



Allergen induces CD11c⁺ dendritic cell autophagy to aggravate allergic rhinitis through promoting immune imbalance

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ARTICLE INFO

Keywords:

Allergen
CD11c⁺ dendritic cells
Autophagy
Allergic rhinitis
T cells
Immune imbalance

ABSTRACT

The level of autophagy in CD11c⁺ dendritic cell (DC) and its contribution to the subsequent immune imbalance are still unclear. The aim of this study was to investigate the role and mechanism of them in promoting the allergic inflammatory response. Nasal mucosa tissues were collected from allergic rhinitis (AR) mice and their control group to detect the expression of LC3II, P62 and ATG5 and CD11c⁺DC autophagy. Different concentration of OVA or the combination of OVA and autophagy inhibitor (3-MA) were used to induce the differentiation of mouse bone marrow-derived DCs (CD11c⁺BMDCs). Differences in LC3II, P62 and ATG5 expression and autophagosome formation were detected. BMDCs in the above groups were cocultured with spleen lymphocytes to detect the proportions of effector T cells and changes in cytokines. OVA-loaded BMDCs were injected intravenously into C57BL/6 mice to develop allergic model. The nasal mucosa of mice in the AR group showed significantly increased LC3II and ATG5 protein expression, whereas showed significantly decreased P62 protein expression. Moreover, LC3II was mainly co-expressed with CD11c⁺ DC markers. In vitro, OVA stimulation induced the increase of autophagosomes and the expression of autophagy-related proteins in BMDCs in a dose-dependent manner, and inhibition of autophagy showed significantly decreased LC3II and ATG5 expression and autophagosome abundance. In addition, OVA-induced BMDC autophagy can affect CD4⁺T cell differentiation and related cytokine levels, however, the Th1/Th2/Th9/Th17/Treg/Tfh cell immune imbalances were significantly reversed after the addition of 3-MA. Adoptive transfer of OVA-loaded BMDCs could promote the allergic inflammation, while the administration of 3-MA on OVA-loaded BMDCs could significantly reduce the AR inflammation. Overall, our findings demonstrate that allergen can induce CD11c⁺DC autophagy in a dose-dependent manner and promote the immune imbalance of downstream T cells towards a proinflammatory phenotype.

1. Introduction

Allergic rhinitis (AR) is a noninfectious inflammatory disease of the nasal mucosa mediated by immunoglobulin (Ig) E that affects approximately 400 million people worldwide [1]. In recent years, the incidence of AR has been increasing annually, and an increasing number of

patients are suffering from this disease [2–4]. In the pathogenesis of AR, allergens contact the body, are ingested by antigen-presenting cells (APCs), bind to major histocompatibility complex (MHC) molecules, and then are presented to Th cells to induce immune imbalance. In particular, excessive and/or abnormal Th2 and Th17 cell responses and attenuated Th1 cell responses are always involved, thus promoting the

Abbreviations: DC, dendritic cell; AR, Allergic rhinitis; LC3II, microtubule-associated proteins light chain 3; P62, Sequestosome 1; ATG5, autophagy related gene 5; 3-MA, 3-Methyladenine; OVA, ovalbumin; BMDC, bone marrow-derived dendritic cell; Th, helper T cell; IL, interleukin; MHC, major histocompatibility complex; CD, clusters of differentiation; APCs, antigen-presenting cells; IgE, immunoglobulin (Ig) E; GM-CSF, granulocyte-macrophage colony-stimulating factor; TNF- α , tumor necrosis factor; IFN- γ , interferon- γ ; CBAs, cytokine microsphere array kits.

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<https://doi.org/10.1016/j.intimp.2022.108611>

Received 9 November 2021; Received in revised form 27 January 2022; Accepted 4 February 2022

Available online 11 February 2022

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allergic inflammatory response. Dendritic cells (DCs) are the strongest antigen-presenting cells in the body, and DCs contacting allergens is the trigger point of the allergic reaction [5], which plays a crucial role in the pathogenesis of allergic diseases.

Autophagy is a normal physiological process in the body that is responsible for maintaining the homeostasis and survival of normal cells. It is mainly responsible for lysosomal degradation of damaged proteins and organelles but also participates in the phagocytosis of foreign bodies (viruses, bacteria, and fungi) in the body. Under normal physiological conditions, intracellular autophagy is usually at a low level, but when cells are under stress, the autophagy level is rapidly upregulated [6]. A series of autophagy genes (such as LC3II and ATG5) induce autophagosome formation under the above conditions. Conversion of LC3I to LC3II is greatly enhanced by culturing cells under starvation conditions that efficiently induce autophagy [7]. A large fraction of p62 bodies are degraded by autophagy. Inhibition of autophagy led to an increase in the size and number of p62 bodies and p62 protein levels [8]. The decrease of P62 protein level is usually closely associated with autophagy activation [9–11].

These autophagosomes promote the phagocytosis of foreign bodies by immune cells and strengthen host immune defense [12]. On the other hand, autophagy plays an important role in regulating the immune response. Especially in the entire respiratory system, autophagy can control adaptive immunity by regulating DC antigen presentation, lymphocyte development and innate immunity by regulating cytokine production [13]. These roles are crucial in host immune defense.

Currently, there are some reports on autophagy and the development of AR [14–16]. Li J et al. [17] reported that Baicalin inhibits autophagy and regulates Treg/Th17 balance to relieve symptoms of AR. Another study [18] reported that IL-33 promoted degranulation of mast cells through autophagy in AR. These studies suggest that autophagy is involved in the pathogenesis of AR. However, the level of autophagy in DCs, which are the trigger during the occurrence of AR, and its contribution to the subsequent immune imbalance are still unclear. In this study, we assessed the role of CD11c⁺ DC autophagy in the pathogenesis of allergic rhinitis and found that allergen-induced CD11c⁺ DC autophagy aggravate allergic rhinitis through promoting immune imbalance.

2. Materials and methods

2.1. Experimental animals

All of the clean-grade (SPF) C57BL/6 female mice aged 4–6 weeks were purchased from Beijing Weitonglihua Experimental Animal Technology Co. Ltd. (License No.: SCXK2016-0006). The mice were kept in the Animal Center of the Renmin Hospital of Wuhan University at a temperature of 18–22 °C. The mice had free access to food and water. All experimental procedures were approved by the Animal Ethics Committee of the Renmin Hospital of Wuhan University (Ethics License No.: WDRM-20161016).

2.2. Animal model

2.2.1. Murine model treated with autophagy inhibitor (3-MA)

Eighteen naive C57BL/6 mice were first fed adaptively in the animal facility for one week and then randomly divided into three groups with 6 mice in each group by the numerical table method. Group A was the control group, group B was the AR group, and group C was the AR + 3-MA group (AR mice treated with 3-MA, Tianshengxing Bio, China, M9281).

The mice in the AR group and the AR + 3-MA group were intraperitoneally injected with 100 µg of OVA + 2 mg of Al(OH)₃ (500 µL) in a saline suspension for basic sensitization on days 1, 7 and 14. On days 15 to 28, mice in the AR group were intranasally challenged with OVA (10% in saline) once a day for 14 consecutive days [19–20], while mice

in the AR + 3-MA group were injected intraperitoneally with 3-MA (15 mg/kg) 30 min before each OVA administration [21–22]. The mice in the normal group received an intraperitoneal injection of normal saline and nasal drops at the above time points. OVA was purchased from Sigma, Inc. Alumina hydroxide was purchased from Pierce Chemical Company, USA.

2.2.2. Adoptive transfer murine model

Whole bone marrow cells isolated from eighteen naive C57BL/6J mice were cultured in RPMI 1640 medium supplemented with GM-CSF (20 ng/mL) and IL-4 (20 ng/mL) at 37 °C in the presence of 5% CO₂. On days 3 and 5, the medium was refreshed. On day 5, TNF-α (15 ng/mL) was added to further induce BMDC differentiation. On day 6, BMDCs were harvested and incubated in 6-well plates in the presence of OVA protein (500 µg/mL) and/or 3-MA for 24 h. On day 7, cells were adoptively transferred to recipient mice for further detection.

Nine naive C57BL/6 mice were randomly divided into the 3 groups (n = 3/group): Control group (BMDCs), OVA group (500 µg OVA-loaded BMDCs), 3-MA group (5 mM 3-MA + 500 µg OVA-loaded BMDCs group).

