Isoalantolactone alleviates ovalbumin-induced asthmatic inflammation by reducing alternatively activated macrophage and STAT6/PPAR-γ/KLF4 signals

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Abstract. Isoalantolactone (IAL), a sesquiterpene lactone, has anti-inflammatory activity in lipopolysaccharide (LPS)-induced sepsis. However, it remains to be elucidated whether IAL influences asthmatic inflammation. The present study found that IAL inhibited ovalbumin (OVA)-induced asthmatic inflammation and attenuated OVA-induced eosinophil infiltration, immunoglobulin E generation and the production of interleukin (IL)-4, IL-5, C-C motif chemokine ligand (CCL)17 and CCL22. In addition, IAL treatment with IL-4 reduced the expression of arginase-1, Ym-1, CCL17 and CCL22 in bone marrow-derived macrophages *in vitro*. Furthermore, IAL inhibited IL-4-induced STAT6 phosphorylation and the expression of peroxisome proliferator-activated receptor γ and Krüppel-like factor 4. Collectively, the results suggested that IAL attenuated asthmatic inflammation and is a potential therapeutic agent for the treatment of asthma.

Introduction

Asthma is a common chronic inflammatory disorder with heterogeneous pathophysiology, characterized by airway inflammation, variable airflow obstruction and bronchial hyperresponsiveness (1). Asthma is thought to affect ~300 million individuals worldwide and the number of patients with asthma is predicted to reach 400 million by 2025 (2,3). Although various compounds have been reported to be effective in the treatment of asthma, inhaled corticosteroids (ICS) remain an irreplaceable pharmacologic treatment for asthma in clinical settings (2). However, some patients with refractory asthma are resistant to corticosteroid treatment and long-term use of ICS causes obvious side effects, such as obesity, diabetes, hypertension and depression (4,5). Therefore, it is important to identify new compounds and develop novel medicines for treating asthma.

The asthmatic pathology is a T helper type 2 (Th2)-mediated immune response that is associated with the production of interleukin (IL)-4, IL-5, IL-13 and immunoglobulin (Ig)E, as well as pulmonary eosinophil infiltration, mast cell activation and Th2 lymphocyte activation (6). In response to allergen stimulation, injured airway epithelial cells generate various bioactive molecules, such as C-C motif chemokine ligand (CCL)5, CCL2, CCL22 and IL-8, all of which promote inflammatory cell infiltration and macrophage polarization (7). In response to type 2 cytokines, recruited macrophages are polarized to alternatively activated (M2) subtypes, which contribute to the overactivation of Th2 cells, inflammatory deterioration and progressive lung injury (8,9). Therefore, manipulating macrophage polarization is a promising treatment strategy for asthma.

Traditional medicine is an important resource for developing new medicines because plant-based natural compounds are successfully used to treat numerous diseases (10). Sesquiterpene lactones are a group of natural compounds with various biological activities, including anti-tumor, anti-inflammatory and mycobacterial activities (11-13). Isoalantolactone (IAL) is a sesquiterpene lactone extracted from the roots of *Inula helenium* L. and has been known to possess several bioactive effects (14), including antibacterial (15) and anti-lipogenic effects (16) and has proven efficacy in treating lung injuries (17) and pulmonary fibrosis (17). Recently, the anti-inflammatory activity of IAL has been identified in lipopolysaccharide (LPS)-induced acute lung injury and sepsis disorders by inhibition of nuclear factor E2-related factor 2 (Nrf2), NF-κB and tumor necrosis factor receptor associated factor 6 (TRAF6) ubiquitination (18-20). However, it is still unknown whether IAL has a pharmacological effect on asthmatic inflammation.

The present study determined the effect of IAL on ovalbumin (OVA)-induced asthmatic inflammation. It was found that treatment with IAL attenuated OVA-induced eosinophil infiltration, IgE generation and the production of IL-4, IL-5,

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CCL17 and CCL22. In addition, IAL alleviated OVA-induced asthmatic inflammation by inhibiting alternatively activated macrophages, with a reduction in STAT6 phosphorylation and the expression of peroxisome proliferator-activated receptor (PPAR)-γ and Krüppel-like factor 4 (KLF4). The results indicated that IAL is a promising agent for the treatment of asthma.

