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FoxO4 mediates macrophage M2 polarization by promoting LXA4R expression in an ovalbumin-induced allergic asthma model in mice

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Abstract
Background: Asthma imposes a heavy burden due to its high prevalence. Forkhead box O4 (FoxO4) proteins participate in the modulation of cell progression. However, the role and mechanism of FoxO4 in asthma remains uncharted.
Methods: An allergic asthma model was constructed by the induction of ovalbumin and interleukin (IL)-4 in mice and monocyte/macrophage-like Raw264.7 cells, respectively. The role and mechanism of FoxO4 in asthma was determined by pathological staining, immunofluorescence assay, measurement of inflammatory cells in the blood, reverse transcription quantitative polymerase chain reaction (RT-qPCR), Western blot analysis, and flow cytometry.
Results: Ovalbumin treatment triggered an obvious inflammatory cell infiltration with a prominent increase in F4/80$^+$ cell numbers. The relative *messenger RNA* (mRNA) and protein expressions of FoxO4 were increased in both ovalbumin-induced mice and interleukin-4 (IL-4)-induced Raw264.7 cells. Inhibition of FoxO4 via AS1842856 reduced inflammatory cell infiltration, the number of Periodic Acid Schiff+ (PAS+) goblet cells, the numbers of inflammatory cells in the blood, and the airway resistance in ovalbumin-induced mice. Besides, interference of FoxO4 decreased the number of F4/80$^+$CD206$^+$ cells, and the relative protein expressions of CD163 and Arg1 *in vivo* and *in vitro*. Mechanically, suppression of FoxO4 diminished the relative mRNA and protein expressions of LXA4R in both ovalbumin-induced mice and IL-4-induced Raw264.7 cells. Overexpression of LXA4R reversed the outcomes caused by repression of FoxO4, including airway resistance, the number of F4/80+ cells, the proportion of CD206+ cells in ovalbumin-induced mice, and the proportion of F4/80$^+$CD206$^+$ cells in IL-4-induced Raw264.7 cells.
Conclusion: FoxO4/LXA4R axis mediated macrophage M2 polarization in allergic asthma.

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Introduction

Asthma is the most common chronic respiratory disorder affecting people of all ages.[1] It is estimated that by 2025. there would be more than 400 million asthma patients globally.[2] Although asthma is generally characterized by wheezing, cough, chest tightness, and shortness of breath, specific differences in relevant risk factors, age of onset, comorbidity, degree of severity, and response to therapy are discovered in most of the patients, indicating that asthma is heterogeneous.[3-6] The pathogenesis of asthma is mainly associated with inflammation, airway remodeling, hyperresponsiveness, reversible airway obstruction, and epigenetics.[7, 8] Importantly, inflammation plays a pivotal role in asthma pathogenesis, among which inflammation of the airways leads to bronchial hyperresponsiveness, remodeling of the airway wall, and mucus production.[9] It has been demonstrated that 60% of asthma is allergic, which is involved in T helper 2 (Th2) cell responses.[9] A series of agent target molecules, cytokines, and effector cells specifically of asthma is approved as alternative treatment of asthma;[10,11] however, the mainstay of clinical treatment remains inhaled corticosteroids, which have certain adverse effects and limitations.[12] Thus, the ongoing exploration of molecular mechanisms and search for potential therapeutic targets of asthma are still urgent for its clinical development.

Forkhead box O (FoxO) proteins are a type of transcription factors (TFs) with a highly conservative forkhead box domain that can directly bind to diverse target DNA sequences in nucleus.[13] FoxO proteins serve as a critical function on cellular adaptation to various stress stimuli, and multiple age-related disorders, which have been demonstrated to be potential targets for the therapeutic development of drugs and geroprotectors.[14] Four members (FoxO1, FoxO3, FoxO4, and FoxO6) mainly comprise the FoxO family in mammals.[15] Among them, FoxO4 is broadly expressed in different tissues, which can act as a transcriptional inhibitor and activator by regulating upstream and downstream gene.[16] Importantly, FoxO4 participates in the modulation of cell cycle arrest, cell proliferation, cell senescence, apoptosis, inflammation, autophagy, energy production, and muscle homeostasis.[17] Several studies have revealed that FoxO signal pathway is involved in asthma.[18,19] An upregulated expression of FoxO3 is observed in nasal epithelium in childhood asthma via transcriptome-wide and differential expression network analyses.[20] However, the role and mechanism of FoxO4 in asthma is still required to be determined.

Lipoxin A4 (LXA4) belongs to the lipoxin family that is produced in human leukocytes through 15-lipoxigenase from arachidonic acid.[21] LXA4 plays an important role in suppressing chemotaxis, adhesion, immune cell recruitment, and transmigration; thus, it generally serves as a vital halt following infection or injury *in vivo* and *in vitro*.[22] LXA4 is strongly involved in anti-inflammatory effects that protect against excessive inflammatory reactions to restrict damage to the host.[23-25] Moreover, LXA4 enhances the recruitment of macrophages to clear cellular debris and suppresses macrophage/microglial activation.[26-28] LXA4 receptor (LXA4R) is one of the G protein-coupled receptors of LXA4. It is initially cloned from human and mouse myeloid cells because of the strength of its high affinity for LXA4.[29,30] LXA4R has been reported to be downregulated in severe asthma,[23] and its activation suppresses airway inflammation in ovalbumin (OVA)-induced mice through toll-like receptor 2–myeloid differentiation primary response 88–nuclear factor *kappa B* (TLR2–MYD88–NF-κB) axis.[31]

In the present study, the role of FoxO4 in asthma was explored *in vitro* and *in vivo*. The expression of FoxO4 was first detected in ovalbumin-induced mice and interleukin-4 (IL-4)-induced Raw264.7 cells. Then, the role of FoxO4 in airway inflammation in ovalbumin-induced mice and in macrophage M2 polarization in IL-4-induced Raw264.7 cells was investigated. In addition, the potential mechanism was surveyed through the assessment of FoxO4–LXA4R axis in both animal and cell models of asthma.

Materials and Methods

Animals

Male C57BL/6 mice, 3 days after birth (P3), were purchased from Cyagen (Jiangsu, China) and raised in a temperature-controlled laboratory conditions with a 12-h light–dark cycle. During the entire experiment, the newborn mice were fed with mother's breast milk. All experiments involving animal were validated by the Guide for the Care and Use of Laboratory Animals,[32] as well as by the Animal Research Ethics Committee of Zhejiang Provincial People's Hospital (Approval No.: A2021121303).