On days 0, and 2, 1 × 10⁷ BMDCs (control group), 1 × 10⁷ OVA-loaded BMDCs (OVA group) or 1 × 10⁷ OVA (500 µg)-loaded BMDCs pretreated with 5 mM 3-MA (3-MA group) in a total volume of 200 µL were injected i.v. (via the tail vein) into naive C57BL/6 mice respectively. Mice were analyzed 24 h after the last transfer [23].

2.3. Animal symptomatic scores

Mice in each group received the last nasal drip (OVA or normal saline) or last transfer, the numbers of nasal scratches and sneezes and features of a runny nose were observed within 30 min, and behavioral symptomatic scores were evaluated. Symptom scores were defined as follows: 0 points: 0 sneezes, no nasal clearing, no nose scratching; 1 point: 1–3 sneezes, runny nose visible in the nasal opening, slight scratching of the nose several times; 2 points: 4 to 10 sneezes, nasal clearing over the nasal opening, repeated nose scratching with both paws; 3 points: >11 sneezes, runny face, scratching nose, rubbing around. A total score > 5 represents successful modeling [19–20].

2.4. OVA-specific serum immunoglobulin E (IgE) assay

Twenty-four hours after administration of the last nasal drip or last transfer, the mice in each group were anesthetized, and blood was collected retro-orbitally. The serum was collected and stored at –80 °C. OVA-specific IgE in the serum was detected according to the reagent instructions for enzyme-linked immunosorbent assay (ELISA). An ELISA kit was purchased from Wuhan Hualianke Technology.

2.5. Serum cytokine detection

Twenty-four hours after administration of the last transfer, spleen samples from each group of mice were harvested and ground into single-cell suspensions. Erythrocytes were removed by ACK lysis buffer and the remaining cells were resuspended in PBS, the single-cell suspensions of lymphocytes were cultured in RPMI1640 medium containing 10% fetal bovine serum, with 1 µL/ml lipopolysaccharide stimulation. Multiplex IL-2, IFN-γ, IL-4, IL-6, and IL-17 analyses were then performed after 12 h incubation with cytokine bead array kit (BD Pharmingen, USA) according to the manufacturer's instructions.

2.6. Morphological observation of the nasal mucosa

Twenty-four hours after administration of the last nasal drip, 3 mice were randomly selected and sacrificed under anesthesia. The nasal bones of the mice were fixed in paraformaldehyde, removed 24 h later and treated in a 10% EDTA decalcification solution. After 2–3 weeks, the samples were embedded in paraffin and sectioned. Morphological

changes in the nasal mucosa were observed by HE staining and PAS staining. Microscopically, the eosinophilic nucleus was blue, and the cytoplasm was light red or red. Eosinophilic granulocytosis was counted in any 5 high-power (HP) fields (400X), and the mean value was taken [19–20].

For adoptive transfer murine model, twenty-four hours after administration of the last transfer, mice were sacrificed under anesthesia. The nasal bones of the mice were fixed in 4% paraformaldehyde for 24 h, followed by decalcification in rapid decalcification reagents (servicebio, China, G1107-500ML) for two days according to the manufacturer's instructions. The samples were embedded in paraffin and sectioned. Morphological changes in the nasal mucosa were observed by HE staining and PAS staining and counted in any 5 high-power (HP) fields (400X) as described above.

2.7. Western blotting

Mouse nasal mucosal tissue samples were placed in a liquid nitrogen grinding apparatus by weight with RIPA sonication buffer (containing a phosphatase inhibitor and protease inhibitor, 2 mg/ml), ultrasonic sonication was performed on ice for 30 min after 1 min, the samples were centrifuged at 4 °C and 12,000 RPM for 30 min, the supernatant was collected, and a defined volume of protein sample buffer was added to the samples and incubated at 100 °C for 10 min. The extracted proteins were electrophoresed in a 10% SDS-PAGE gel for 2 h at 90 V and then transferred to a PVDF membrane (GE Life, USA, #10600023) under a 200-mA current. The PVDF membrane was placed in 5% skim milk for blocking for 1 h, washed 3 times with TBS-Tween 20, and then put into a primary antibody solution prepared according to the specified antibody concentration for anti-LC3II (1:1000; ServiceBio, China, GB11124), anti-P62 (1:1000; Servicebio, GB11239-1) or anti-ATG5 (1:1000; ServiceBio GB11401). The membrane was incubated overnight on a shaker at 4 °C. The next day, the PVDF membrane was washed three times with TBS-Tween 20 and then incubated with a secondary antibody (1:20000) at room temperature for 1 h. Then, the PVDF membrane was washed three times with TBST on a shaker. An ECL chemiluminescence solution was added to each membrane, and 0.5 mL of ECL A solution and 0.5 mL of ECL B solution were taken with a pipetting gun and mixed in an EP tube. The membrane was developed in a chemiluminescence imager, ImageJ software was used for quantitative analysis, and the ratio of a detected target protein to the internal reference GAPDH was calculated [24]. Western blotting of cellular proteins also followed the above methods.

2.8. Immunofluorescence

Mouse tissue samples were placed in 4% paraformaldehyde for fixation, followed by permeabilization in Triton X-100 (0.2%) for 10 min. After incubation with an anti-LC3II antibody (CST, USA, 3868S) and an anti-CD11c antibody (Service, GB11059) for 2 h, the samples were incubated with IgG and Hoechst 33,342 for 2 h. Images were observed and collected under an upright microscope.

2.9. Culture and treatment of bone marrow dendritic cells in vitro

After euthanization of naive C57BL/6 mice aged 8–12 weeks by cervical dislocation, the femurs were taken, and both ends of the femurs were cut off. Primary mouse bone marrow cells were injected into culture plates with a 1-ml syringe containing PBS. Primary bone marrow cells were collected after red blood cell lysis, added into the culture medium, and cultured in 6-well plates. The number of cells in each well was 1×10^6 cells/ml. GM-CSF (20 ng/mL) and IL-4 (20 ng/mL) were added to each well, and then the plates were placed in a 37 °C cell incubator. On the 3rd and 5th days of culture, half the cell medium was replaced and supplemented with the corresponding cytokines, and on the 5th day, TNF- α (15 ng/mL) was added to further induce differentiation

[25].

The experiment was divided into a blank group (bone marrow cells), a control group (BMDCs, no OVA treatment), a low-concentration group (OVA, 100 μ g/mL), a high-concentration group (OVA, 500 μ g/mL), and an autophagy inhibition group (500 μ g/mL OVA + 5 mM 3-MA). OVA or the autophagy inhibitor 3-MA was added according to the above groups on the 6th day, and related tests were carried out on the 7th day. The experiments were repeated three times. Twelve mice were used.

On the 7th day, naive C57BL/6 mice aged 8–12 weeks were sacrificed by cervical dislocation under anesthesia, and the spleen was collected. Then, the spleens were ground, erythrocytes were lysed, and lymphocytes were collected. The experiments were repeated three times. Six mice were used.

The lymphocytes were cocultured with BMDCs 1 day after treatment with the above different factors, the BMDCs were washed before coculture to avoid the presence of IL-4, GM-CSF and TNF- α in the culture medium, and the relevant experiments were conducted after continuous culture for 48 h.

2.10. Flow analysis

To evaluate the maturity of CD11c⁺BMDCs cultured in vitro, 1×10^6 cells were collected on day 7 of BMDCs culture and processed into a single-cell suspension, and the related flow cytometric antibodies APC-conjugated anti-CD11c (BD, USA, 561119), FITC-conjugated anti-CD86 (BD, USA, 561962), PE-conjugated anti-CD80 (BD, USA, 561955), and CY5.5-conjugated anti-MHC II (BD, USA, 562363) were added. The cells were incubated in the dark for 30 min at 4 °C. Then, the single-cell suspension was washed twice with PBS, and after completion, a 200-mesh filter was used to filter the cells into a new EP tube for flow cytometric detection [25].

To detect the effect of BMDC autophagy on the quantities of Th1, Th2, Th9, Treg, Th17, and Tfh cells, BMDC treated with different factors were cocultured with lymphocytes for 48 h, and then the cells and cell supernatants were collected by centrifugation. A total of 1×10^6 cells were prepared into a single-cell suspension, and the flow cytometric antibodies BV-421-conjugated anti-T-BET (BD, USA, 563318), BV-421-conjugated anti-GATA3 (BD, USA, 563349), PE-conjugated anti-PU-1 (CST, USA, 81886S), BV-650-conjugated anti-ROR γ T (BD, USA, 564723), BB700-conjugated anti-CXCR5 (BD, USA, 566492) and APC-conjugated anti-Foxp3 (BD, USA, 563318) were added. The cells were incubated in the dark for 30 min at 4 °C. The single-cell suspension was washed twice with PBS and then filtered into a new EP tube with a 200-mesh filter for flow cytometric detection. Additionally, the cell culture supernatant was collected, and the changes in related cytokines in the supernatant were measured with various cytokine microsphere array kits (CBAs) as described in Section 2.5, including those for the cytokines IL-2, IFN- γ , TNF, IL-4, IL-6, IL-10 and IL-17A related to Th1/Th2/Th17 cells [19,26].

