGAACCGTCCCTCGCGAAA
GATA3	GCTTCACAATATTAACAGACCC	TAAACGAGCTGTTCTTGGG
RORα	TAGGATCCACCATGGAGTCAGCTCCG	TCGGAATTCTTACCCATCAATTTGC
GAPDH	TCAAGATCATCAGCAATGCC	CGATACCAAAGTTGTCATGGA

RT-qPCR, reverse transcriptase quantitative polymerase chain reaction; NLRP3, NOD-like receptor family pyrin domain containing 3; IL-1β, interleukin-1β; IL-18, interleukin-18; IL-5, interleukin-5; IL-13, interleukin-13; GATA3, GATA binding protein 3; RORα, retinoic acid-related orphan receptors α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
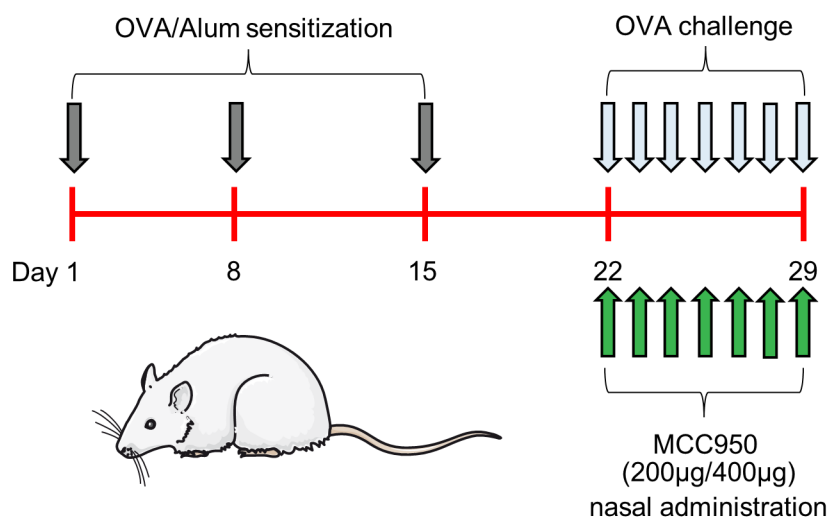
Fig. 1. A schematic diagram of the model establishment. OVA, ovalbumin

The schematic diagram of the model establishment was described in Fig. 1. All the animal experiments were approved by the Ethics Committee of Wuhan Myhalic Biotechnology Co., Ltd. (Wuhan, China) (Approval number: 202005019).

Histological Examination

After the final intranasal sensitization, the nasal mucosa samples were removed once the animals were euthanized. The tissue samples were fixed with 4% paraformaldehyde, dehydrated in gradient ethanol, and paraffin-embedded. Afterward, 4-μm-thick nasal mucosa sections were used for hematoxylin and eosin (H&E) staining as per the standard protocols.

ILC2 Detection in the Nasal Mucosa of Mouse Models

The cells from nasal tissue were stained with Lin⁻ (FcεR1α (AER-37), Gr-1 (RB6,8C5), CD11c (N418), NK1.1 (PK136), CD5 (53,7.3), B220 (RA3,6B2), TER-119 (TER,119), CD3ε (145,2C11), TCRβ(H57,597), TCRγδ(eBioGL3), and CD11b (M1/70)), CD127⁺(A7R34), and ST2⁺(RMST2,2) lymphocytes. The ILC2s in murine PBMCs were identified as Lin⁻ ST2⁺CD127⁺lymphocytes. Cytofix (BD Biosciences) was used for cellular staining to detect IL-5 (JES1-39D10) or IL-13 (JES10-5A2)-positive ILC2 cells in PBMCs following

the manufacturer's protocol.

Statistical Analysis

SPSS 22.0 Software (IBM, Chicago, USA) was utilized to conduct statistical analyses. $p<0.05$ was considered statistically significant. Statistical values are expressed as the mean±standard deviations. Spearman's test was employed for correlations. Intergroup comparisons were assessed by one-way ANOVA test.

RESULTS

Activation of NLRP3 Inflammasome in the AR Patients

First, we investigated whether NLRP3 inflammasome is activated in AR or not. ELISA was conducted to detect pro-inflammatory cytokine release. As shown in Figs. 2A-2B, IL-1β (125.67±11.32 vs. 39.26±3.15, $p=0.0002$) and IL-18 (164.09±12.28 vs. 55.70±4.23, $p=0.0001$) concentrations in the serum of the AR patients were higher than in those of the healthy controls. We further measured the IL-1β and IL-18 mRNA levels in the nasal mucosa using RT-qPCR. The results showed that the AR patients exhibited upregulated IL-1β (3.26±0.21 vs. 1.00±0.00, $p=0.0000$) and IL-18 (2.99±0.26 vs. 1.00±0.00, $p=0.0002$) in the nasal mucosa, compared with the healthy
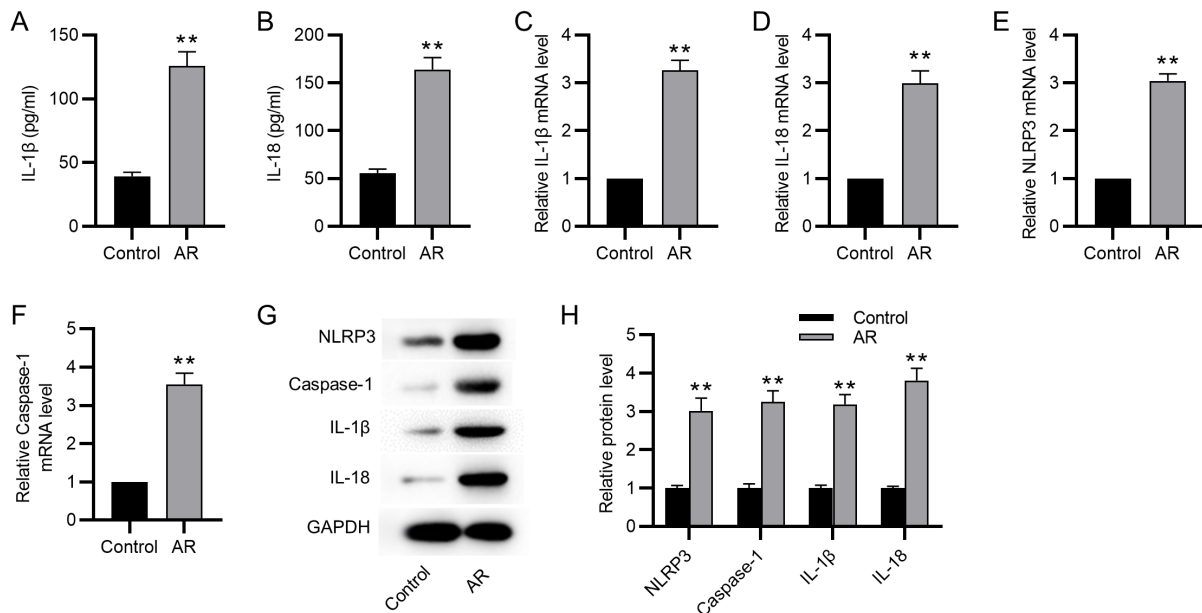
Fig. 2. Activation of NLRP3 inflammasome pathway in patients with AR. (A-B) IL-1β and IL-18 concentrations in the serum of the AR patients and the healthy controls were measured using ELISA. (C-F) IL-1β, IL-18, NLRP3, and caspase-1 mRNA levels in the nasal mucosa of the AR patients and the healthy controls were assessed using RT-qPCR. (G-H) Western blotting analysis was performed to determine the protein levels of NLRP3, caspase-1, L-1β, and IL-18 in the nasal mucosa of the AR patients and in the healthy controls. ${}^{**}p<0.01$. AR, allergic rhinitis; NLRP3, NOD-like receptor family pyrin domain containing 3; IL-1β, interleukin-1β; IL-18, interleukin-18; ELISA, enzyme-linked immunosorbent assay; RT-qPCR, reverse transcriptase quantitative polymerase chain reaction.