Animal treatment

Mice were randomly allotted into five groups (n = 5), including sham mice, OVA, OVA+12 mg/kg AS1842856, OVA+25 mg/kg AS1842856, and OVA+25 mg/kg AS1842856+OE-LXA4R. After 2 days of acclimatization, mice were intraperitoneally injected with 10-μg ovalbumin (01641, 97–100% [High-performance liquid chromatography, HPLC]; Sigma, St. Louis, MO) on postnatal day 5 (P5) and P10, followed by a daily administration of 3% nebulized ovalbumin for 10 min up to P20. The sham mice were treated with the same dose of normal saline (IN9000; Solaibio, Beijing, China). In addition, mice in the OVA+12 mg/kg AS1842856 and OVA+25 mg/kg AS1842856 groups were intraperitoneally administrated with 12- and 25-mg/kg FoxO4-specific inhibitor AS1842856 (344355, ≥98% HPLC; Sigma) 48 h prior to modeling, while mice in the sham group were intraperitoneally injected with the same volume of normal saline. Mice in the OVA+25 mg/kg AS1842856+OE-LXA4R group were intraperitoneally administrated with 25-mg/kg AS1842856 48 h prior to modeling, and then received 1×10^8 plaque-forming units (PFU) of adenovirus-OE-LXA4R (GenePharma, Shanghai, China) through the caudal vein. Mice were intraperitoneally injected with 50-mg/kg sodium pentobarbital for sacrifice 24 h after the last ovalbumin stimulation. The blood was harvested for the analysis of inflammatory cells. Lung tissues were also collected for the subsequent assays and the bronchoalveolar lavage fluid (BALF) was rendered using 1 mL of 1× Hanks buffered salt solution (HBSS, H1025; Solaibio) to lavage lung tissues.

Pathological staining

Perfused lung tissues were immobilized with 4% paraformaldehyde (P1110; Solaibio). After dehydration and embeddedness, tissues were sectioned to 5-μm slices. Then, slices were dewaxed, hydrated, and stained with hematoxylin and eosin (H&E) kit (G1120; Solaibio) and periodic acid schiff (PAS) kit (G1280; Solaibio) to evaluate airway inflammation and mucus gland hyperplasia, respectively. After being mounted with neutral resin, slices were photographed using a digital trinocular camera microscope (CX23; Olympus, Tokyo, Japan).

Immunofluorescence assay

After flushed with pre-cold 0.1-M phosphate buffer saline (PBS; P1020; Solarbio) transcardially, mice were further flushed with pre-cold 4% paraformaldehyde. Subsequently, lung tissues were promptly extirpated and fixed in paraformaldehyde overnight. Next, lung tissues were embedded in optimal cutting temperature (OCT) compound (4583; Sakura, CA, USA) and sectioned to 5-μm slices. Slices were incubated with *bovine serum albumin* (BSA) blocking buffer (SW3015; Solarbio) and 0.2% Triton X-100 (T8200; Solarbio), and treated with the antibody targeting F4/80 protein (1:1000, ab6640; Abcam, Cambridge, UK) overnight at 4°C. Following the administration of goat anti-rat immunoglobulin G (IgG) H&L (Alexa Fluor® 647; 1:1000, ab150167; Abcam) for 1 h at room temperature, slices were stained with mounting medium, anti-fading (with 4′,6-diamidino-2-phenylindole [DAPI], S2110; Solarbio), and projected with a fluorescence microscopy (IX71; Olympus).

Measurement of inflammatory cells in the blood

Inflammatory cells in the blood, including white blood cells, monocytes, neutrophils, eosinophils, lymphocytes, and basophils, were detected by an automated multi-species hematology analyzer, HEMAVET 950 FS (Drew Scientific Inc., Oxford, UK).

Detection of airway responsiveness

The methacholine-elicited airflow obstruction was used to assess airway responsiveness based on the previous study.[33] Mice were intraperitoneally injected with 50-mg/kg sodium pentobarbital, and stimulated with elevating concentrations of methacholine varied from 0 to 50 mg/mL. Lung airway resistance of mice with cannula was determined by the Buxco Pulmonary Mechanics System (Buxco Electronics, NC, USA).

Cell treatment and transfection

Murine macrophage cell line Raw264.7 were obtained from Procell (CL-0190; Wuhan, China) and grown in Dulbecco's modified eagle *medium* (DMEM, PM150210; Procell) with 10% fetal bovine serum (FBS, 164210-50; Procell) and 1% streptomycin–penicillin (PB180120; Procell). Cells were exposed to 20-ng/mL IL-4 for 48 h to induce macrophage M2 differentiation.[34] To explore the role of FoxO4 in IL-4-induced Raw264.7 cells, short hairpin (sh)RNA targeting FoxO4 (shFoxO4) and relevant negative control (shNC) were acquired from Genechem (Shanghai, China) and transfected into Raw264.7 cells with Lipofectamine 3000 (L3000001; Invitrogen, Carlsbad, CA). Besides, the overexpressed plasmids of LXA4R (GenePharma) were also transfected into Raw264.7 cells to address the role of LXA4R in the regulation of FoxO4 inhibition *in vitro*.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The relative *messenger RNA* (mRNA) levels of FoxO4 and LXA4R were examined by RT-qPCR experiment in keeping with the previous description.[35] Lung tissues and Raw264.7 cells were introduced with TRIzol reagent (TaKaRa Biotechnology, Dalian, China) to extract total RNA, which was then reversely transcribed by Bio-Rad Scrip™ complementary DNA (cDNA) synthesis kit (1708890; Bio-Rad Laboratories, Hercules, CA, USA). RT-qPCR was performed with 25-μL mixture, including 1-μL cDNA templates, 12.5-μL 2×SYBR Green PCR Mastermix (SR1110; Solarbio), 1-μL of 10-μM forward and reverse primers, and 10.5-μL diethyl pyrocarbonate (DEPC) double-distilled water (ddH_2O) on the Bio-Rad CFX Manager software (Bio-Rad Laboratories). The primer sequences were: 5′-GAATCCTGGGGGCTGTAAC-3′ (FoxO4 forward), 5′-CCTTGATGAACTTGCTGTGC-3′ (FoxO4 reverse), 5′-CCTGGGGCAACTCTGTTGAG-3′ (LXA4R forward), 5′-AGTCCTGGCCCATGAAAACA-3′ (LXA4R reverse), 5′-CATGTACGTTGCTATCCAGGC-3′ (ß-actin forward), and 5′-CTCCTTAATGTCACGCACGAT-3′ (ß-actin reverse).