2.11. Quantitative real time PCR assay

Total RNA from cell lysates was extracted with Trizol (Invitrogen) and cDNA was reverse transcribed with a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Real-time quantitative PCR was performed using FastStart Universal SYBR Green Master (Rox; Roche). The primers were shown in Table 1:

2.12. Statistical analysis

All data were analyzed using SPSS software v. 20.0 (IBM Corp, NY, USA). Western blot and confocal immunofluorescence images were analyzed using ImageJ software. All data were first subjected to descriptive analysis, normal distribution analysis, and analysis of variance. For data consistent with a normal distribution and homogeneity of variance, one-way analysis of variance was used for comparisons among

Table 1

Name		Sequence (5' -3')
T-bet	Forward	CCACCTGTTGTGGTCCAAGTTC
	Reverse	CCACAAACATCTGTAATGCTTG
GATA3	Forward	CCTCTGGAGGAGGAACGCTAAT
	Reverse	GTTTCGGGTCTGGATGCTTCT
PU-1	Forward	GAGGTGTCTGATGGAGAAGCTG
	Reverse	ACCCACAGATGCTGTCCTTCA
RORyt	Forward	GTGGAGTTTGCCAAGCGGCTTT
	Reverse	CCTGCACATTCTGACTAGGACG
CXCR5	Forward	ATCGTCCATGCTGTTACGCCT
	Reverse	CAACCTTGCAAGAGGAGTTC
FOXP3	Forward	CCTGGTGTGAGAAGGTCTTCG
	Reverse	TGCTCCAGAGACTGCACACTT
GAPDH	Forward	CATCACTGCCACCCAGAAGACTG
	Reverse	ATGCCAGTGAGCTTCCCGTTCAG

Samples were run in triplicate and relative expression of T-bet, GATA3, PU-1, RORyt, CXCR5 and FOXP3 was normalized to GAPDH with the $2^{-\Delta\Delta Ct}$ method [27]. Data were collected from three independent experiments.

multiple groups, and an LSD *t*-test was used for comparisons between two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. AR mice showed an obvious allergic inflammatory response, and the expression of autophagy proteins in the nasal mucosa was increased.

To investigate dendritic cell autophagy in an AR animal model, we used C57BL/6 mice as the model. Compared with the control group, mice in the AR group demonstrated increased allergic symptoms scores, including frequent scratching of the nose surface, a large number of nasal effusions from the front nostril, and continuous sneezing, while mice in AR + 3-MA group showed less nasal scratching, sneezing, and nose running ($P < 0.05$, Fig. 1B). Compared with the control group, AR mice also showed upregulated OVA-sIgE levels, increased eosinophil infiltrates as determined by HE staining, and elevated goblet cells hyperplasia as determined by PAS staining ($P < 0.05$, Fig. 1C-G). Notably, mice treated with 3-MA displayed markedly reduced serum levels of OVA-sIgE, as well as significant reductions in nasal eosinophil infiltration and mucus hypersecretion ($P < 0.05$, Fig. 1C-G). These experimental results all confirmed that the AR mouse model was successfully established in this study and 3-MA treatment attenuates the inflammatory responses in an AR model.

Compared with the control group, the AR group showed significantly up-regulated expression of LC3II and ATG5, while the expression of LC3II and ATG5 were decreased in the AR + 3-MA group ($P < 0.05$, Fig. 1H). In addition, P62 expression was significantly decreased in the AR group compared with the control group, and the expression of P62 was increased in the AR + 3-MA group ($P < 0.05$, Fig. 1H).

These results confirmed that a large number of autophagy reactions existed in the nasal mucosa of AR animal models and 3-MA treatment attenuates autophagy.

3.2. Autophagy was significantly increased in CD11c⁺ DCs in the nasal mucosa of AR mice, 3-MA treatment attenuates CD11c⁺ DC autophagy.

To study CD11c⁺ DC autophagy in AR model mice, LC3II expression in CD11c⁺ DCs in the nasal mucosa of AR model mice was tested. Compared with the control group, the AR group showed significantly increased LC3II expression in CD11c⁺ DCs in the nasal mucosa, while mice in AR + 3-MA group demonstrated markedly decreased LC3II expression in CD11c⁺ DCs (Fig. 2A, C, E). Statistical analysis found that the number of LC3II-positive cells, the number of CD11c-positive cells, and the number of CD11c and LC3II double-positive cells in the nasal mucosa of AR mice were significantly increased, however, all of these cells were decreased in the AR + 3-MA group ($P < 0.01$, Fig. 2B, D, F).

These results showed that the activation of CD11c⁺ DC autophagy was involved in the Th2 inflammation in the nasal mucosa of the AR animal model.

3.3. OVA stimulation induced increases in autophagosomes and the expression of autophagy-related proteins in BMDCs in a dose-dependent manner.

To investigate the direct effect of OVA on autophagy in BMDCs, BMDCs were induced in vitro, with the purity of CD11c⁺ BMDCs over than 92.3%. We observed the levels of autophagosomes and autophagy-related proteins in BMDCs. Compared with control treatment, OVA stimulation led to the appearance of a large number of autophagosomes in BMDCs, which increased with increasing OVA concentration ($P < 0.05$, Fig. 3A-B). However, the number of autophagosomes in BMDCs stimulated with 500 μ g/mL OVA was significantly reduced by 3-MA ($P < 0.01$, Fig. 3A-B).

Western blot results showed that compared with control group, increasing OVA concentrations gradually increased the autophagy marker proteins LC3II/LC3I and ATG5, and the highest expression was observed in the high-concentration group. However, increasing OVA concentrations gradually reduced the expression of P62 protein ($P < 0.05$, Fig. 3C-E). Moreover, after 3-MA-mediated inhibition of autophagy, the expression of LC3II and ATG5 decreased significantly compared with the OVA group, whereas the expression of P62 protein was increased compared with the OVA group ($P < 0.05$, Fig. 3C-E). These results confirmed that OVA induced autophagy in BMDCs in a dose-dependent manner and that autophagy inhibitors effectively blocked OVA-induced autophagy in BMDCs.

3.4. OVA induced BMDC autophagy by increasing the surface level of MHC II molecules to enhance DC antigen-presenting function

To study the effect of BMDC autophagy on the AR inflammatory response, we stimulated cultured CD11c⁺ BMDCs with OVA and studied their antigen-presenting function. In the blank group without the addition of the cytokines GM-CSF, IL-4, and TNF- α , bone marrow cells almost failed to differentiate into CD11c⁺ BMDCs (Fig. 4A). However, in the control group treated with several cytokines, including GM-CSF, IL-4, and TNF- α , 90% of the bone marrow cells were converted into CD11c⁺ BMDCs ($P < 0.05$, Fig. 4A). Similarly, 90% of the bone marrow cells in the low- and high-concentration groups and the 3-MA group were converted into CD11c⁺ BMDCs ($P < 0.05$, Fig. 4A) with normal morphology (Fig. 4E). These experimental results confirmed that we successfully induced CD11c⁺ BMDCs in vitro.

To further verify the influence of OVA and 3-MA on the maturity and function of BMDCs, we tested the BMDC surface expression of CD80, CD86, and several types of MHC II molecules. CD80 and CD86 reflect the maturity of DCs [28–29], and MHC II reflects the antigen-presenting function of DCs [30]. We found no significant difference in the expression of CD80 or CD86 on the CD11c⁺ BMDC surface among the control group, low-concentration group, high-concentration OVA group, and 3-MA group ($P > 0.05$, Fig. 4B-C), suggesting that OVA-induced autophagy does not affect the maturation of CD11c⁺ BMDCs. However, in the high-concentration OVA group, MHC II expression on the surface of CD11c⁺ BMDCs was increased compared with that in any other group ($P < 0.05$, Fig. 4D). MHC II expression was not different among the control group, the low-concentration group and the 3-MA group ($P > 0.05$, Fig. 4F). These results confirmed that a high concentration of OVA could induce autophagy in CD11c⁺ BMDCs and promote BMDC antigen-presenting function, which could be blocked by autophagy inhibitors.