controls (Figs. 2C-2D). Additionally, compared with the control group, high mRNA levels of NLRP3 (3.04±0.15 vs. 1.00±0.00, $p=0.0000$) and caspase-1 (3.55±0.29 vs. 1.00±0.00, $p=0.0001$) were detected in the nasal mucosa of the AR patients (Figs. 2E-2F). Western blotting showed higher protein levels of NLRP3, caspase-1, L-1β, and IL-18 in the AR patients than in the healthy controls (Figs. 2G-2H), suggesting that the NLRP3 inflammasome pathway was activated in the AR patients.
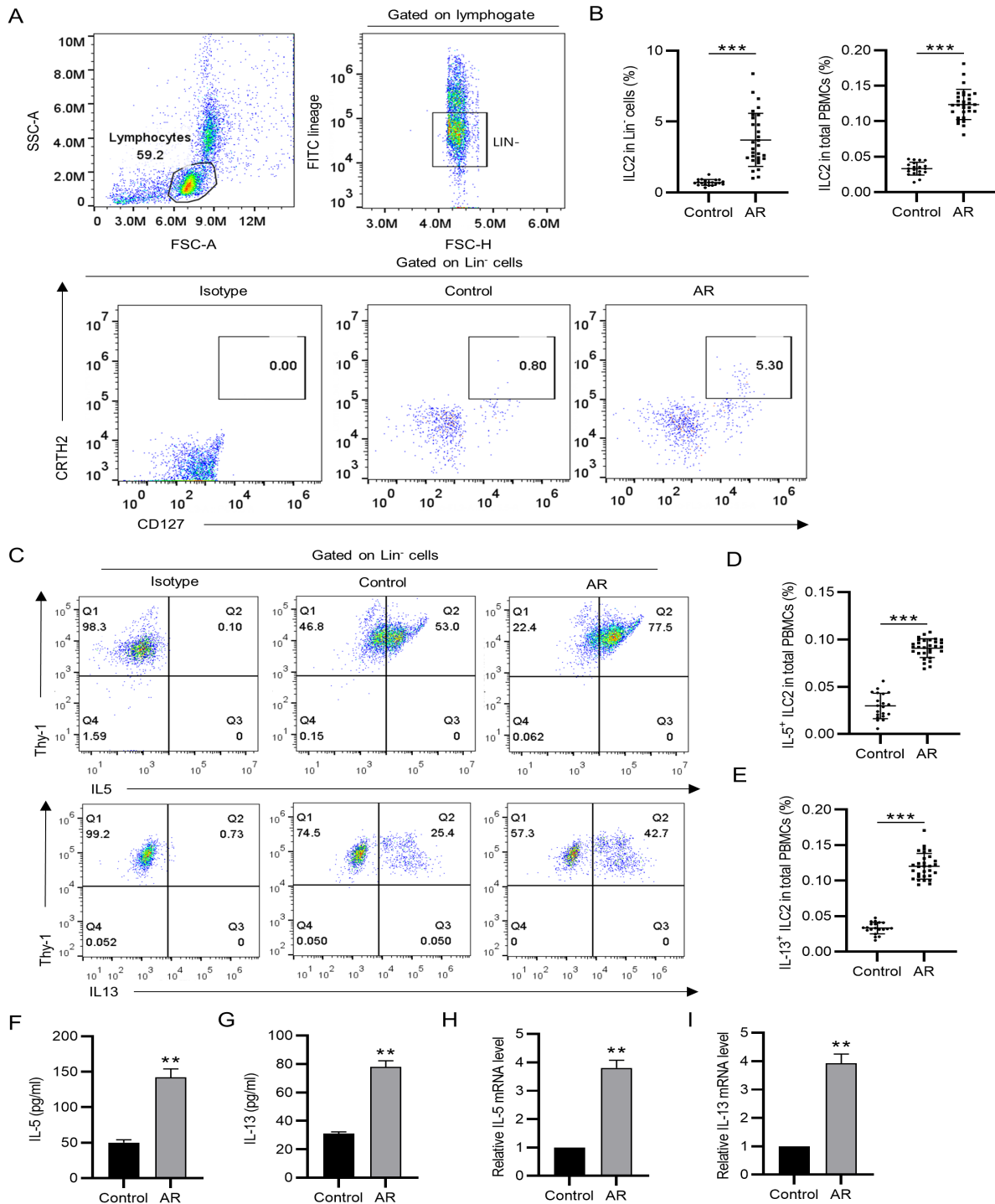
Increased Proportion of ILC2, IL-5⁺ILC2, IL-13⁺ILC2, and Th2-related Factors in the AR Patients

ILC2s were identified as Lin⁻CRTH2⁺CD127⁺lymphocytes (Fig. 3A). ILC2 proportion in Lin⁻ cells in the healthy controls was low. However, the percentage of ILC2s in the AR patients was markedly high (Fig. 3B). As flow cytometry analysis showed, both IL-5⁺ILC2 and IL-13⁺ILC2 proportions were elevated in the AR patients compared with those in the healthy controls (Figs. 3C-3E). Additionally, the

results of ELISA showed that the AR patients displayed higher serum concentrations of IL-5 (142.33±11.67 vs. 50.03±4.11, $p=0.0002$) and IL-13 (78.00±4.26 vs. 31.00±1.25, $p=0.0001$) than in the healthy controls (Figs. 3F-3G). Consistent with the results of ELISA, RT-qPCR showed significantly upregulated mRNA levels of IL-5 (3.80±0.27 vs. 1.00±0.00, $p=0.0001$) and IL-13 (3.92±0.33 vs. 1.00±0.00, $p=0.0001$) in the AR patients compared with the control (Figs. 3H-3I).