Materials and methods

Mice. A total of 60 Specific pathogen-free (SPF) female BALB/c mice, 6-8 weeks of age, with an average weight of 20-25 g, were purchased from Slac Laboratory Animal Corporation and housed in SPF conditions (~19-27˚C, 12-h light/dark cycle and ~40-70% humidity). All experimental protocols described in the present study were approved by the Institutional Animal Care and Use Committee of Shanghai Pudong Hospital and the procedures were approved by the Biological Research Ethics Committee of Shanghai Pudong Hospital.

Compound and reagents. IAL (cat. no. C15H20O2; MW, 232.31; purity >98%) was purchased from TargetMol; OVA (Grade V) was purchased from Sigma-Aldrich (Merck KGaA); Dulbecco's modified Eagle's medium (DMEM), antibiotics (10,000 units/ml penicillin and 10,000 μg/ml streptomycin), 0.25% trypsin and phosphate buffered saline (PBS) were purchased from Thermo Fisher Scientific, Inc.; Fetal bovine serum (FBS) was purchased from EVERY GREEN (Zhejiang Tianhang Biotechnology Co., Ltd.); and bicinchoninic acid (BCA) protein assay kit was purchased from Beyotime Institute of Biotechnology. The primary antibodies used in the present study were synthesized by Shanghai HuaGen Biotech Co., Ltd.. Phosphorylated (p)-STAT6 (Tyr641; cat. no. 56554), total (t)-STAT6 (cat. no. 5397), PPAR-γ (cat. no. 2443), KLF4 (cat. no. 12173) and β-actin (cat. no. 3700) were purchased from Cell Signaling Technology, Inc. The peroxidase AffiniPure goat anti-rabbit IgG (H + L; cat. no. 111-035-003) and peroxidase AffiniPure goat anti-mouse IgG (H + L) (cat. no. 115-035-003) secondary antibodies were purchased from Jackson ImmunoResearch Laboratories and diluted in EZ-Buffers (cat. no. C520011-0100; Sangon Biotech Co., Ltd.). Other chemical reagents were obtained from Sigma-Aldrich. Loading buffer was prepared by 1M TrisCl (cat. no. A610195; Sangon Biotech Co., Ltd.), 10% SDS (cat. no. B548118; Sangon Biotech Co., Ltd.), 7.5 mM bromophenol blue (cat. no. 60503ES08; Shanghai Yeasen Biotechnology Co., Ltd.) 25% glycerin (cat. no. A501745; Sangon Biotech Co., Ltd.) and 35.75 μM β-mercaptoethanol (cat. no. 200-464-6; Sigma-Aldrich; Merck KGaA).

OVA-induced asthma mouse model. Mice were randomly divided into three groups as follows: PBS + vehicle group, OVA + vehicle group and OVA + IAL group. IAL was dissolved in a vehicle comprising castor oil:ethanol: Physiological saline at a 1:1:8 ratio. For the OVA + vehicle group, mice were sensitized on days 0, 7 and 14 [40 μg OVA and 40 mg $Al(OH)_3$ in 0.2 ml PBS, intraperitoneal (i.p.)] and challenged at days 25, 26 and 27 [10 μg OVA in 0.2 ml PBS, intranasal (i.n.)]. For the OVA + IAL group, following sensitization, mice were administered IAL (20 mg/kg, i.p.) once daily from days 22 to 27, together with challenging at days 25, 26 and 27 (10 μg OVA in 0.2 ml PBA, i.n.). For the PBS + vehicle group, mice were injected with PBS (0.2 ml, i.p.) on days 0, 7 and 14 and the same volume of vehicle as the mice in the OVA + IAL group. Mice were anesthetized with sodium pentobarbital (50 mg/kg; i.p.) to collect samples on day 28 and blood, bronchoalveolar lavage fluid (BALF) and lung tissues were collected for further analysis (20).

Analysis of protein concentration in BALF. The BALF was centrifuged at 500 x g, 4˚C following collection. The supernatant was collected for analysis of protein concentration using the BCA protein assay kit according the manufacturer's protocols.

Lung histology. After fixing with 4% paraformaldehyde (Sangon Biotech Co., Ltd.) overnight, the left lung was embedded in paraffin and dehydrated using graded ethanol. Then the tissues were cut into 5 μm thick sections for haematoxylin and eosin (H&E) staining (Nanjing Jiancheng Bioengineering Institute). Images were captured using a microscope (RX51; Olympus Corporation).