3.5. OVA-induced BMDC autophagy can promote the immune imbalance of CD4⁺ T cell subtypes

In view of the immunological characteristics of AR, Th2 and Th17

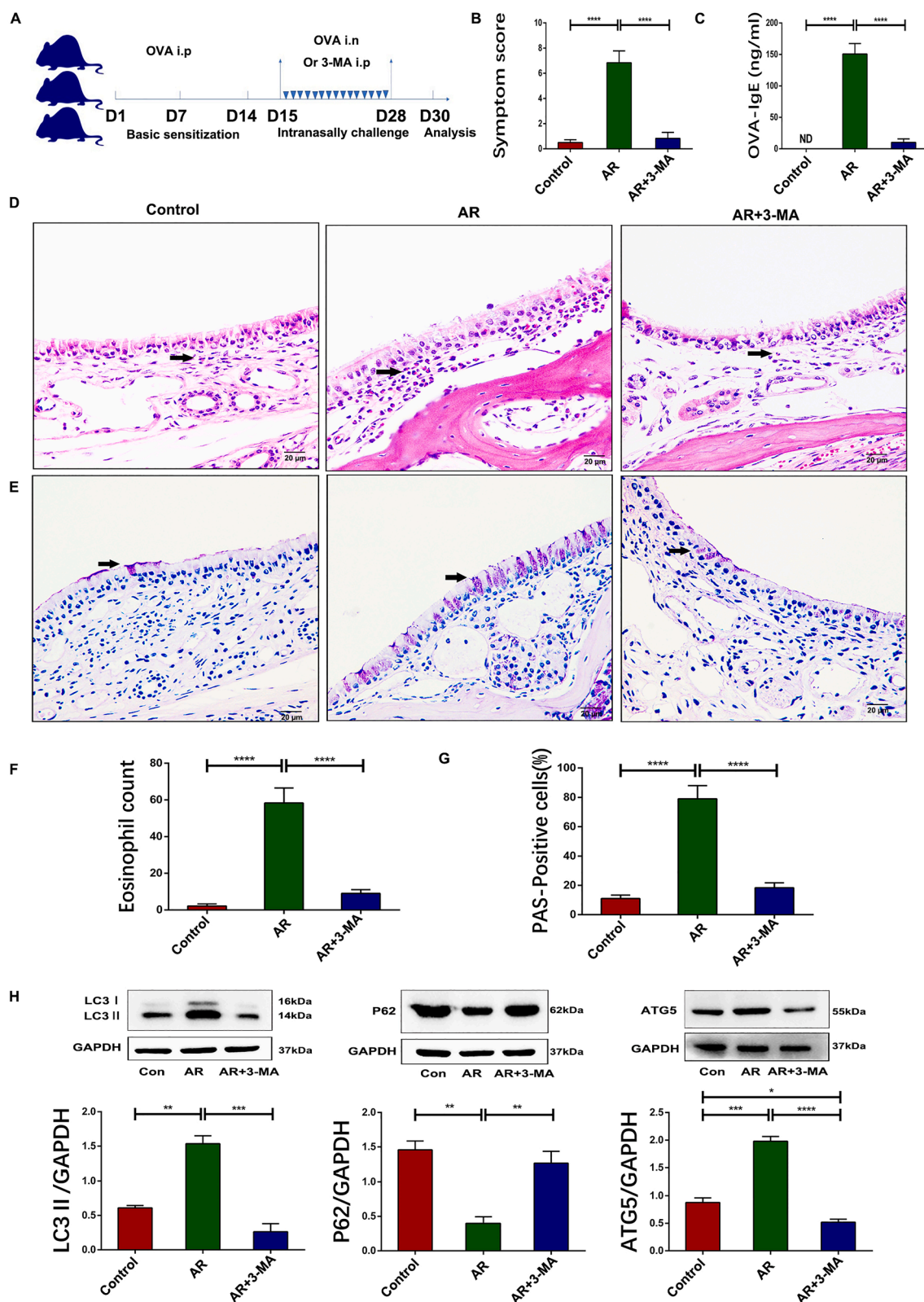


Fig. 1. Inflammatory response and expression of autophagy proteins in the nasal mucosa of AR mice. **A:** The mouse model of allergic rhinitis was established with intraperitoneal (I. P) injection and nasal drip (I. N) treatment. **B:** Symptomatic scores for each group. **C:** Levels of OVA-sIgE in the serum of mice in each group. **D:** Histopathological changes in the nasal mucosa; the arrow indicates eosinophils (400X). **E** Proliferation of goblet cells in the nasal mucosa. The arrows indicate proliferating goblet cells (400X). **F:** Eosinophils in the nasal mucosa of mice in each group were counted at high magnification. **G:** Proliferating goblet cells were counted in the nasal mucosa of mice in each group under high magnification. **H:** LC3II/GAPDH expression, P62/GAPDH expression and ATG5/GAPDH expression. Data were collected from three independent experiments, and the values represent the mean value. By independent t-test, * $P < 0.05$ and ** $P < 0.01$ *** $P < 0.001$ **** $P < 0.0001$.

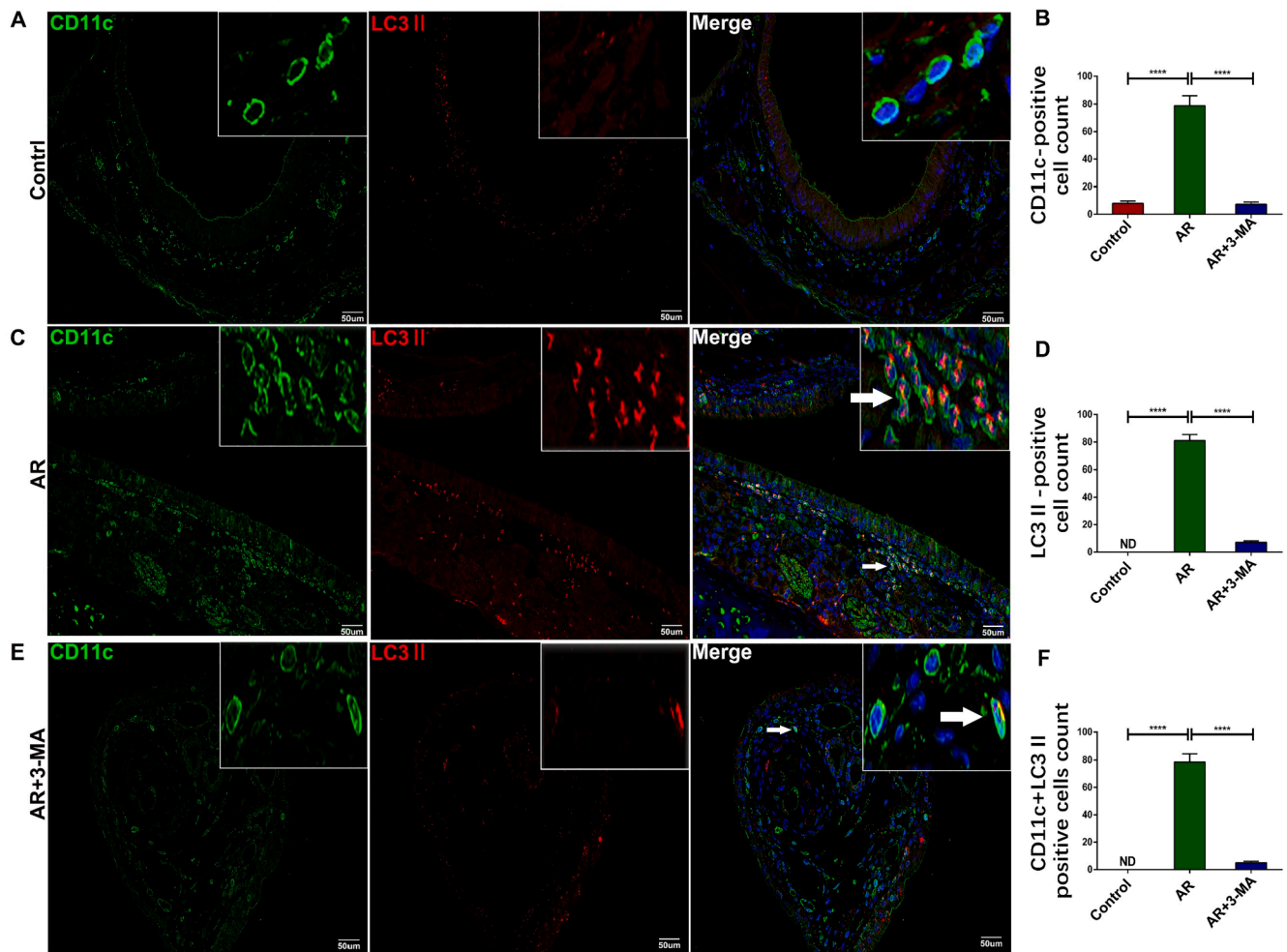


Fig. 2. LC3II fluorescence signals in CD11c⁺ DCs in the nasal mucosa of AR mice increased significantly. A: Staining for CD11c and LC3II in the nasal mucosa of control mice. C: Staining for CD11c and LC3II in the nasal mucosa of AR mice. E: Staining for CD11c and LC3II in the nasal mucosa of AR+3-MA mice. B: The number of CD11c-positive cells in the nasal mucosa of mice in each group. D: The number of LC3II-positive cells in the nasal mucosa of mice in each group. F: The number of CD11c and LC3II double-positive cells in the nasal mucosa of mice in each group. Data were collected from three independent experiments, and the values are mean values. By independent *t*-test, **** *P* < 0.0001. The arrows indicate CD11c⁺DCs autophagy.