Correlation Analysis of ILC2 Proportion in PBMCs and NLRP3 Inflammasome in the AR Patients

Next, we detected the correlation between NLRP3 inflammasome and ILC2 proportion. As Fig. 4A showed, ILC2s in PBMCs were positively related to TNSS score ($R^2=0.4460$, $p<0.01$). Additionally, IL-1β expression ($R^2=0.3269$, $p<0.01$) and IL-18 expression ($R^2=0.3944$, $p<0.01$) in the AR patients were proportional to ILC2 proportion (Figs. 4B-4C). There was a positive correlation between

Fig. 3. Increased proportions of ILC2s, IL-5+ILC2s, IL-13+ILC2s, and Th2-related factors in the AR patients. (A) Lymphocytes were identified from the whole PBMCs, and lineage-negative (Lin-) cells were gated and further assessed for the co-expression of CD127 and CRTH2. ILC2s were identified as Lin-CRTH2+CD127+lymphocytes. (B) Quantitative analysis of the proportion of ILC2 cells. (C) Flow cytometry analysis of the proportion of IL-5+ILC2s and IL-13+ILC2s. (D) Quantitative analysis of the proportion of IL-5+ILC2s in total PBMCs in the AR patients and in the healthy controls. (E) Quantitative analysis of the proportion of IL-13+ILC2s in total PBMCs in the AR patients and in the healthy controls. (F-G) ELISA of serum concentrations of IL-5 and IL-13 in the AR patients and in the healthy controls. (H-I) RT-qPCR analysis of IL-5 and IL-13 mRNA levels in the nasal mucosa of the AR patients and in the healthy controls. $^{**}p<0.01$, $^{***}p<0.001$. ILC2, type II innate lymphoid cell; PBMCs, peripheral blood mononuclear cells; AR, allergic rhinitis; ELISA, enzyme-linked immunosorbent assay; RT-qPCR, reverse transcriptase quantitative polymerase chain reaction.
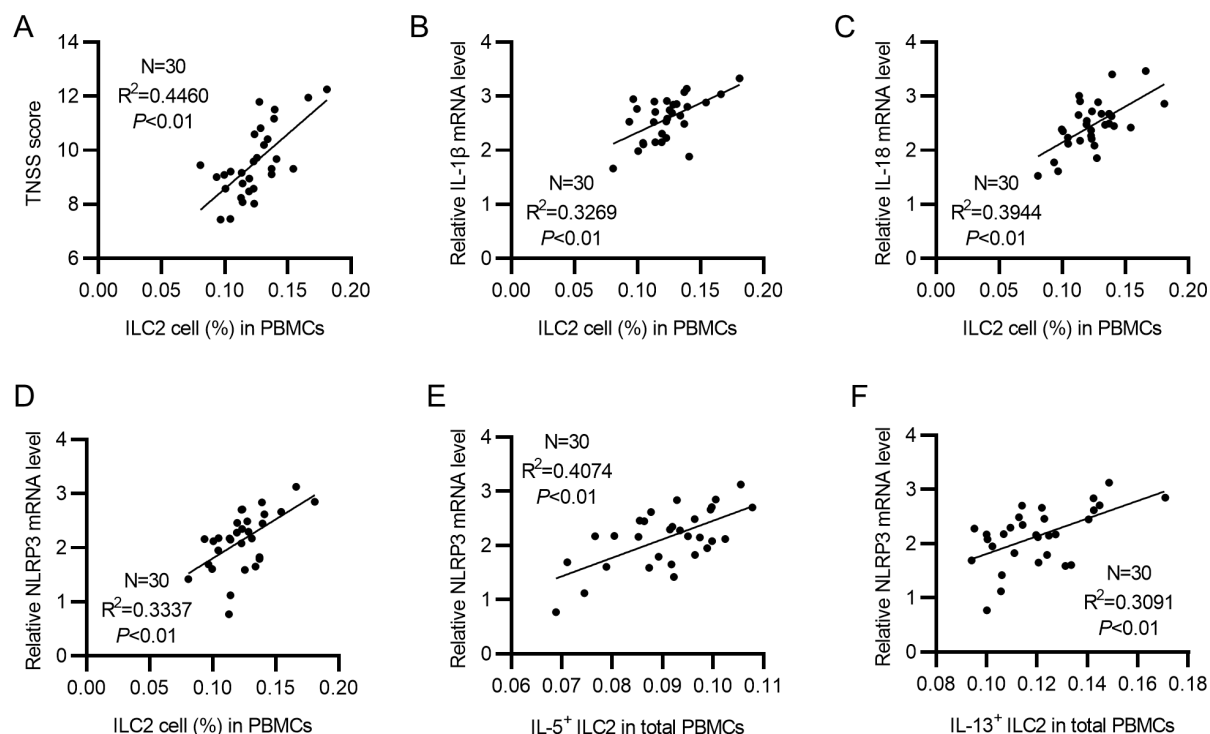
Fig. 4. Correlation analysis of ILC2 proportion in PBMCs and NLRP3 inflammasome pathway in PBMCs in the AR patients. The correction of ILC2s in total PBMCs with (A) TNSS score, (B) IL-1β mRNA level, (C) IL-18 mRNA level, and (D) NLRP3 mRNA level. The correction of NLRP3 mRNA level with (E) IL-5+ILC2s, IL-13+ILC2s (F) in total PBMCs. N=30. ILC2, type II innate lymphoid cell; PBMCs, peripheral blood mononuclear cells; NLRP3, NOD-like receptor family pyrin domain containing 3; AR, allergic rhinitis; TNSS, total nasal symptom score; ILC2, type II innate lymphoid cells; IL-1β, interleukin-1β; IL-18, interleukin-18.

the proportion of ILC2 (R^2=0.3337, $p<0.01$), IL-5+ILC2 (R^2=0.4074, $p<0.01$), and IL-13+ILC2 (R^2=0.3091, $p<0.01$) in PBMCs and NLRP3 expression in the AR patients (Figs. 4D-4F). This suggested a positive correlation between ILC2 proportion in PBMCs and NLRP3 inflammasome in the AR patients.