Preparation of bone marrow-derived macrophages. Mice were sacrificed by cervical dislocation following anesthesia as aforementioned. Mouse bone marrow-derived macrophages (BMDMs) were isolated from BALB/c mice and incubated with DMEM supplemented with 10% FBS, 1% penicillin/streptomycin at 37˚C and 10 ng/ml macrophage colony-stimulating factor (M-CSF; PeproTech, Inc.) for 6 days. BMDMs were incubated at 37˚C, 5% CO_2 and stimulated with mouse 10 ng/ml IL-4 (mIL-4; PeproTech, Inc.) and 0, 3, 10, or 30 μM IAL. Cells and culture supernatants were collected for RNA and protein expression analyses.

Western blotting. Western blotting was conducted as previously described (9). Briefly, BMDMs were plated in 6-well plates ($1x10^6$/well) and incubated overnight at 37˚C, 5% CO_2. BMDMs were pre-treated with IAL (0, 3, 10, or 30 μM) for 30 min and challenged with mIL-4 (10 ng/ml) for 30 min or 24 h, with dimethyl sulfoxide (DMSO) as a solvent control. Cells were washed twice with PBS, harvested using RIPA lysis buffer (Beyotime Institute of Biotechnology) and protein concentration was determined using the BCA method. Subsequently, the samples were loaded on 10% SDS/PAGE gels with 20 μg protein loaded per lane, transferred to nitrocellulose filter membranes and blocked with 5% milk at room temperature for 2 h. The membrane was incubated with primary antibodies (1:1,000) overnight at 4˚C. Blots were then incubated with secondary antibodies (1:10,000) for 1 h at room temperature. The antibody-specific proteins were visualized using ECL western blotting substrate (Thermo Fisher Scientific, Inc.) β-actin was used as loading control. Quantification of western blots was performed using ImageJ software 1.4.3.67 (National Institute of Mental Health).

Isolation of total RNA and reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from BMDMs and lung tissues using TRIzol reagent (Thermo Fisher Scientific, Inc.). cDNA was prepared using the ReverTra Ace RT kit (Toyobo Life Science) according to the manufacture's protocol. Gene expression values were quantified by PCR using StepOne Plus (Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for PCR: Initial denaturation at 94˚C for 30 sec; 35 cycles of 94˚C for 30 sec, 55˚C for 30 sec and