cells are hyperactive, and Th1 cells are inhibited. In this study, the effects of OVA-induced BMDC autophagy on the immune imbalance of Th1/Th2/Th9/Th17/Treg/Tfh cell were studied. The results showed that the numbers of Th1 cells (CD4 + T-bet⁺) and Treg cells (CD4 + CD25 + Foxp3⁺) in the low-concentration group and high-concentration group gradually decreased compared with those in the control group (*P* < 0.05, Fig. 5A, F), while the numbers of Th2 cells (CD4 + GATA3⁺), Th9 cells (CD4 + PU-1⁺), Th17 cells (CD4 + RORγ T⁺) and Tfh cells (CD4 + CXCR5⁺) gradually increased (*P* < 0.05, Fig. 5B-E). These results suggest that BMDC autophagy can directly induce an allergic inflammatory response via promoting the immune imbalance of Th1/Th2/Th9/Th17/Treg/Tfh cells.

Compared with the low- and high-concentration groups, the 3-MA group, which included the autophagy inhibitor, showed significantly increased numbers of Th1 cells (CD4 + T-bet⁺) and Treg cells (CD4 + CD25 + Foxp3⁺) (*P* < 0.05, Fig. 5A, F), while the numbers of Th2 cells (CD4 + GATA3⁺), Th9 cells (CD4 + PU-1⁺), Th17 cells (CD4 + RORγ T⁺) and Tfh cells (CD4 + CXCR5⁺) were significantly decreased (*P* < 0.05, Fig. 5B-E).

The qRT-PCR results showed the same trends of key transcriptional factors of Th1/Th2/Th9/Th17/Treg/Tfh cells among these groups. Briefly, compared with the Control group, the expression of T-bet, Foxp3 were decreased and the expression of GATA3, PU-1, RORγt and CXCR5 were increased in both low- and high- concentration OVA groups.

Moreover, markedly increased T-bet, Foxp3 levels, as well as significantly decreased GATA3, PU-1, RORγt and CXCR5 expression, were observed in 3-MA group (Fig. 5G; *p* < 0.05). These results suggest that autophagy inhibitors can effectively block autophagy in BMDCs and their induction of allergic inflammation via reversing the immune imbalance of CD4⁺ T cell subtypes.

3.6. OVA-induced BMDC autophagy can aggravate an allergic inflammatory response by promoting Th1/Th2/Th17 cytokine imbalance

To understand the effect of BMDC autophagy on the allergic inflammatory response, we detected the levels of Th1 (IL-2, TNF-α, and IFN-γ), Th2 (IL-4, IL-6, and IL-10), and Th17 (IL-17) cytokines. The contents of IL-2, TNF-α, and IFN-γ in the supernatant of lymphocyte cocultures were significantly dose-dependently decreased after the addition of OVA-stimulated BMDCs compared to control group (*P* < 0.05, Fig. 6A-B). However, the levels of the cytokines IL-4 and IL-6 were significantly increased in a dose-dependent manner (*P* < 0.05, Fig. 6B). Compared with those in the control group, the levels of IL-17 and IL-10 in the OVA (100 μg/mL) group were decreased, but the difference was not statistically significant (*P* > 0.05); the levels of IL-17 and IL-10 in the OVA group (500 μg/mL) were significantly increased (*P* < 0.05, Fig. 6A-B). These results suggest that OVA-induced BMDC autophagy can directly induce an allergic inflammatory response by promoting Th1/

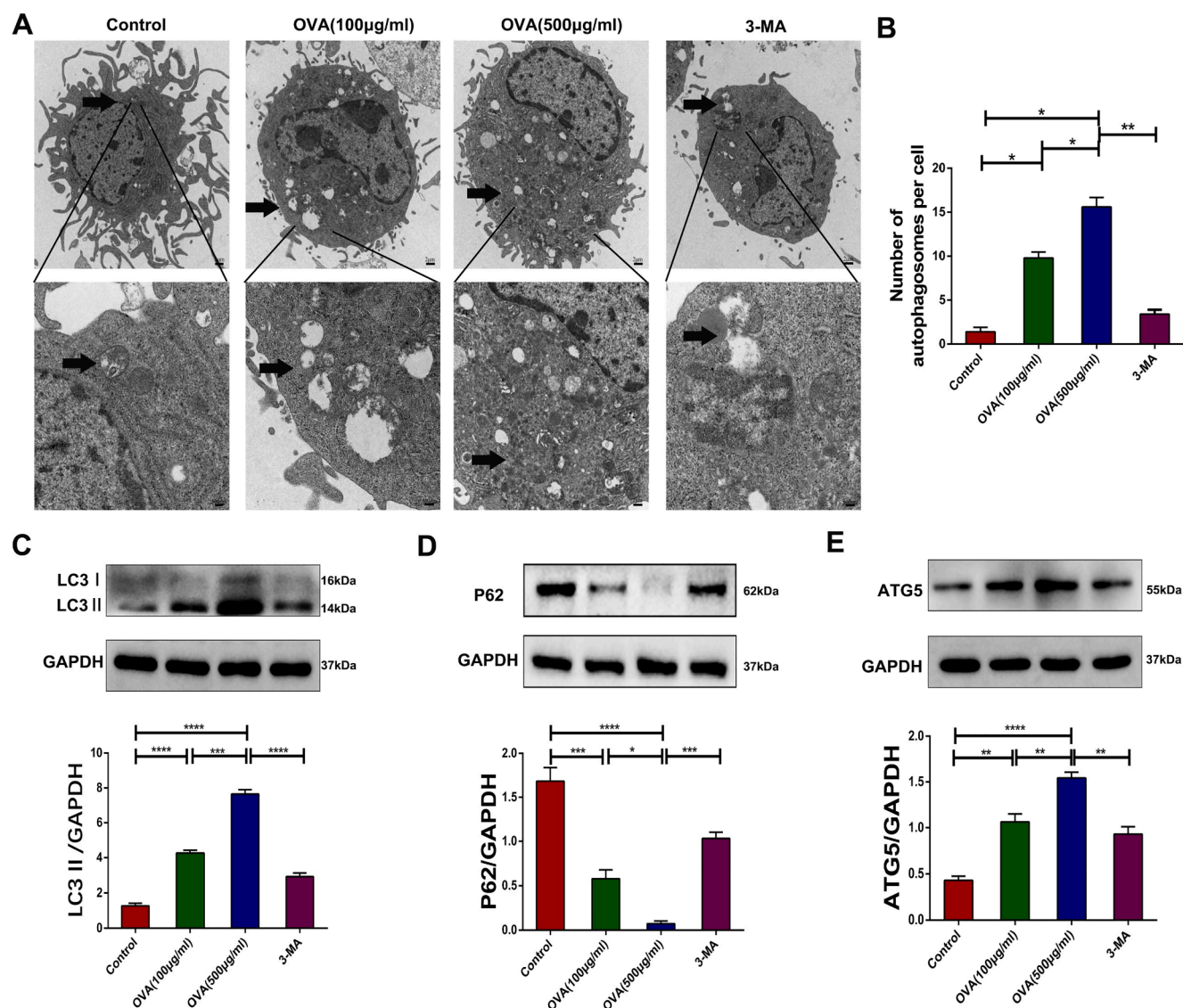


Fig. 3. OVA can induce autophagy in BMDCs cultured in vitro in a dose-dependent manner. A: Transmission electron microscopy. The arrow points to the vacuolar, bilateral membranous autophagosomes (8000 X) in BMDCs. B: Statistical analysis of the number of autophagosomes in each group. C: The LC3II expression in each group. D: The P62 expression in each group. E: The ATG5 expression in each group. Data were collected from three independent experiments, with values representing the mean; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Th2/Th17 cytokine imbalance.