Western blot analysis

The relative protein expressions were determined by Western blot analysis according to the previous study.[36] Total protein from lung tissues and Raw264.7 cells was isolated by radioimmunoprecipitation (RIPA) lysis buffer (R0010; Solarbio), and quantified with bicinchoninic acid (BCA) protein assay kit (PC0020; Solarbio). Protein samples were dissolved in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane electrically for 4 h at 150 V. Following blocking with skimmed milk (D8340; Solarbio) for 1 h at room temperature, the membrane was treated with the following primary antibodies: anti-FoxO4 (1:3000, ab154520; Abcam); anti-FoxO1 (1:1000, 2880; Cell Signaling Technology, Danvers, MA, USA); anti-FoxO3 (1:1000, 2497; Cell Signaling Technology); anti-LXA4R (1:1000, 720293; Thermo Fisher Scientific, Waltham, MA, USA); anti-CD163 (1:1000, ab182422; Abcam); anti-Arg1 (1:1000, 93668; Cell Signaling Technology); and ß-actin (1:5000, ab8227; Abcam) overnight at 4°C. Then the membrane was rinsed thrice, hatched with goat anti-rabbit IgG H&L (human retinal pericytes [HRPs]) (1:50,000, ab205718; Abcam) for 1 h at 37°C, and visualized with 3,3'-diaminobenzidine (DAB) kit (DA1010; Solarbio).

Flow cytometry

Raw264.7 cells and cells from BALF were analyzed by flow cytometry by using F4/80 antibody (12-4801-82; Thermo Fisher Scientific) and CD206 antibody (MA5-16870; Thermo Fisher Scientific) on a FACScan flow cytometry with the CellQuest software (BD Biosciences, NJ, USA) as reported previously.[37] The percentage of macrophages (F4/80+) and M2 macrophages (CD206+) was calculated.

Statistical analysis

All statistical analyses were done by the SPSS 26.0 software (IBM, Armonk, New York, USA). Data were exhibited as mean ± standard deviation (SD), and analyzed by Student's *t*-test for two groups, or one-way analysis of variance (ANOVA) for more than two groups, followed by *post hoc* Bonferroni test. $P < 0.05$ was considered as statistically significant.

Results

FoxO4 was highly expressed in ovalbumin-induced mice

The airway inflammation was assessed by H&E staining after mice were treated with and without ovalbumin (Figure 1A). The results showed that inflammatory cells obviously infiltrated into perivascular and peribronchial connective tissue in mice with ovalbumin treatment, compared to those without ovalbumin treatment. Meanwhile, a prominent increase in the number of F4/80$^+$ cells was found in lung tissues from mice with ovalbumin treatment, compared to those without ovalbumin treatment (Figure 1B), indicating an enhancement in macrophage numbers. Compared to the sham group, the relative mRNA and protein expressions of FoxO4 in ovalbumin-induced mice were significantly elevated (Figures 1C and 1D). Similar results in the relative mRNA and protein expressions of FoxO4 were also observed in IL-4-induced Raw264.7 cells (Figures 1E and 1F). Thus, these results suggested that FoxO4 was highly expressed in ovalbumin-induced mice accompanied with an increase in the number of macrophages.

Inhibition of FoxO4 alleviated airway inflammation in ovalbumin-induced mice

Two doses of AS1842856 (12 and 25 mg/kg), commercial inhibitor of FoxO family were used to suppress the level of FoxO4 in ovalbumin-treated mice. As shown in Figure 2A, both doses of AS1842856 markedly counteracted the ovalbumin-induced relative protein expression of FoxO4 in mice lung tissues. However, no statistical difference was observed in the relative protein expressions of FoxO1 and FoxO3 in ovalbumin-treated mice, compared to that in the sham group. However, both doses of AS1842856 treatment observably reduced the ovalbumin-induced relative protein expressions of FoxO1 and FoxO3, indicating an effectively inhibitory effect of AS1842856 on the expression of FoxO family (Figure 2A). Enhancement in inflammatory cell infiltration (Figure 2B) and the number of PAS$^+$ goblet cells (Figure 2C) in lung tissues from mice with ovalbumin treatment, compared to those without ovalbumin treatment, was distinctly neutralized by the inhibition of FoxO4. Besides, the number of inflammatory cells, including white blood cells, monocytes, neutrophils, eosinophils, lymphocytes, and basophils, was significantly increased in the blood of the mice with ovalbumin treatment, compared to those without ovalbumin treatment, which was markedly decreased by the inhibition of FoxO4 (Figure 2D). Moreover, inhibition of FoxO4 significantly reduced the ovalbumin-induced airway resistance (Figure 2E). Altogether, these data indicated that inhibition of FoxO4 alleviated airway inflammation in ovalbumin-induced mice.

Inhibition of FoxO4 improved macrophage M2 polarization both in vivo and in vitro

The results shown in Figure 3A revealed that the increased number of F4/80$^+$ cells in lung tissues from ovalbumin-induced mice was markedly decreased with the inhibition of FoxO4. Prominently, inhibition of FoxO4 significantly reduced the ovalbumin-induced proportion of CD206$^+$ cells (Figure 3B) as well as the relative protein expressions of CD163 and Arg1 (Figure 3C). In addition, inhibition of FoxO4 (Figure 3D) markedly declined IL-4-induced proportion of F4/80$^+$CD206$^+$ Raw264.7 cells (Figure 3E). In addition, the relative protein expressions of CD163 and Arg1 were significantly enhanced in IL-4-induced Raw264.7 cells, which were markedly reduced by the inhibition of FoxO4 (Figure 3F). Therefore, these results expounded that inhibition of FoxO4 improved macrophage M2 polarization both *in vivo* and *in vitro*.

FoxO4 regulated the transcription of LXA4R

Mechanically, the relative mRNA and protein expressions of LXA4R were significantly elevated in both ovalbumin-induced mice and IL-4-induced Raw264.7 cells (Figures 4A-4D). Yet, both the doses of AS1842856 markedly reduced the ovalbumin-induced relative mRNA and protein expressions of LXA4R *in vitro* (Figures 4A and 4B). Inhibition of FoxO4 also significantly decreased the relative mRNA and protein expressions of LXA4R in IL-4-induced Raw264.7 cells (Figures 4C and 4D). Thus, FoxO4 modulated the transcription of LXA4R.