Inhibition of NLRP3 Inflammasome Inhibits ILC2 Cell Proliferation and Th2-Related Factors

After ILC2 cells were stimulated, the cells were divided into PBS, LPS+ATP, and LPS+ATP+MCC950 groups. LPS plus ATP treatment induced activation of NLRP3 inflammasome, and MCC950 (100 ng/mL) was used to inhibit NLRP3 inflammasome. As Figs. 5A-5B shows, NLRP3, caspase-1, IL-1β, and IL-18 protein levels in the LPS+ATP group increased compared with the other groups, suggesting that NLRP3 inflammasome was activated in ILC2s. However, this increase

was offset by the MCC950 administration. The results of tritiated thymidine incorporation revealed that ILC2 proliferation was enhanced by LPS plus ATP treatment compared with the other groups and was inhibited in the LPS+ATP+MCC950 group (Fig. 5C). This suggested that blocking NLRP3 inflammasome inhibited the proliferation of ILC2s. GATA-3 is essential for IL-5 and IL-13 production by ILC2 cells. RORα plays a crucial role in the ILC2 development (21). We found that MCC950 inhibited mRNA expression of both GATA-3 (1.28±0.11 vs. 3.41±0.26, p=0.0002) and RORα (1.22±0.08 vs. 3.68±0.21, p=0.0000) in stimulated ILC2s (Figs. 5D-5E). As expected, the concentration of the type II cytokines IL-5 (337.00±29.00 vs. 545.00±38.00, p=0.0017) and IL-13 (158.00±12.00 vs. 290.00±15.40, p=0.0003) by ILC2s also downregulated after MCC950 treatment (Figs. 5F-5G). These data suggested that blocking NLRP3 inflammasome might
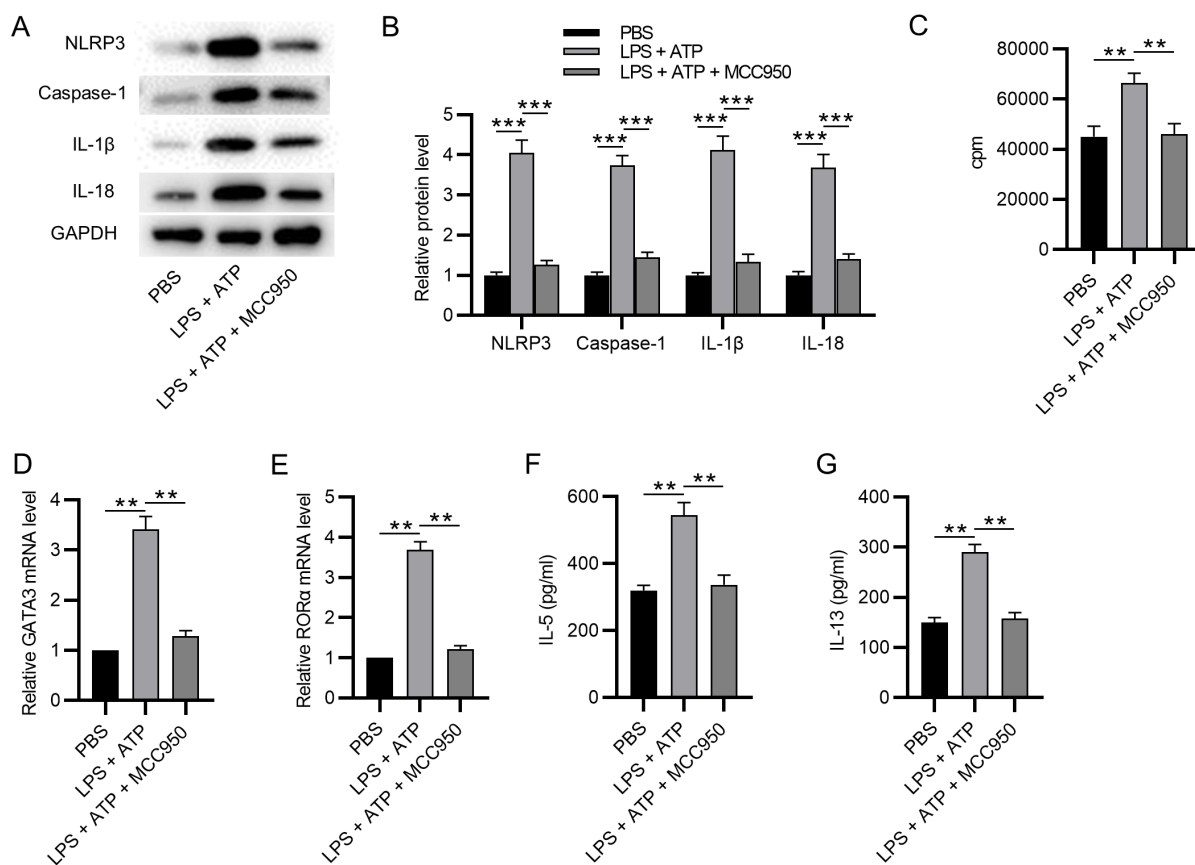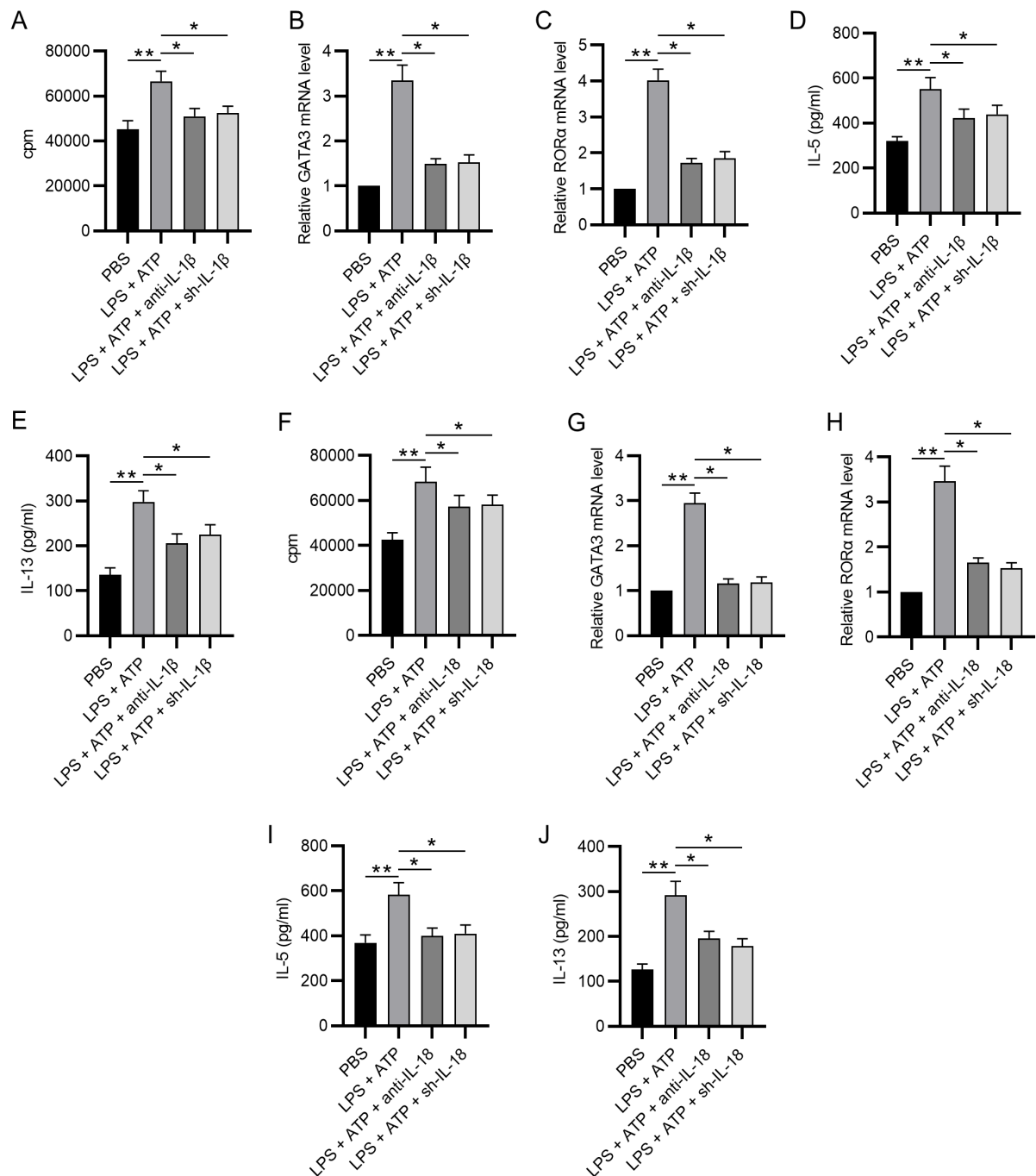
Fig. 5. Activation of NLRP3 inflammasome promotes ILC2 cell proliferation and Th2-related cytokine expression. (A-B) Western blotting was used to assess the protein levels of NLRP3, caspase-1, IL-1β, and IL-18 in ILC2s of each group. (C) Tritiated thymidine incorporation was used to detect the proliferation of ILC2s in each group. (D-E) RT-qPCR analysis of GATA3 and RORα mRNA levels in ILC2s of each group. (F-G) ELISA of IL-5 and IL-13 levels in ILC2s of each group. $^{**}p<0.01$, $^{***}p<0.001$. NLRP3, NOD-like receptor family pyrin domain containing 3; ILC2, type II innate lymphoid cell; IL-1β, interleukin-1β; IL-18, interleukin-18; Th2, type 2 T helper; GATA3, GATA binding protein 3; RORα, retinoic acid-related orphan receptors α; ELISA, enzyme-linked immunosorbent assay; RT-qPCR, reverse transcriptase quantitative polymerase chain reaction.