However, compared with those in the coculture supernatant in the OVA (100 µg/mL) and OVA (500 µg/mL) groups, the contents of IL-2, TNF- α , and IFN- γ in the coculture supernatant in the 3-MA group were increased, while the contents of the cytokines IL-4, IL-6, IL-17, and IL-10 were significantly decreased ($P < 0.05$, Fig. 6A-B). These results confirm that autophagy inhibitors can effectively block OVA-induced BMDC autophagy and the subsequent induction of allergic inflammation via reversing Th1/Th2/Th17 cytokines imbalance.

3.7. Adoptive transfer of OVA-loaded BMDCs could significantly promote the allergic inflammation, while the administration of 3-MA on OVA-loaded BMDCs could significantly reduce the AR inflammation

Since it has been proved that OVA induced BMDCs autophagy in a dose-dependent manner, adoptive transfer murine model was further conducted to study the role of BMDCs autophagy on allergic inflammation (Fig. 7A). Compared with the Control group, mice in OVA group exhibited increased allergic symptom scores (Fig. 7B; $p < 0.05$), OVA-specific serum IgE titres (Fig. 7C; $p < 0.05$), eosinophil infiltration,

and goblet cell hyperplasia (Fig. 7D-F; $p < 0.05$). However, compared with the OVA group, mice in 3-MA group displayed markedly reduced allergic symptom scores, serum OVA-specific IgE titres, nasal eosinophil infiltration, and mucus hypersecretion (Fig. 7B-F; $p < 0.05$).

Furthermore, compared with the Control group, the levels of the cytokines IL-4, IL-6 and IL-17 were significantly increased in OVA group, while the levels of IL-2 and IFN- γ were decreased. The administration of 3-MA on OVA-loaded BMDCs could significantly reversed the cytokine imbalance (Fig. 7G; $p < 0.05$). These results further indicated the important role of DC autophagy in the development of AR.

4. Discussion

The occurrence of autophagy requires several stages, such as the formation of autophagosome precursors, the formation of autophagosomes, and the completion of substrate degradation by the fusion of autophagosomes and lysosomes [12]. In the whole process, LC3II on autophagosome precursors has been indicated to play a key role in the extension of autophagosome precursors [31]; its expression can indirectly reflect the degree of autophagy [32]. A study on the AR mouse

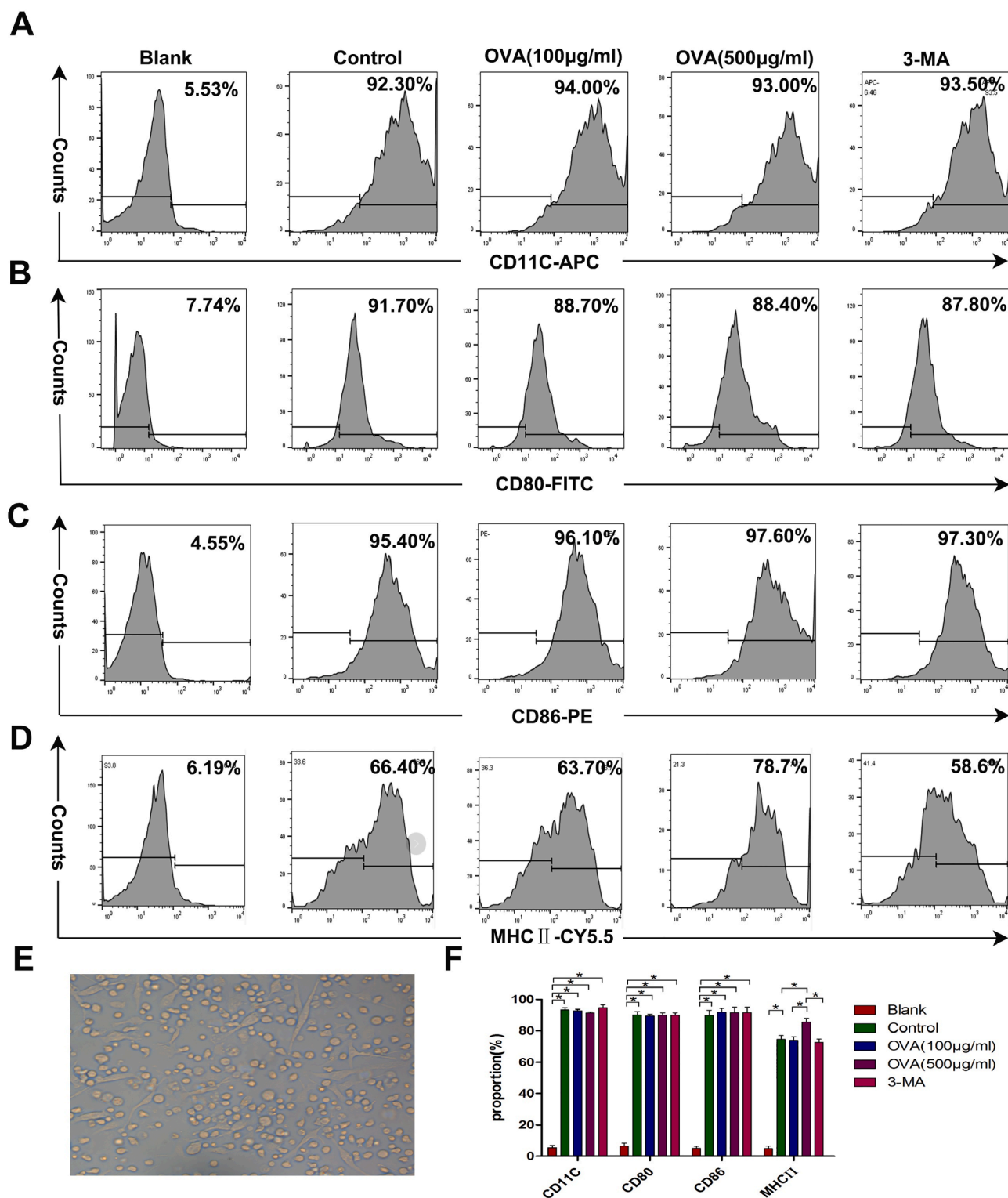


Fig. 4. Autophagy promotes antigen presentation by CD11c⁺BMDCs. A: Expression of CD11c on the cell surface in each group. B: Expression of CD80 on the cell surface in each group. C: Expression of CD86 on the cell surface in each group. D: Expression of MHC II on the cell surface in each group. E: Mature CD11c⁺ BMDCs (200X) were observed under a microscope. F: Statistical analysis of CD11c, CD80, CD86, and MHC II. Data were collected from three independent experiments, and the value represents the mean; independent *t*-test, * *P* < 0.05.

model showed an increase in OVA-specific IgE in AR mice, a large amount of eosinophilic infiltration, and goblet cell proliferation in the nasal mucosa of AR mice, with typical characteristics of Th2 polarization, which confirmed the successful construction of the AR mouse model. Furthermore, the expression of the autophagy protein LC3II was

significantly increased in CD11c⁺ DCs in the nasal mucosa of AR mice. These results jointly confirm that allergens may promote the occurrence and development of AR by inducing CD11c⁺ DC autophagy.