Overexpression of LXA4R reversed the role of FoxO4 inhibition

To further verify the direct role of LXA4R in the regulation of FoxO4 inhibition, the level of LXA4R was upregulated both *in vitro* and *in vivo*. Overexpression of LXA4R markedly rescued the AS1842856-reduced relative protein level of LXA4R in ovalbumin-induced mice, while no impact was discovered on the relative protein level of FoxO4 with the overexpression of LXA4R in ovalbumin-induced mice treated with AS1842856 (Figure 5A). The decreased

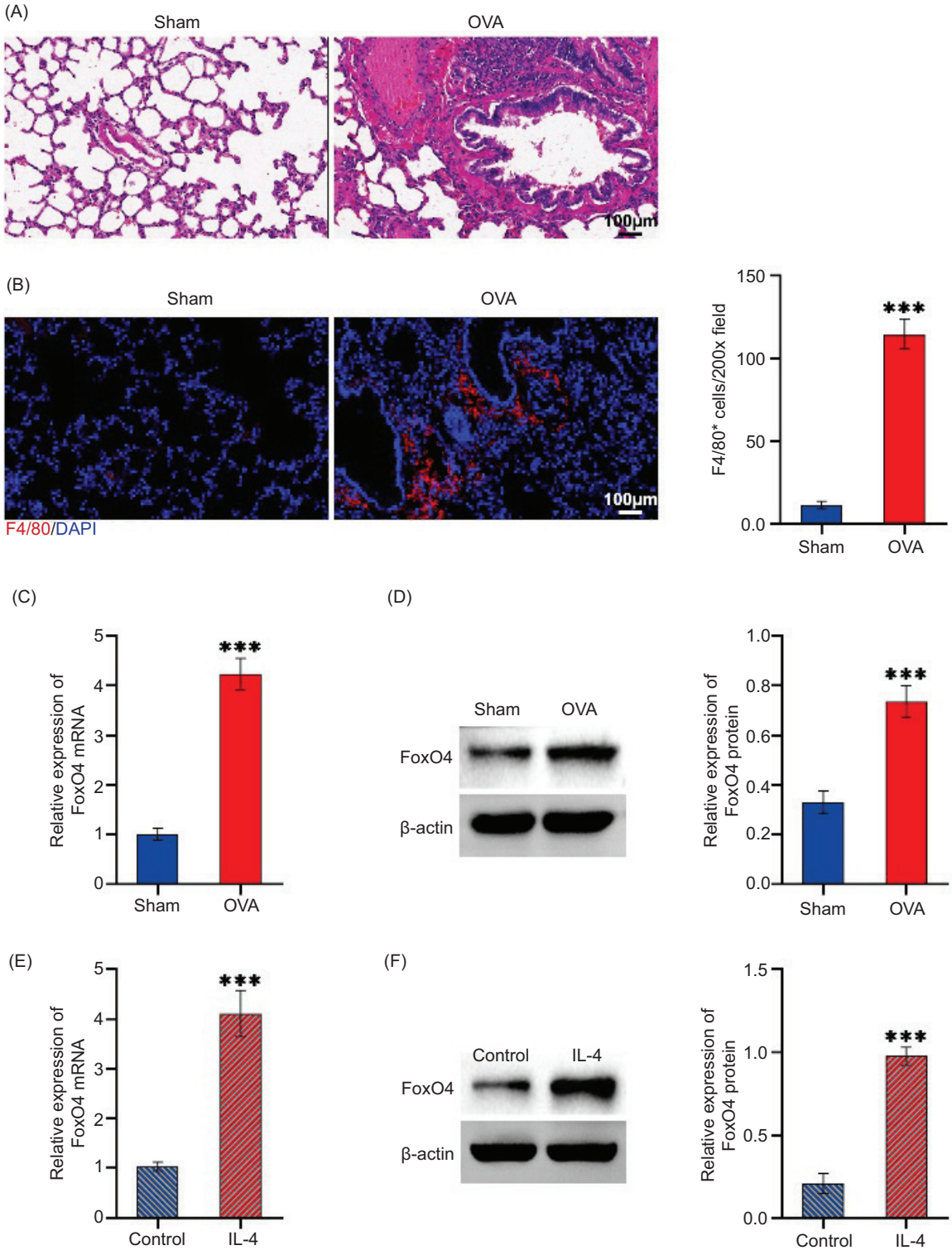

Figure 1 Increase in the expression of FoxO4 and the number of macrophages in ovalbumin-induced mice. (A) The pathological examination of lung tissues was determined by H&E staining. Scale bar: 100 μm. (B) Lung tissues were marked with F4/80 antibody by immunofluorescence, and the number of F4/80^{+} cells in lung tissues was counted. ***P < 0.001 vs sham group. Scale bar: 100 μm. (C) The relative mRNA expression of FoxO4 in lung tissues was measured by RT-qPCR. The results were normalized with β-actin. ***P < 0.001 vs Sham group. (D) The relative protein expression of FoxO4 in lung tissues was quantified by Western blot analysis. The results were normalized with β-actin. ***P < 0.001 vs Sham group. (E) The relative mRNA expression of FoxO4 in Raw264.7 cells was measured by RT-qPCR. The results were normalized with β-actin. ***P < 0.001 vs control. (F) The relative protein expression of FoxO4 in Raw264.7 cells was quantified by Western blot analysis. The results were normalized with β-actin. ***P < 0.001 vs control. All data were presented as mean ± SD.