contribute to decreased proliferation and cytokine production of ILC2s by inhibiting GATA-3 and RORα.

NLRP3 Inflammasome Promotes ILC2 Cell Proliferation to Produce Th2-related Factors by Secreting IL-1β/IL-18

To test whether NLRP3 inflammasome promotes ILC2s through IL-1β/IL-18, 100 ng/mL anti-IL-1β/18 or sh-IL-1β/18 was utilized to treat ILC2s after LPS plus ATP stimulation. As Fig. 6A shows, the proliferative abilities of ILC2s enhanced by LPS plus ATP and were attenuated by anti-IL-1β or sh-IL-1β treatment. The results of RT-qPCR showed that anti-IL-1β reversed the effect of LPS plus ATP on GATA3 mRNA level (1.49±0.12 vs.

3.35±0.34, $p=0.0009$, Fig. 6B), RORα mRNA level (1.73±0.12 vs. 4.02±0.31, $p=0.0003$, Fig. 6C), as well as the concentration of IL-5 (422.00±41.00 vs. 552.00±51.00, $p=0.0263$, Fig. 6D) and IL-13 (206.00±21.00 vs. 298.00±25.00, $p=0.0082$, Fig. 6E). Consistent with the effect of anti-IL-1β, sh-IL-1β yielded the same results (Figs. 6B-6E). Additionally, anti-IL-18 or sh-IL-18 reversed increased ILC2 proliferation by LPS plus ATP (Fig. 6F). GATA3 and RORα mRNA levels (Figs. 6G-6H) and IL-5 and IL-13 concentrations (Figs. 6I-6J) upregulated in ILC2s after treatment with LPS plus ATP, which was offset by anti-IL-18 or sh-IL-18. Overall, NLRP3 inflammasome promoted ILC2 proliferation to produce Th2-related factors by secreting IL-1β/IL-18.