MHC II expression mainly occurs on the surface of DCs and other antigen-presenting cells, and MHC II can be seen as a marker of the

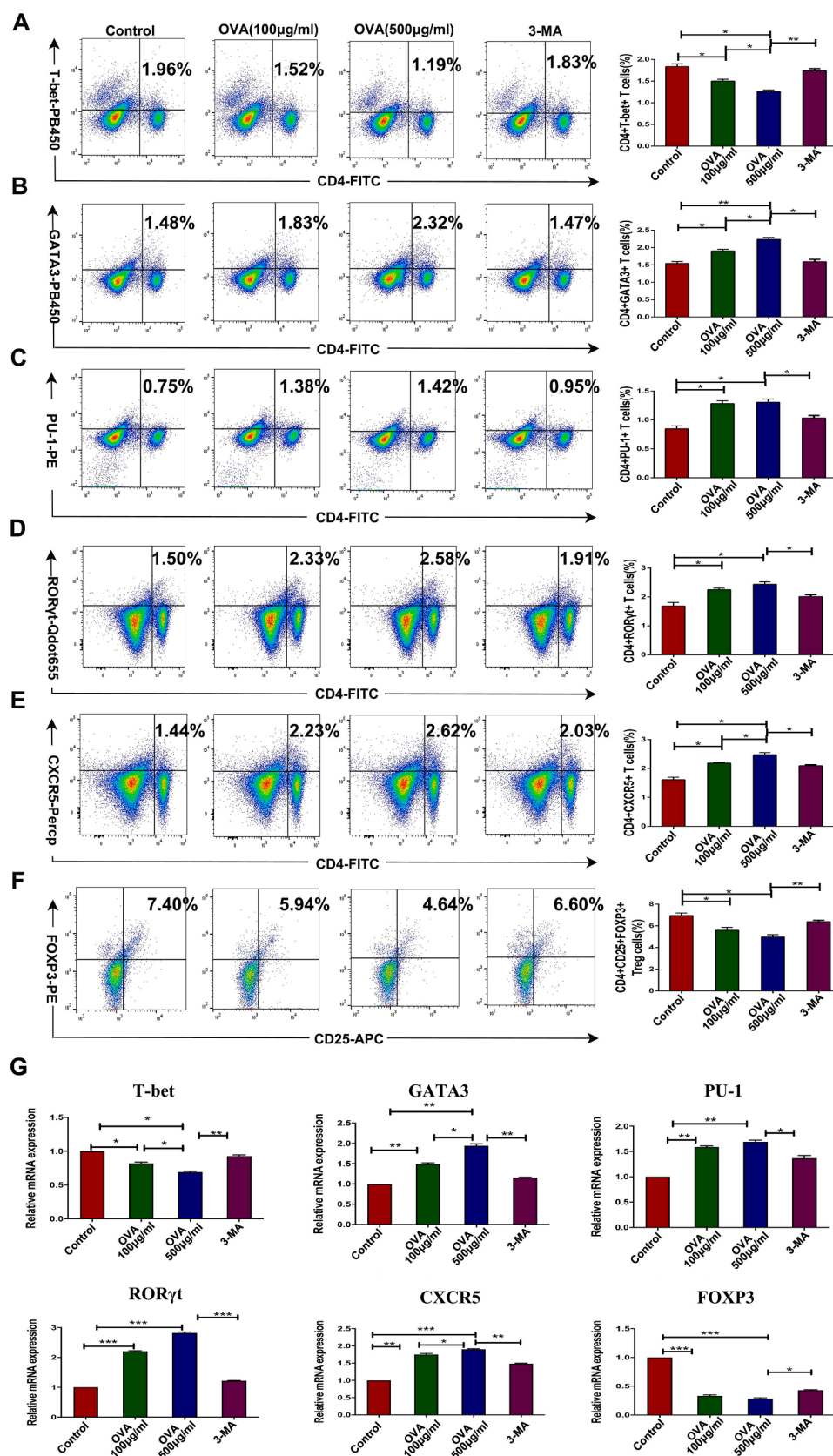


Fig. 5. Effects of BMDC autophagy on the immune imbalance of Th1/Th2/Th9/ Th17/Treg/ Tfh cells. A: The number of Th1 cells (CD4+T-bet+) in the lymphocyte population in each group. B: The number of Th2 cells (CD4+GATA3+) in the lymphocyte population in each group. C: The number of Th9 cells (CD4+PU-1+) in the lymphocyte population in each group. D: The number of Th17 cells (CD4+RORγt+) in each group. E: The number of Tfh cells (CD4+CXCR5+) in each group. F: The number of Treg cells (CD4+CD25+Foxp3+) in the lymphocyte population in each group. G: Gene expression of T-bet, GATA3, PU-1, RORγt, CXCR5 and FOXP3 in cocultures was determined by quantitative real-time PCR. Data were collected from three independent experiments, with values representing the mean; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

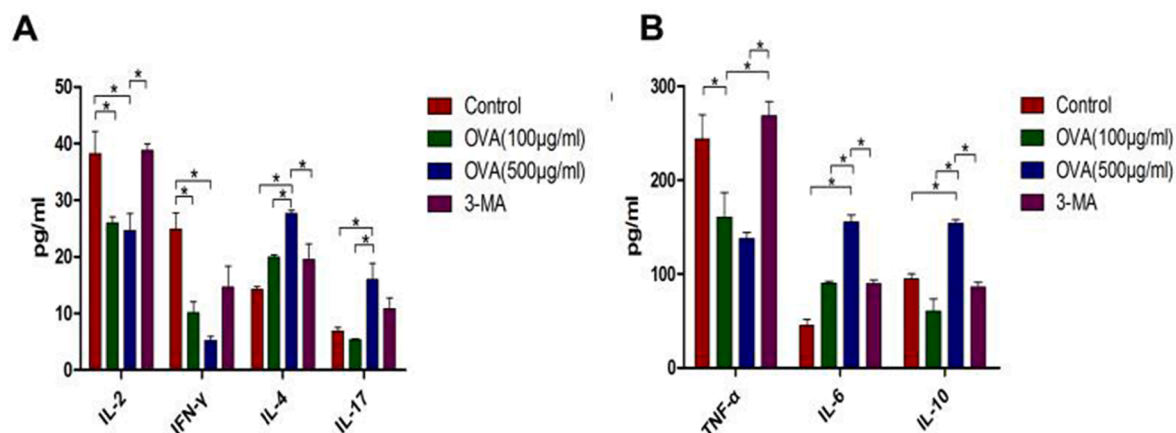


Fig. 6. BMDC autophagy can induce an allergic inflammatory response by promoting Th1/Th2/Th17 cytokine imbalance. A: Levels of the cytokines IL-2, IFN- γ , IL-4, and IL-17 in the supernatant of cells cocultured with lymphocytes in each group. B: Levels of the cytokines TNF- α , IL-6, and IL-10 in the supernatant of cells in each group. Data were collected from three independent experiments, and the value represents the mean; *, $P < 0.05$.

antigen-presenting ability of a cell [33]. Autophagy may play an important role in the antigen-presenting function of DCs, and DC autophagy provides a large amount of antigens, which can be loaded onto MHC II molecules to play an important role in initiating the next step of the T cell-mediated immune reaction [5,34–37]. To date, there has been no systematic study on the correlations among allergens, CD11c⁺ DC autophagy, and antigen-presenting function. In vitro experiments in this study showed that OVA could induce CD11c⁺BMDC autophagy in a dose-dependent manner. Additionally, a high concentration of OVA induced BMDC autophagy and could promote MHC II molecule expression on the BMDC surface, enhancing BMDC antigen-presenting function. We speculated that BMDC autophagy and the associated enhancement of antigen presentation by BMDCs might be important reasons for the occurrence and development of AR.

Studies have shown that DC autophagy can regulate the T cell response through multiple pathways, such as MHC antigen presentation, inflammatory signal transduction, production of related cytokines, and the influence of related metabolism in T cells [38]. Persistent and uncontrolled immune imbalance of Th cell responses are often associated with the pathological states of allergic rhinitis. At present, there are few reports on the effects of DC autophagy on Th1, Th2, Th9, Th17, and Tfh cells. Studies have reported only that DC autophagy can promote Th17 cell differentiation and IL-17 cytokine secretion via IL-1 cytokine secretion [39]. In this work, we investigated the effects of allergen-induced CD11c⁺DC autophagy on downstream effector T cells. Considering that the spleen lymphocytes of mice can relatively comprehensively simulate the immune system of mice in vitro, we selected the spleen lymphocytes of mice and CD11c⁺BMDCs for a coculture experiment according to Delamarre's method [40–44] to better understand the effect of BMDC autophagy on the immune imbalance of Th cell responses.

In this study, OVA-stimulated BMDCs were cocultured with lymphocytes, and the differentiation of the CD4⁺ T cell subtypes Th2, Th9, Th17, and Tfh was increased, while the differentiation of Th1 and Treg cells was decreased. Concordant with this, the levels of the Th2/Th17-related cytokines IL-4, IL-6 and IL-17 in the supernatant of cocultures increased, while the levels of the Th1-related cytokines IL-2, TNF- α , and IFN- γ decreased, showing typical Th2 cell polarization. However, the autophagy inhibitor 3-MA [45] could block OVA-induced BMDC autophagy and subsequent inflammation. This is different from the results of Suzuki, Y et al. [46], who found that deletion of ATG5, a key protein in autophagy initiation, in DCs promoted the differentiation of Th17 cells. The possible reason is that CD11c⁺DC autophagy involves multiple genes, and other autophagy genes may be activated and lead to the differentiation of Th17 cells after ATG5 knockout in DCs. 3-MA used in

this study could inhibit all related proteins in autophagy pathway, which may explain the different results between the two studies. In addition, the different results might also be due to different mouse strains and experimental conditions. The BMDCs obtained from C57BL/6 mice were cocultured with lymphocytes in the presence of 500 μ g/mL OVA in this study, while BMDCs obtained from OT-II mice were cocultured with CD4⁺T cells in the presence of 10 mg/mL OVA₃₂₃₋₃₃₉ in Suzuki, Y' research [46].