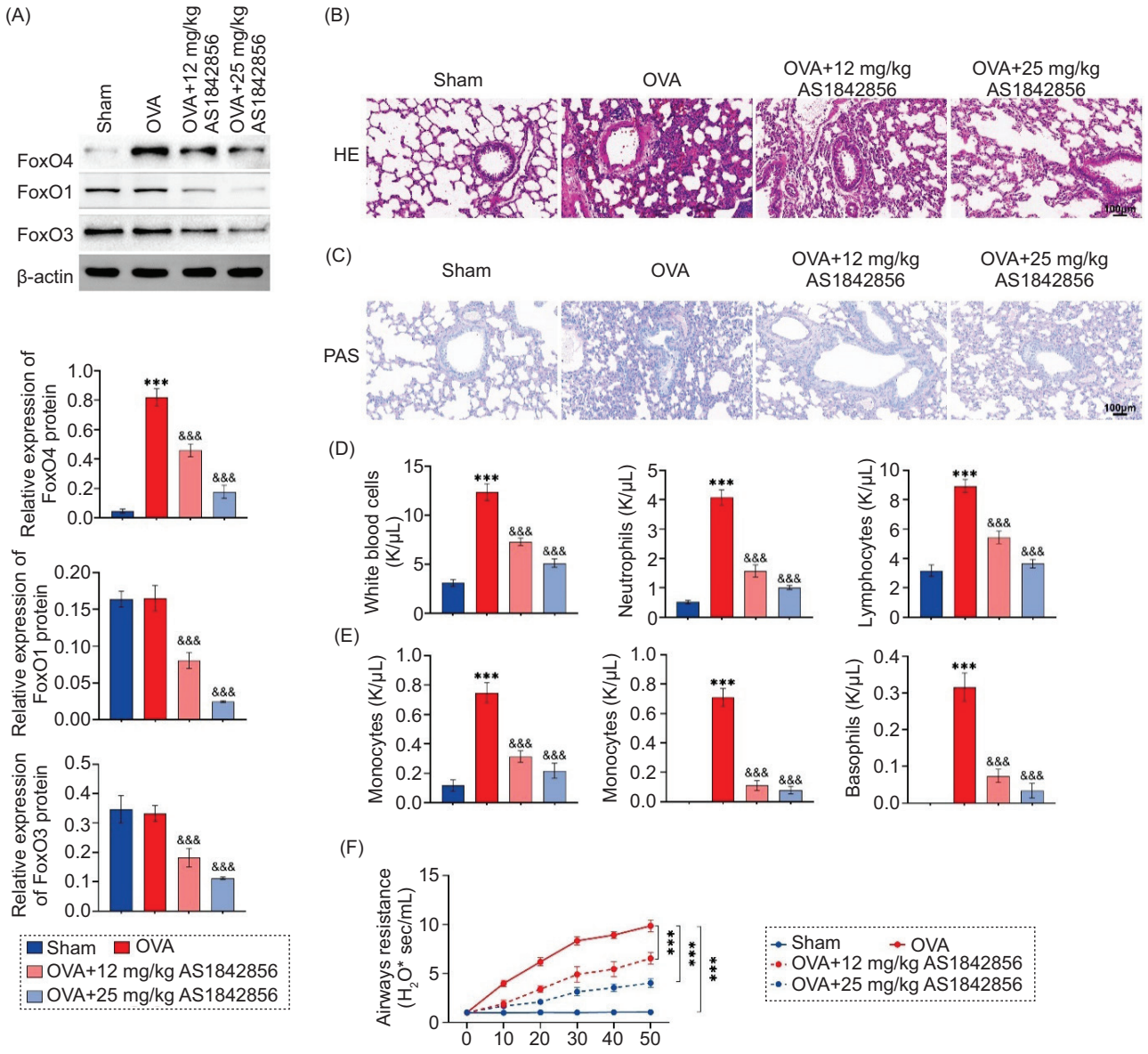

Figure 2 Inhibition of FoxO4 improved airway inflammation in ovalbumin-induced mice. (A) The relative protein expressions of FoxO4, FoxO1, and FoxO3 were examined by Western blot analysis. The results were normalized with ß-actin. ***P < 0.001 vs. Sham; &&&P < 0.001 vs OVA. (B) The pathological examination of lung tissues was determined by H&E staining. Scale bar: 100 μm. (C) The mucus gland hyperplasia was assessed by PAS staining. Scale bar: 100 μm. (D) Inflammatory cells in the blood, including white blood cells, monocytes, neutrophils, eosinophils, lymphocytes, and basophils, were detected by a HEMAVET 950 FS. ***P < 0.001 vs sham. &&&P < 0.001 vs OVA. (E) Airway responsiveness of mice stimulated to enhance concentrations of methacholine (0–50 mg/mL) under anesthesia. ***P < 0.001. All data were presented as mean ± SD.

airway resistance with the administration of AS1842856 in ovalbumin-induced mice was almost completely restored by the upregulation of LXA4R (Figure 5B).

Similar outcomes were also observed in the number of F4/80$^+$ cells (Figure 5C) and the proportion of CD206$^+$ cells (Figure 5D) in lung tissues. In IL-4-induced Raw264.7 cells transfected with shFoxO4, overexpression of LXA4R consistently recovered the relative protein level of LXA4R, but no statistical difference was observed in the relative protein level of FoxO4 (Figure 5E). The proportion of F4/80$^+$CD206$^+$ Raw264.7 cells was markedly decreased following the transfection of shFoxO4 in IL-4-induced Raw264.7 cells, which was significantly saved by the overexpression of LXA4R (Figure 5F). Taken together, overexpression of LXA4R inverted the role of FoxO4 inhibition.

Discussion

In this study, an allergic asthma model was constructed by the induction of ovalbumin and IL-4 in mice and Raw264.7 cells, respectively. Ovalbumin treatment triggered an