Fig. 6. NLRP3 inflammasome activation promotes ILC2 cell proliferation to produce Th2-related factors through secretion of IL-1β/IL-18. (A) Tritiated thymidine incorporation was used to detect the proliferation of ILC2s of indicated groups (PBS, LPS+ATP, LPS+ATP+anti-IL-1β, LPS+ATP+sh-IL-1β). (B-C) RT-qPCR analysis of GATA3 and RORα mRNA levels in ILC2s of indicated groups (PBS, LPS+ATP, LPS+ATP+anti-IL-1β, LPS+ATP+sh-IL-1β). (D-E) ELISA of IL-5 and IL-13 concentrations in ILC2s of indicated groups (PBS, LPS+ATP, LPS+ATP+anti-IL-1β, LPS+ATP+sh-IL-1β). (F) Proliferation of ILC2s of indicated groups (PBS, LPS+ATP, LPS+ATP+anti-IL-18, LPS+ATP+sh-IL-18). (G-H) GATA3 and RORα mRNA levels in ILC2s of indicated groups (PBS, LPS+ATP, LPS+ATP+anti-IL-18, LPS+ATP+sh-IL-18). (I-J) IL-5 and IL-13 concentrations in ILC2s of indicated groups (PBS, LPS+ATP, LPS+ATP+anti-IL-18, LPS+ATP+sh-IL-18). $^*p<0.05$, $^{**}p<0.01$. NLRP3, NOD-like receptor family pyrin domain containing 3; ILC2, type II innate lymphoid cells; IL-1β, interleukin-1β; IL-18, interleukin-18; Th2, type 2 T helper; GATA3, GATA binding protein 3; RORα, retinoic acid-related orphan receptors α; ELISA, enzyme-linked immunosorbent assay; RT-qPCR, reverse transcriptase quantitative polymerase chain reaction; PBS, phosphate buffer saline; LPS, lipopolysaccharide, ATP, adenosine triphosphate.
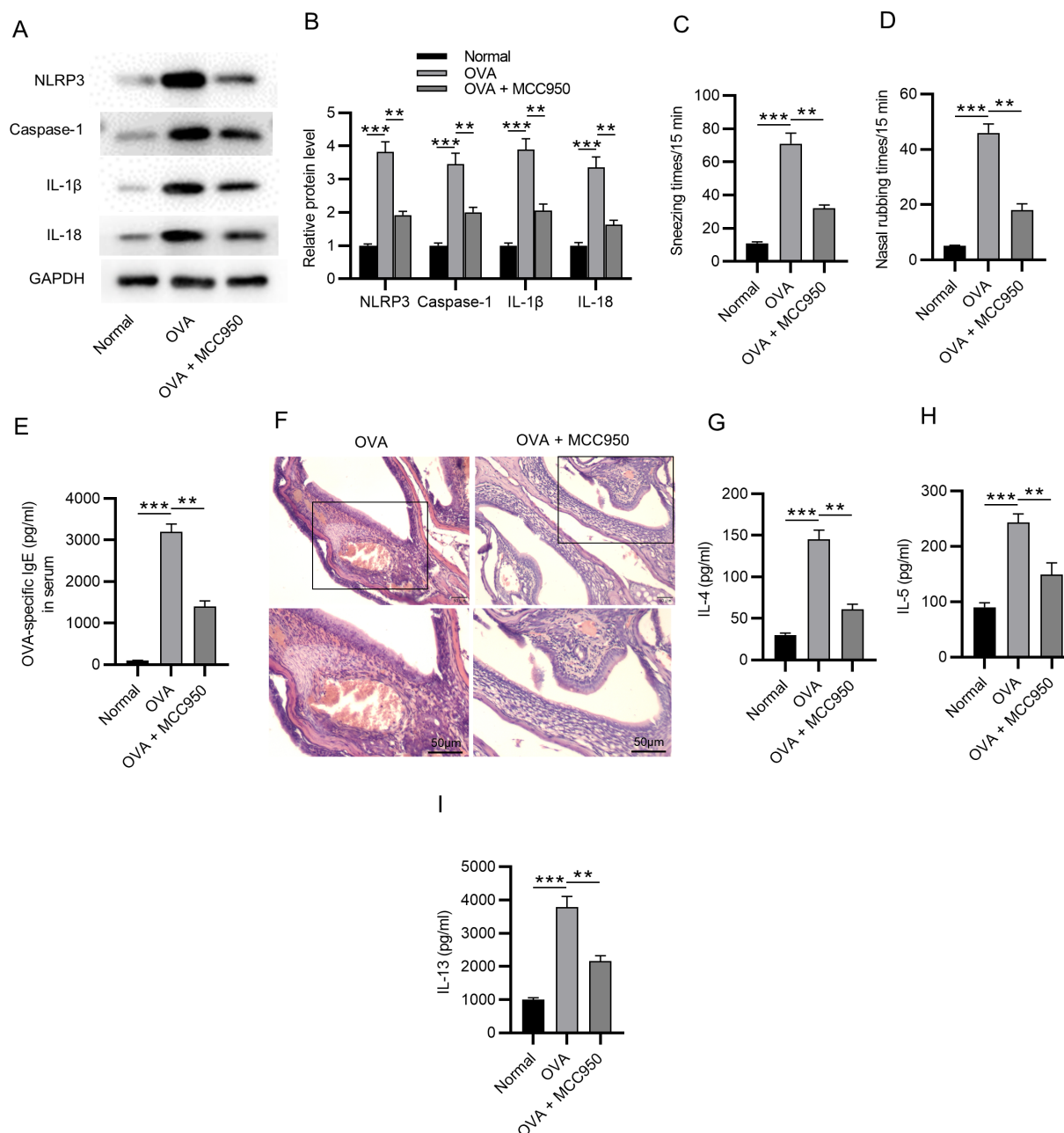
Fig. 7. MCC950 alleviates nasal symptoms of the AR mice. (A-D) Western blotting analysis of the protein levels of NLRP3, caspase-1, IL-1β, and IL-18 in the nasal mucosa of mice in the normal, OVA, and OVA+MCC950 groups. (E) Sneezing times/15 min of indicated groups. (F) Nasal rubbing times/15 min of indicated groups. (G) ELISA of OVA-specific IgE level in serum of indicated groups. (H) H&E staining on murine nasal mucosa for histological examination in indicated groups. (H-I) ELISA of the concentrations of IL-4, IL-5, and IL-13 in the nasal mucosa of indicated groups. $^{**}p<0.01$, $^{***}p<0.001$. AR, allergic rhinitis; NLRP3, NOD-like receptor family pyrin domain containing 3; IL-1β, interleukin-1β; IL-18, interleukin-18; OVA, ovalbumin; H&E, hematoxylin and eosin; ELISA, enzyme-linked immunosorbent assay.