In addition, adoptive transfer murine model was further conducted to study the role of BMDCs autophagy in allergic inflammation. Interestingly, i.v. injection of OVA-BMDCs alone 2 times, without i.p. OVA sensitization, over 3 days elicited an allergic phenotype. The administration of 3-MA on OVA-loaded BMDCs could significantly reduce allergic symptom scores, serum OVA-specific IgE titres, nasal eosinophil infiltration, and mucus hypersecretion. These results further indicated the important role of DC autophagy in AR.

Since autophagy is a physiological process that widely exists in many immune cells, such as macrophages, lymphocytes, and neutrophils [34], the production of the Th2 inflammatory response in mice observed in the animal experiment in this study might also be the result of multiple factors in addition to CD11c⁺ DC autophagy. In this study, the overall autophagy level in nasal mucosa tissues and the expression of CD11c⁺ DC autophagy marker proteins were detected, but autophagy in other immune cells was not comprehensively detected, so other immune cells undergoing an autophagy reaction and making a contribution to the subsequent allergic inflammatory reaction could not be ruled out. Although CD11c is considered as the maker for DC [47–48], recent studies have revealed that CD11c is also expressed by certain activated neutrophil, macrophage and even B cells [49–50]. So the role of autophagy in other CD11c⁺ cells, such as neutrophil, macrophage and even B cells, on the development of AR is worthy of further study.

Some studies have reported that autophagy plays a protective role in some proinflammatory immune cells and innate immune cells [51], but when activated in some cells, such as macrophages in pulmonary fibrosis, autophagy may aggravate disease occurrence [52]. These studies suggest that autophagy may play a dual role in the immune system. The results of this study indicate that allergens can induce autophagy in BMDCs and that autophagy in BMDCs will lead to an immune imbalance of Th1/Th2/Th9/Th17/Treg/Tfh cell, thus causing subsequent AR inflammation, which is a factor that aggravates the occurrence of AR.

In conclusion, this study found that OVA-induced CD11c⁺DC autophagy aggravated allergic rhinitis by increasing the antigen-presenting function of CD11c⁺DCs and promoting the immune imbalance of CD4⁺ T cell subtypes. Specifically, inhibiting CD11c⁺ DC autophagy

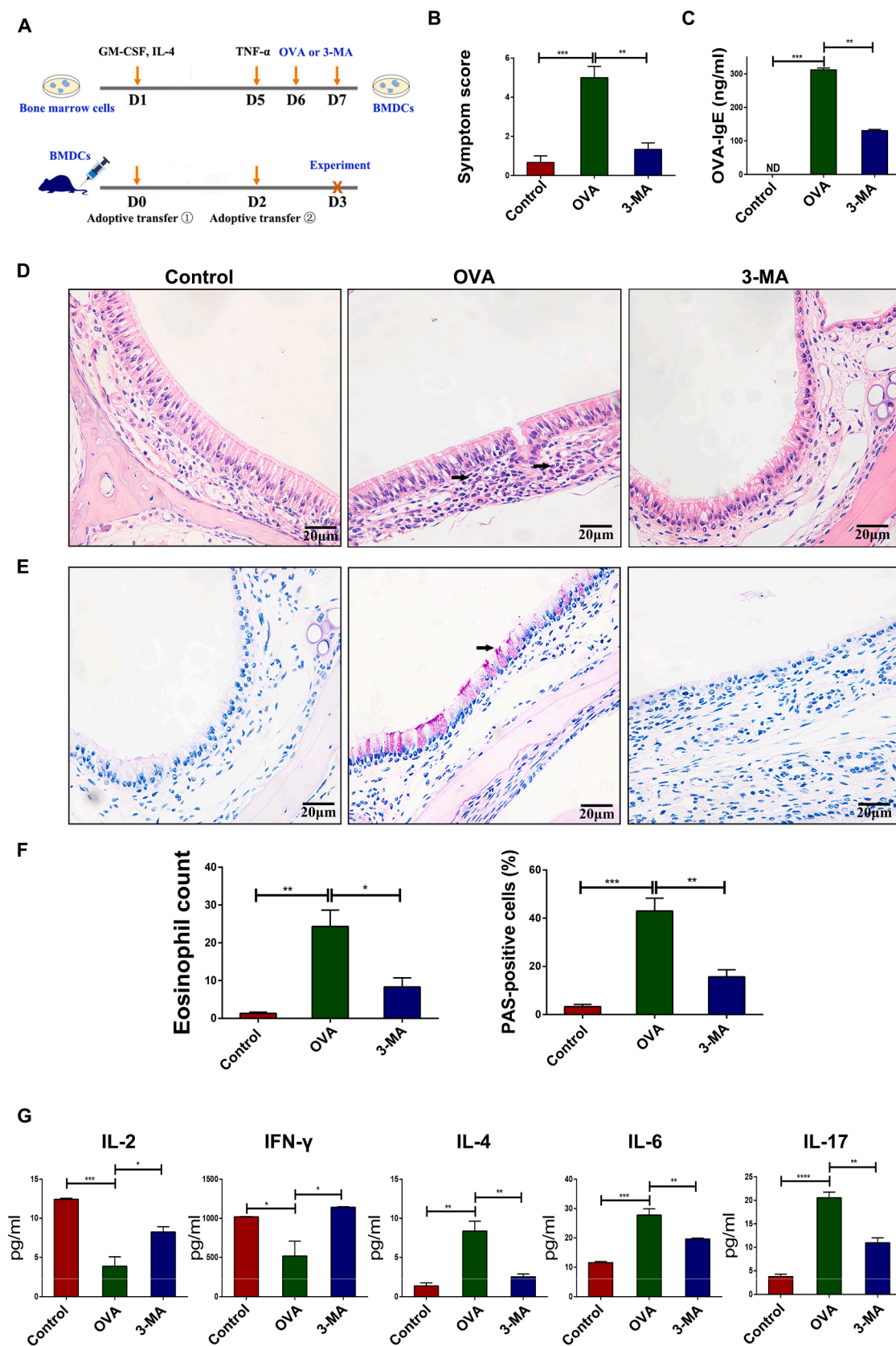


Fig. 7. Adoptive transfer of OVA-loaded BMDCs could significantly promote the allergic inflammation. A: Experimental protocol for the development of adoptive transfer animal model. B: Symptomatic scores for each group. C: Levels of OVA-sIgE in the serum of mice in each group. D: Histopathological changes in the nasal mucosa; the arrow indicates eosinophils (400X). E: Proliferation of goblet cells in the nasal mucosa; the arrow indicates proliferating goblet cells (400X). F: Eosinophils or proliferating goblet cells were counted at high magnification. G: Levels of the cytokines IL-2, IFN-γ, IL-4, IL-6, and IL-17 in each group. * $P < 0.05$ and ** $P < 0.01$ *** $P < 0.001$ **** $P < 0.0001$.

might be a new target to treat AR.

Author Contributions

SC, RY and ZT designed the research. YH, YQ and SX conducted the research. SX, YK and WJ analyzed the data. YQ wrote the manuscript, SC and WJ revised the manuscript. SX and RY provided language help. All authors contributed to the article and approved the submitted version.

Funding information

This work was supported by the National Natural Science Foundation of China [grant numbers: 81770981; 81870705], by the Fundamental Research Funds for the Central Universities of China [grant numbers: 2042019kf0086], and by Hubei Province health and family planning scientific research project [grant numbers: WJ2019M186].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the National Natural Science Foundation of China [grant numbers: 81770981; 81870705], by the Fundamental Research Funds for the Central Universities of China [grant numbers: 2042019kf0086], and by Hubei Province health and family planning scientific research project [grant numbers: WJ2019M186]. We thank all the funding sources.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics Statement

The animal study was reviewed and approved by the Animal Ethics Committee of the Renmin Hospital of Wuhan University (Ethics License No.: WDRM-20161016). All efforts were made to minimize the suffering of laboratory animals.

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