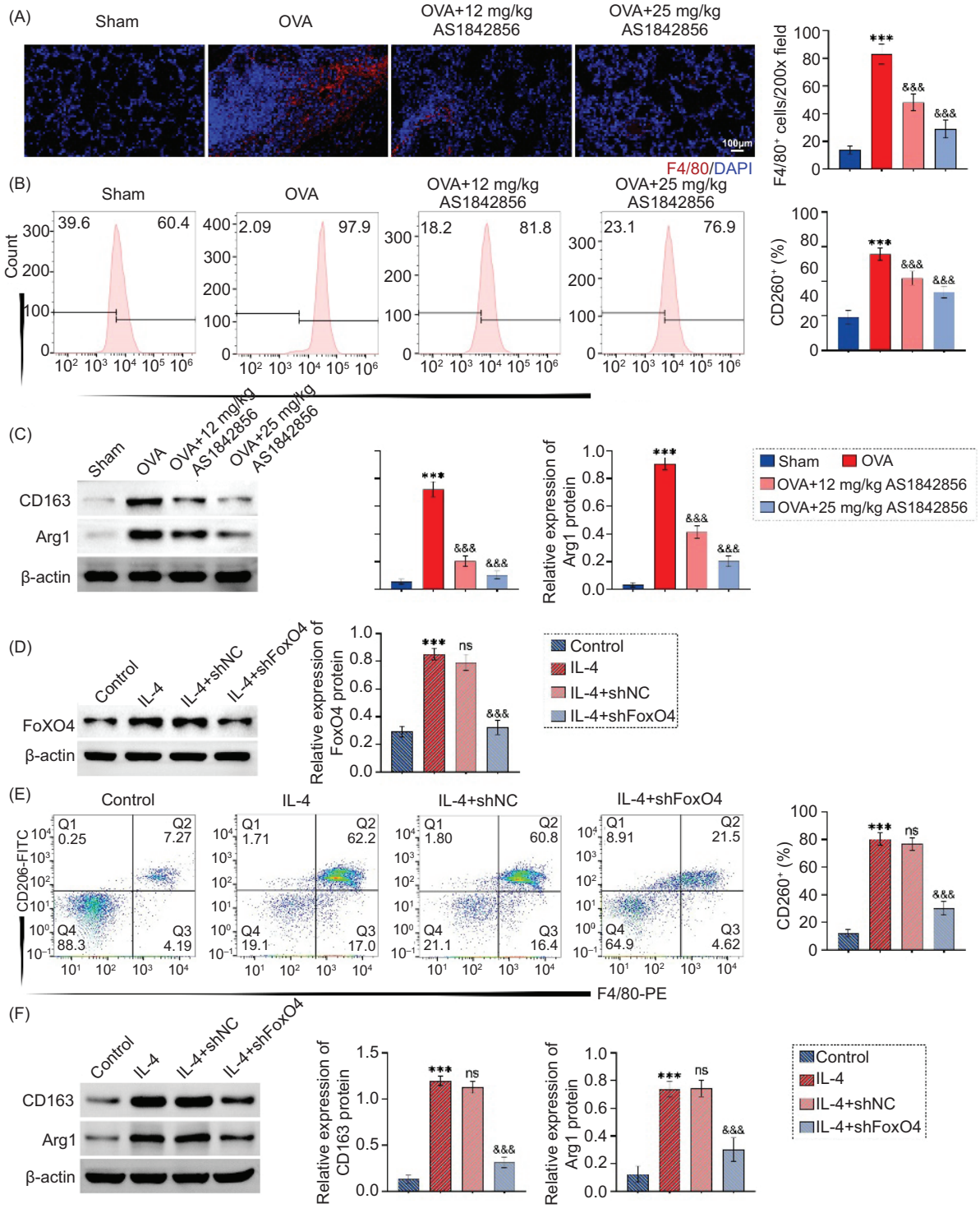

Figure 3 Inhibition of FoxO4 improved macrophage M2 polarization both *in vivo* and *in vitro*. (A) The lung tissues were marked with F4/80 antibody by immunofluorescence, and the number of F4/80⁺ cells were counted in lung tissues. ***P < 0.001 vs sham; &&&P < 0.001 vs OVA. Scale bar: 100 μm. (B) The proportion of CD206⁺ cells in lung tissues was measured by flow cytometry. ***P < 0.001 vs sham; &&&P < 0.001 vs OVA. (C) The relative protein expressions of CD163 and Arg1 in lung tissues were examined by Western blot analysis. The results were normalized with ß-actin. ***P < 0.001 vs sham; &&&P < 0.001 vs OVA. (D) The relative protein expression of FoxO4 was determined by Western blot analysis after Raw264.7 cells were transfected with shFoxO4. The results were normalized with ß-actin. ***P < 0.001 vs control; &&&P < 0.001 vs IL-4; ns indicated nonsignificance, compared to IL-4. (E) The percentage of F4/80⁺CD206⁺ Raw264.7 cells was quantified by low cytometry. ***P < 0.001 vs control; &&&P < 0.001 vs IL-4; ns indicated nonsignificance, compared to IL-4. (F) The relative protein expressions of CD163 and Arg1 in Raw264.7 cells were examined by Western blot analysis. The results were normalized with ß-actin. ***P < 0.001 vs control; &&&P < 0.001 vs IL-4; ns indicated nonsignificance, compared to IL-4. All data were presented as mean ± SD.

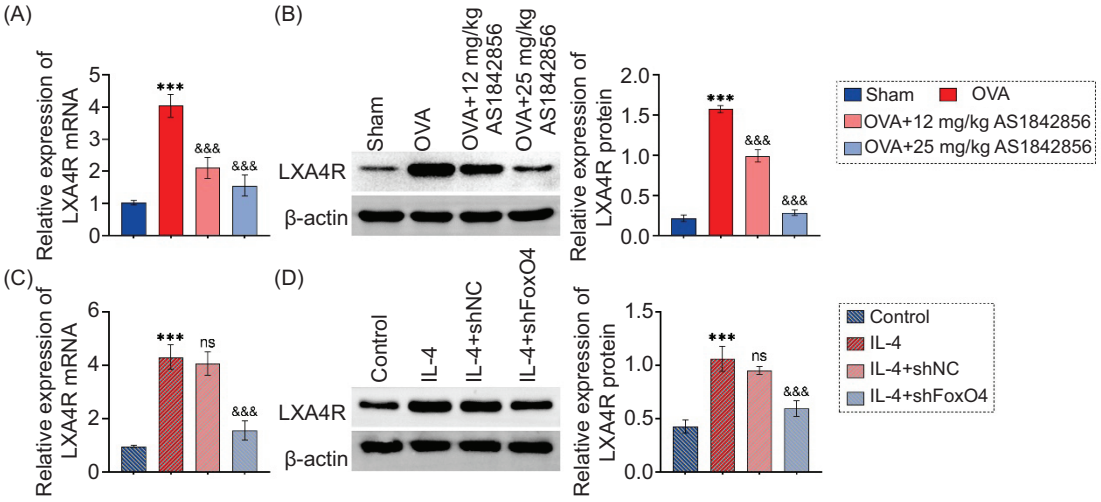

Figure 4 FoxO4 mediated the transcription of LXA4R. (A) The relative mRNA expression of LXA4R in lung tissues was measured by RT-qPCR. The results were normalized with ß-actin. ***P < 0.001 vs sham; &&&P < 0.001 vs OVA. (B) The relative protein expression of LXA4R in lung tissues was examined by Western blot analysis. The results were normalized with ß-actin. ***P < 0.001 vs sham; &&&P < 0.001 vs OVA. (C) The relative mRNA expression of LXA4R in Raw264.7 cells was measured by RT-qPCR. The results were normalized with *ß-actin*. ***P < 0.001 vs. control; &&&P < 0.001 vs IL-4; ns indicated nonsignificance, compared to IL-4. (D) The relative protein expression of LXA4R in Raw264.7 cells was examined by Western blot analysis. The results were normalized with ß-actin. ***P < 0.001 vs control; &&&P < 0.001 vs IL-4; ns indicated nonsignificance, compared to IL-4. All data were presented as mean ± SD.