MCC950 Improves Nasal Symptoms of the AR Mice

Moreover, we investigated the effect of MCC950 on the AR symptoms and ILC2 activation *in vivo*. Western blotting illustrated that the OVA group showed upregulated NLRP3, caspase-1, IL-1β, and IL-18 levels in the nasal mucosa in comparison with the normal group, while this change was reversed by MCC950 treatment (Figs. 7A-7B), suggesting that NLRP3 inflammasome in the AR mouse models was effectively
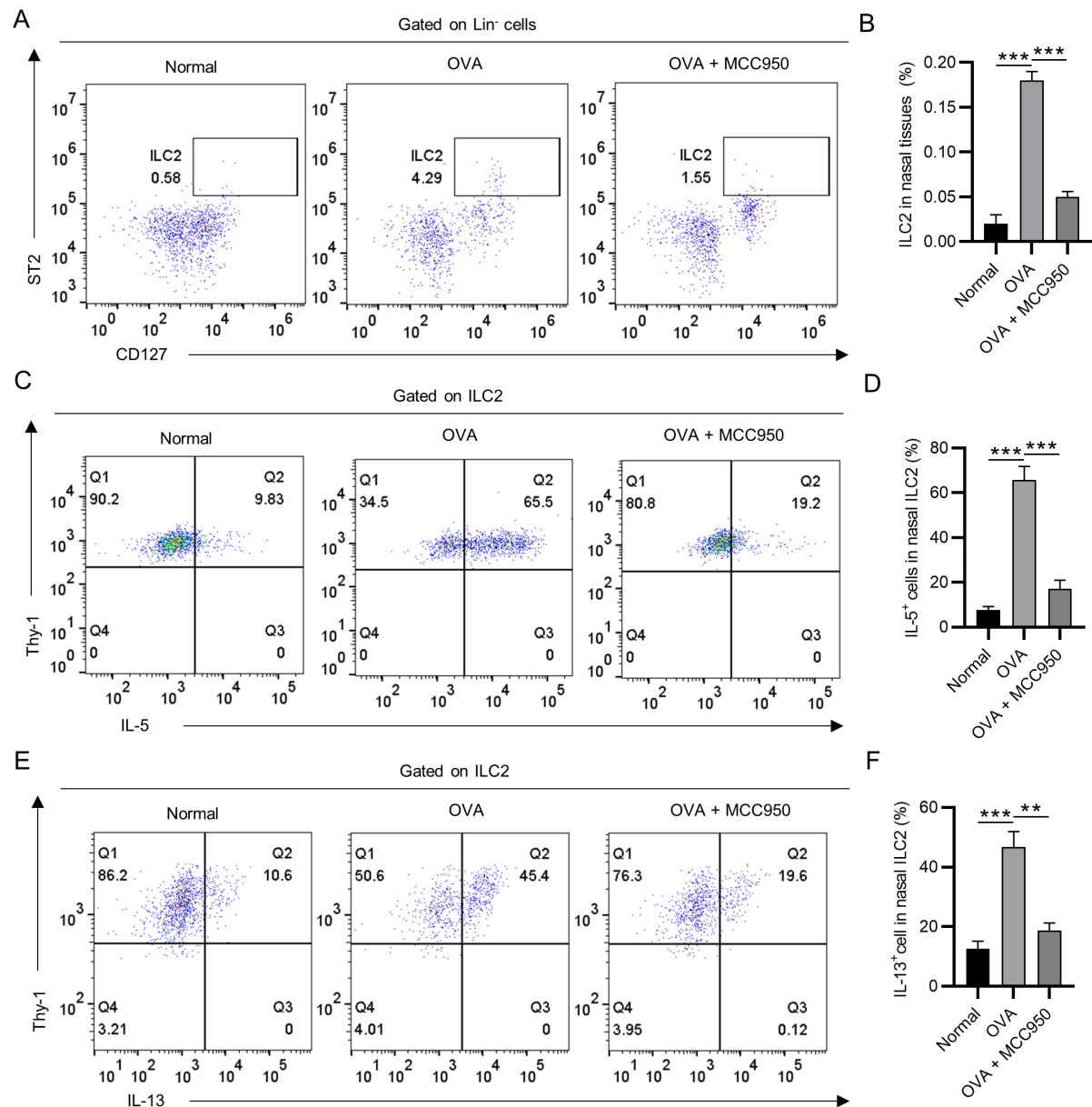
Fig. 8. MCC950 represses ILC2 responses in the AR mice model. (A-B) Flow cytometry analysis of the proportion of ILC2s in total cells of the inferior turbinate tissue of each group. (C-D) Flow cytometry analysis of the proportion of the IL-5⁺ILC2s in total cells of the inferior turbinate tissue of each group. (E-F) Flow cytometry analysis of the proportion of IL-13⁺ILC2s in total cells of the inferior turbinate tissue of each group. $^{**}p<0.01$, $^{***}p<0.001$. ILC2, type II innate lymphoid cell; AR, allergic rhinitis; OVA, ovalbumin.

blocked. In addition, compared with the mice in the normal group, significant increases in frequencies of nasal rubbing and sneezes were observed in the OVA mice, and these effects were attenuated in MCC950-treated AR mice (Figs. 7C-7D). Increased serum level of OVA-specific IgE caused by OVA treatment was also reversed by MCC950 treatment (Fig. 7E). Subsequently, H&E staining of nasal sections in the indicated groups was conducted. The results of histopathological examination

revealed that obvious pathological changes in nasal tissues were observed in OVA-induced AR mice, manifested as capillary edema, disorderly arranged epiderm, inflammatory cell infiltration, and nasal mucosal hyperemia. However, all these lesions were attenuated by MCC950 treatment (Fig. 7F). Moreover, MCC950 treatment reduced the concentrations of type II cytokines IL-4 (61.00 ± 6.00 vs. 145.00 ± 11.38, $p=0.0003$), IL-5 (149.00 ± 21.00 vs. 243.00 ± 15.66,

p=0.0034), and IL-13 (2160.00±159.00 vs. 3780.00±325.00, *p*=0.0015) in murine nasal mucosa elevated by OVA challenge (Figs. 7G-7I).

MCC950 Represses ILC2 Responses in the AR Mouse Models

As flow cytometry indicated in Figs. 8A-8F, ILC2, IL-5+ILC2, and IL-13+ILC2 proportions in total cells of the inferior turbinate were notably high in the OVA group, but reduced by MCC950 treatment, suggesting that MCC950 repressed ILC2 responses in the AR mice *in vivo*.

MCC950 Inhibits NLRP3 Inflammasome Pathway in IL-13-treated hNECs

We also verified whether MCC950 inhibits the inflammasome pathway in human cell lines. The hNECs were stimulated by IL-13 (10 ng/ml). As exhibited in Supplementary Fig. 1A-1B, NLRP3, caspase-1, L-1β, and IL-18 expression were found upregulated in IL-13-treated hNECs compared with the control, implying IL-13 activated NLRP3 inflammasome pathway in hNECs. In the MCC950-treated hNECs, NLRP3 inflammasome was inhibited compared with the hNECs treated with IL-13 only (Supplementary Figs. 1A-1B). Therefore, MCC950 could inhibit the NLRP3 inflammasome pathway in the *in vitro* models of AR.