obvious inflammatory cell infiltration accompanied with a prominent increase in the number of F4/80+ cells. The relative mRNA and protein expressions of FoxO4 were increased in both ovalbumin-induced mice and IL-4-induced Raw264.7 cells. Inhibition of FoxO4 via AS1842856 improved airway inflammation in ovalbumin-induced mice. In addition, inhibition of FoxO4 decreased the macrophage M2 polarization both *in vivo* and *in vitro*. Mechanically, inhibition of FoxO4 diminished the relative mRNA and protein expressions of LXA4R in both ovalbumin-induced mice and IL-4-induced Raw264.7 cells. Overexpression of LXA4R reversed the outcomes caused by inhibition of FoxO4. Collectively, FoxO4 mediated macrophage M2 polarization by promoting LXA4R expression in an allergic asthma model.

FoxO4 has been reported in a variety of disease models because it is widely expressed in various tissues[16] as well as because of its multiple roles in cell progression.[17] For instance, abundant expression of FoxO4 is involved in developing hemochorial placenta.[38] Increased expression of FoxO4 resists oxidative damage in H_2O_2-treated HLEpiC cells[39] and high glucose (HG)-induced HRPs.[40] FOXO4 is upregulated and may act as a biomarker of postmenopausal osteoporosis.[41] Inhibition of FoxO4 dampens hippocampal neuronal apoptosis, which participates in the protective role of sevoflurane against intracerebral hemorrhage.[42] The decreased mRNA expression is shown in a vasculitis model via the tumor necrosis factor-α (TNF-α) stimulation of primary human coronary artery endothelial cells (HCAECs), in which FoxO4 stabilizes endothelial cell homeostasis.[43] In the present study, the relative mRNA and protein expressions of FoxO4 were increased in both ovalbumin-induced mice and IL-4-induced Raw264.7 cells. Thus, FoxO4 could also serve as a biomarker of asthma for its diagnosis and treatment.

Airway obstruction is the main characteristic of asthma, which results because of a decrease in the diameter of the airways. Importantly, chronic inflammation of airway wall mediates the narrowing of the airways, which is generally featured by the infiltration and activation of inflammatory cells, such as neutrophils, lymphocytes, dendritic cells, innate lymphoid cells, eosinophils, and mast cells.[9] The results of the present study established that inhibition of FoxO4 via AS1842856 reduced inflammatory cell infiltration, the number of PAS+ goblet cells, the number of inflammatory cells in the blood, and airway resistance in ovalbumin-induced mice. Several studies have revealed that FoxO proteins are involved in the progression of asthma. Choi et al. showed that FoxO-1 inhibition takes part in the functioning of tranilast, an anti-allergic drug in the treatment of bronchial asthma.[44] Barkund et al. discovered through the single nucleotide polymorphism studies that the gene polymorphism of FoxO3a was related to asthma in the Indian population.[45] In addition, FoxO4 attenuates CD4+ Th cell responsiveness in fasting, in which intermittent fasting passivates asthma inflammation.[46] Therefore, interference of FoxO4 relieved airway inflammation in ovalbumin-induced mice model.

Macrophages are innate immune cells that mediate initial response to immune stimulation and are involved in the development of innate and adaptive immune responses.[47] Moreover, macrophages are abundantly distributed in the lung, which capably mounts different responses required for defense and homeostasis against pathogens.[47] In bronchial asthma, macrophages play different roles in phagocytosis, exocytosis, production of inflammatory mediators, and polarization. Depending on microenvironmental stimulation, macrophages may polarize classical pro-inflammatory M1 macrophages or alternative M2 macrophages.[48] Among