DISCUSSION

As specialized intracellular recognition receptors, Nod-like receptors can provide immediate responses to the invasion of pathogens or allergens during the processes of innate immunity and inflammation (22). NLRP3 inflammasome is a multiprotein complex (23). Enhancement of NLRP3 inflammasome activity triggers the release of pro-inflammatory cytokines in chronic obstructive pulmonary disease, pulmonary inflammation, and asthma (24). NLRP3

participates in fighting against pathogens or allergens in the air (25) and NLRP3 activation leads to the aggregation of apoptosis-associated speck-like protein with CARD domain (ASC) as a speck, activating caspase-1 and subsequently facilitating IL-18 and IL-1β secretion (26). Furthermore, a recent study has demonstrated the pro-inflammatory role of NLRP3 inflammasome signaling pathways in activating IL-18, IL-1β, and caspase-1 in chronic rhinosinusitis with nasal polyps (27). Scientific evidence has shown that bronchial hyperresponsiveness and airway inflammation can be attributed to NLRP3 inflammasome activation in asthma (28), while airway inflammation can be attenuated by blocking NLRP3 inflammasome (29, 30). In the current work, we found the activation of NLRP3 inflammasome in the AR patients, evidenced by the upregulation of inflammasome-related proteins in the serum and nasal mucosa of the AR patients compared with the healthy controls. Additionally, in human nasal epithelial cells, blocking NLRP3 inflammasome reverses inflammatory response induced by the activation of IL-18, IL-1β, and caspase-1 (27). As a diaryl sulfonylurea-based compound, MCC950 can suppress the activities of ASC, caspase-1, and NLRP3 to restrain NLRP3 inflammasome activation, thus decreasing the production of IL-18 and IL-1β in numerous inflammatory and allergic disorders (31-33). A previous work indicated that the activities of NLRP3 inflammasome signaling pathways can be inhibited by MCC950 treatment in murine allergic airway inflammation (31).

ILC2s are distributed in the skin, lung, intestine, and lymphoid tissue, playing pivotal roles in the immune response. Allergens-induced IL-33 and IL-25 activate ILC2s in a direct way and facilitate type II cytokine secretion (34), Doherty T.A. et al. demonstrated that the AR patients allergic to cats exhibit markedly increased ILC2s in the peripheral blood after nasal stimulation (35) However, a report provided preliminary evidence that ILC2s in the peripheral blood

had no notable alternations in the AR patients compared with the controls (36). According to a previous study, ILC2 function can be negatively regulated by IL-35 (18). ILC2s are dependent on acid receptor-related orphan receptors and GATA-3 for differentiation and secrete type 2 cytokines. The proliferative ability of peripheral blood ILC2s can be promoted by nasal stimulation in the AR patients allergic to cats (35). In this study, the AR patients had a higher proportion of ILC2s, IL-5$^+$ILC2s, and IL-13$^+$ILC2s than in the healthy controls. Additionally, the AR patients showed elevated IL-5 and IL-13 production by ILC2s in their serum and nasal mucosa, compared with the healthy controls.

IL-18 is secreted from macrophages and monocytes and binds to IL-18 receptors expressed by smooth muscle cells, endothelial cells, and lymphoid cells to plays its role (37). Increased evidence has suggested that Th2 inflammation is accompanied by elevated IL-18 expression in atopic dermatitis, allergic rhinitis, and asthma (38-40). Many studies on AR have highlighted the role of IL-18. Yamagata discovered that IL-4, IL-13, and OVA-specific IgE levels decreased in the bronchoalveolar lavage fluid (BALF) of IL-18-deficient mice (41). In allergic mice, IL-18 upregulates TNF-α production induced by antigens to promote airway response (42). Kodama et al. found that the intervention of IL-18 in OVA-induced asthmatic mice elevates IL-5 expression in BALF (43). In this study, IL-18 levels increased in the AR patients. Moreover, upregulated IL-18 was detected in ILC2s after LPS stimulation and nasal mucosa of OVA mice, which was reversed by MCC950. 100 ng/mL anti-IL-1β/18 or sh-IL-1β/18 reversed the increase in ILC2 cell proliferation induced by LPS and ATP. Additionally, GATA-3 and RORα mRNA expression as well as IL-5 and IL-13 concentrations upregulated in ILC2s treated with LPS and ATP, which was offset by anti-IL-1β/18 or sh-IL-1β/18. These results suggested that NLRP3 inflammasome activation induces ILC2 cell proliferation

to release GATA3, RORα, IL-5, and IL-13 through the secretion of IL-1β/IL-18.

Here, OVA was utilized to establish an AR mouse model, and intranasal administration of MCC950 into AR mouse models was performed to evaluate the impact of MCC950 on the AR symptoms and ILC2 response in AR. We observed the increased NLRP3, caspase-1, IL-1β, and IL-18 levels in murine nasal mucosa after the OVA challenge, while this increase was offset by MCC950 administration. Moreover, in the *in vitro* experiments, the concentrations of NLRP3, caspase-1, IL-1β, and IL-18 in cultured ILC2 cells were elevated by LPS, then suppressed by MCC950. Furthermore, MCC950 alleviated nasal pathological changes and allergic symptoms in the AR mice. OVA-specific IgE in serum and Th2 cytokines in nasal mucosal tissue increased after the OVA challenge, which was reversed by MCC950. More importantly, MCC950 also repressed ILC2 response in the AR models.

To sum up, MCC950 inhibits the activation of NLRP3 inflammasome signaling, ILC2 proliferation, and Th2 cytokines to alleviate AR *in vitro*. In the *in vivo* experiments, MCC950 mitigates nasal symptoms and ILC2 response in the AR mice. All these findings suggest that blocking NLRP3 inflammasome could alleviate AR via inhibition of ILC2 responses.

ACKNOWLEDGMENTS

AUTHORS' CONTRIBUTION

Guoqing Gong conceived and designed the

experiments. Guoqing Gong, Peng Huang, Changliang Yang, Chengcheng Huang, Zhao Zhang, Ruiyao Chen, Tingfeng Sun, and Guang Yang carried out the experiments. Guoqing Gong and Guang Yang analyzed the data. Guoqing Gong and Guang Yang drafted the manuscript. All authors agreed to be accountable for all aspects of the work. All authors have read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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