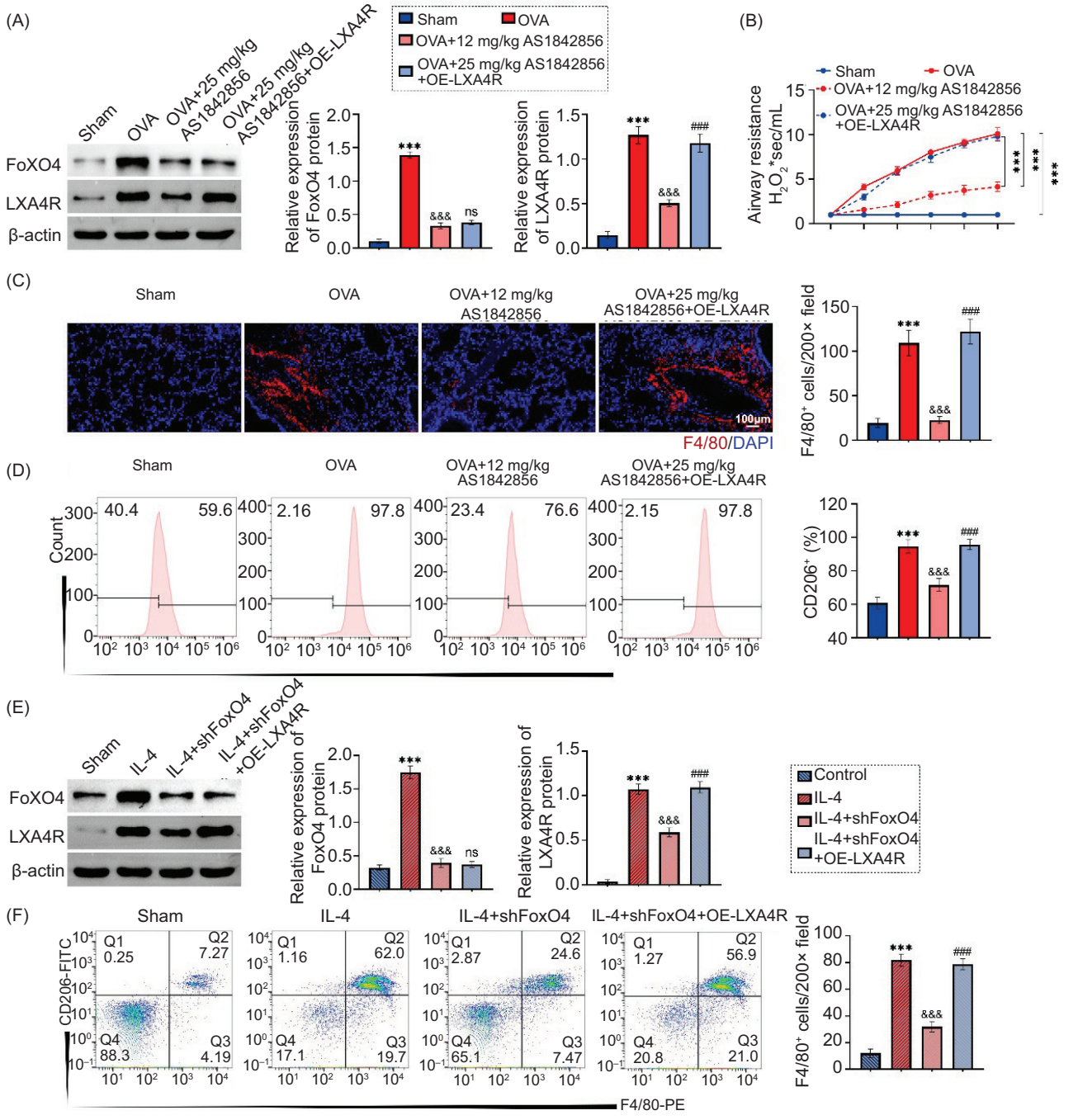

Figure 5 Overexpression of LXA4R reversed the role of FoxO4 inhibition. (A) The relative protein levels of LXA4R and FoxO4 in lung tissues were examined by Western blot analysis. The results were normalized with β-actin. ***P < 0.001 vs sham; &&&P < 0.001 vs OVA; ###P < 0.001 vs OVA+25 mg/kg AS1842856; ns indicated nonsignificance, compared to OVA+25 mg/kg AS1842856. (B) Airway responsiveness of mice was stimulated to enhance the concentration of methacholine (0–50 mg/mL) under anesthesia. ***P < 0.001. (C) The lung tissues were marked with F4/80 antibody by immunofluorescence, and the number of $F4/80^+$ cells was counted in lung tissues. ***P < 0.001 vs sham; &&&P < 0.001 vs OVA; ###P < 0.001 vs OVA+25 mg/kg AS1842856. Scale bar: 100 μm. (D) The percentage of $CD206^+$ cells in lung tissues was measured by flow cytometry. ***P < 0.001 vs sham; &&&P < 0.001 vs OVA; ###P < 0.001 vs OVA+25 mg/kg AS1842856. (E) The relative protein levels of LXA4R and FoxO4 in Raw264.7 cells were examined by Western blot analysis. The results were normalized with β-actin. ***P < 0.001 vs control; &&&P < 0.001 vs IL-4; ###P < 0.001 vs IL-4+shFoxO4; ns indicated nonsignificance, compared to IL-4+shFoxO4. (F) The percentage of $F4/80^+CD206^+$ Raw264.7 cells was quantified by flow cytometry. ***P < 0.001 vs control; &&&P < 0.001 vs IL-4; ###P < 0.001 vs IL-4+shFoxO4. All data were presented as mean ± SD.

these, M2 macrophages have antiparasitic and tissue remodeling functional activities to produce Arg-1, CD206, FIZZ1, Ym1, CCL17, and CCL24 factors.[49] M2 macrophages promote Th2 immune response and secretion of chemokines and cytokines that regulate airway inflammation, tissue repair, and airway remodeling in the lung.[34,50] Holtzman et al. also reported that differentiation and accumulation of M2 macrophages could be a characteristic feature of allergic airway disease.[51] Interference of FoxO4 decreased the number of $F4/80^{+}CD206^{+}$ cells as well as the relative protein expressions of CD163 and Arg1 both *in vivo* and *in vitro*. Hence, inhibition of FoxO4 suppressed macrophage M2 polarization in asthma.

Mechanically, suppression of FoxO4 diminished the relative mRNA and protein expressions of LXA4R in both ovalbumin-induced mice and IL-4-induced Raw264.7 cells. LXA4 is predicted as the downstream regulatory gene of FoxO4 through the transcription factor TF2DNA database. LXA4 suppresses airway remodeling and inflammation in ovalbumin-induced mouse model of asthma.[52] The exhalation of LXA4, combined with LTB4, FeNO, and FEV1, has been reported as an "asthma classification ratio" characterizing childhood asthma.[53] Also, the level of LXA4 is decreased in severe asthma.[23] Thus, LXA4 is proposed as a new direction in asthma therapy.[54] More importantly, LXA4 has been demonstrated to promote polarization of M2 macrophages.[55,56] LXA4R, the receptor of LXA4, is downregulated in severe asthma,[23] whose activation inhibits airway inflammation in ovalbumin-induced mice through TLR2/MYD88/NF-κB axis.[31] Kong et al.[57] report that the inhalation of LXA4 analog 5(S), 6(R)-LXA4 methyl ester, and LXA4R agonist BML-111 is effective and safe in asthmatic children with acute moderate asthma. In the present study, overexpression of LXA4R reversed the outcomes caused by inhibition of FoxO4, including airway resistance, numbers of F4/80+ cells, and proportion of CD206+ cells in ovalbumin-induced mice, and the proportion of $F4/80^{+}CD206^{+}$ cells in IL-4-induced Raw264.7 cells. Thus, FoxO4 mediated macrophage M2 polarization by promoting LXA4R expression in asthma.

Conclusion

In summary, FoxO4 was upregulated in asthma both in vivo and in vitro, and inhibition of FoxO4 alleviated airway inflammation in ovalbumin-induced mice, and suppressed polarization of macrophage M2 in both ovalbumin-induced mice and in IL-4-induced Raw264.7 cells, which is strongly associated with the expression of LXA4R. However, several limitations remain to be addressed in the future. For instance, more shRNAs targeting FoxO4 should be applied in transfection assays to avoid the off-target effect. Additionally, more preclinical trials must be executed in the future study for the clinical application of FoxO4. Briefly, the results provided an alternative target for the diagnosis and treatment of asthma.

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Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Competing Interests

The authors state that there were no conflicts of interest to disclose.

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Author Contributions

Tong Yu designed the study, completed the experiment, and supervised data collection. Yiping Yu analyzed and interpreted the data. Yingyu Ma and Guoqing Chen prepared the manuscript for publication and reviewed draft of the manuscript. All authors read and approved the final manuscript.

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