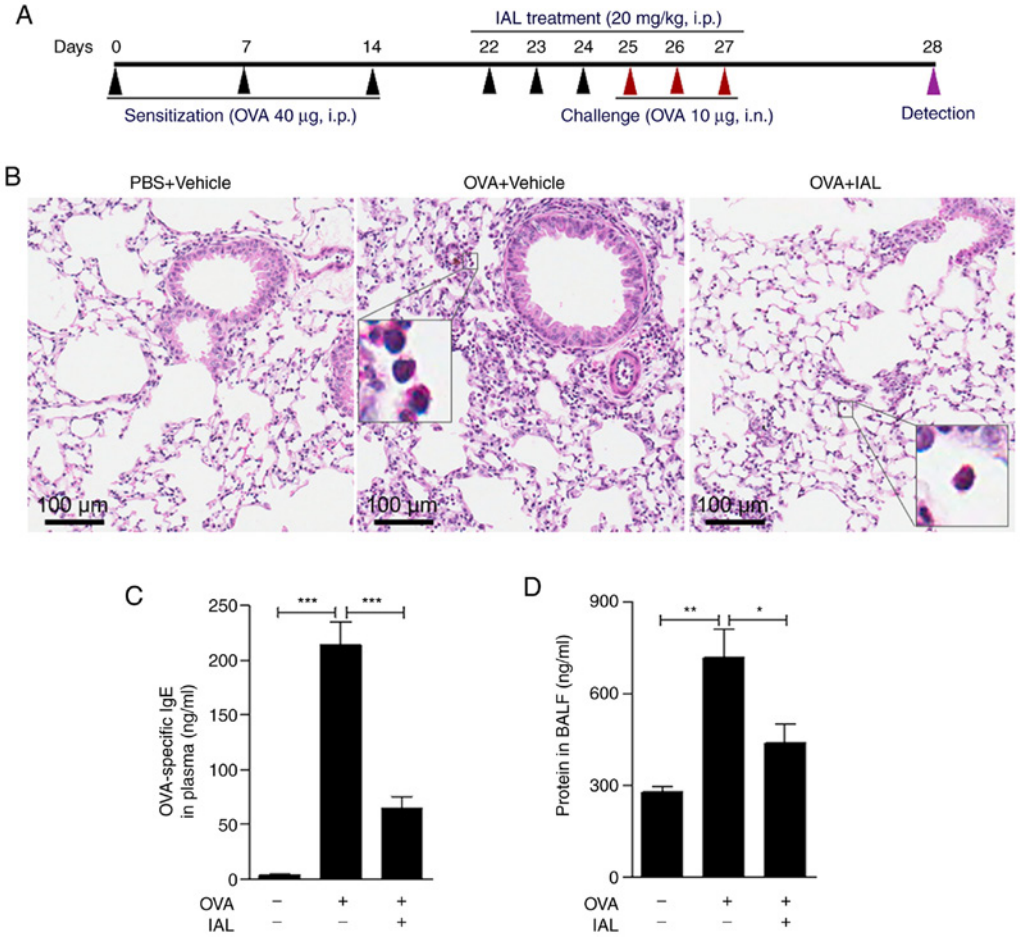

Figure 1. IAL ameliorates pathologic injury in OVA-induced asthma. (A) Experimental scheme of the OVA-induced asthma mouse model and IAL treatment. Eight-week-old BALB/c mice were i.p. injected with 40 μg OVA or PBS at days 0, 7 and 14 for sensitization and then i.n. challenged with 10 μg OVA or PBS at days 25, 26 and 27. Mice received IAL (20 mg/kg, i.p.) or vehicle at days 22 to 27 and were killed at day 28 for further analysis. (B) The lung lobes were excised and subject to hematoxylin and eosin staining. Scale bars, 100 μm; magnification of inserts, x400. (C) The content of IgE protein in plasma and the (D) concentration of total protein in BALF were measured. Values represent means ± standard error of the mean, n=5 mice in each group, *P<0.05, **P<0.01, ***P<0.001. IAL, isoalantolactone; OVA, ovalbumin; i.p., intraperitoneal; i.n., intranasal; BALF, bronchoalveolar lavage fluid.

72˚C for 2 min; and a final extension at 72˚C for 3 min and stored at 4˚C. The primers were synthesized by Huagene Biosciences. Relative mRNA levels were normalized to GAPDH mRNA levels. The quantification of RT-qPCR was based on the ΔΔCq (21). The sequences of primers used for each gene were as follows: Arginase-1 (Arg-1) (forward, 5'-CAATGAAGAGCTGGCTGGTGT-3', reverse, 5'-GTG TGAGCATCCACCCAAATG-3'), Ym-1 (forward, 5'-TAC TCACTTCCACAGGAGCAGG-3', reverse, 5'-CTCCAGTGT AGCCATCCTTAGG-3'), CCL17 (forward, 5'-CGAGAGTGC TGCCTGGATTACT-3', reverse, 5'-GGTCTGCACAGATGA GCTTGCC-3'), CCL22 (forward, 5'-GTGGAAGACAGTATC TGCTGCC-3' reverse, 5'-AGGCTTGCGGCAGGATTTTGA G-3') and GAPDH (forward, 5'-CATCACTGCCACCCAGAA GACTG-3' reverse, 5'-ATGCCAGTGAGCTTCCCGTTC AG-3').

Flow cytometry assay. After blocking Fc receptors with Fc blocking anti-mouse CD16/32 antibody (BD Biosciences), cells were collected from BALF and incubated with phycoerythrin-conjugated anti-Siglec-F antibodies (BD Biosciences) and allophycocyanin-conjugated anti-CD11c antibodies (BioLegend) at 4˚C for 30 min. Samples were analyzed using a flow cytometer (LSRFortessa; BD Biosciences) and FlowJo v10 software (FlowJo LLC).

Enzyme-linked immunosorbent assay (ELISA). The concentrations of IL-4 (cat. no. M4000B), IL-5 (cat. no. M5000), CCL17 (cat. no. MCC170) and CCL22 (cat. no. MCC220) were quantified using ELISA kits from R&D Systems, Inc. and OVA-specific IgE (cat. no. 88-50460-88) was quantified using an ELISA kit from Thermo Fisher Scientific, Inc., according to the manufacturer's instructions.

Statistical analysis. GraphPad Prism 5 (GraphPad Software, Inc.) was used to perform the data analysis. All data, which were obtained from at least three independent experiments, were evaluated with one-way ANOVA and Tukey's test was used to determine the difference between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

IAL ameliorates pathological injury in OVA-induced asthma. To investigate the effect of IAL on asthmatic inflammation, an OVA-induced mouse asthma model (Fig. 1A) was

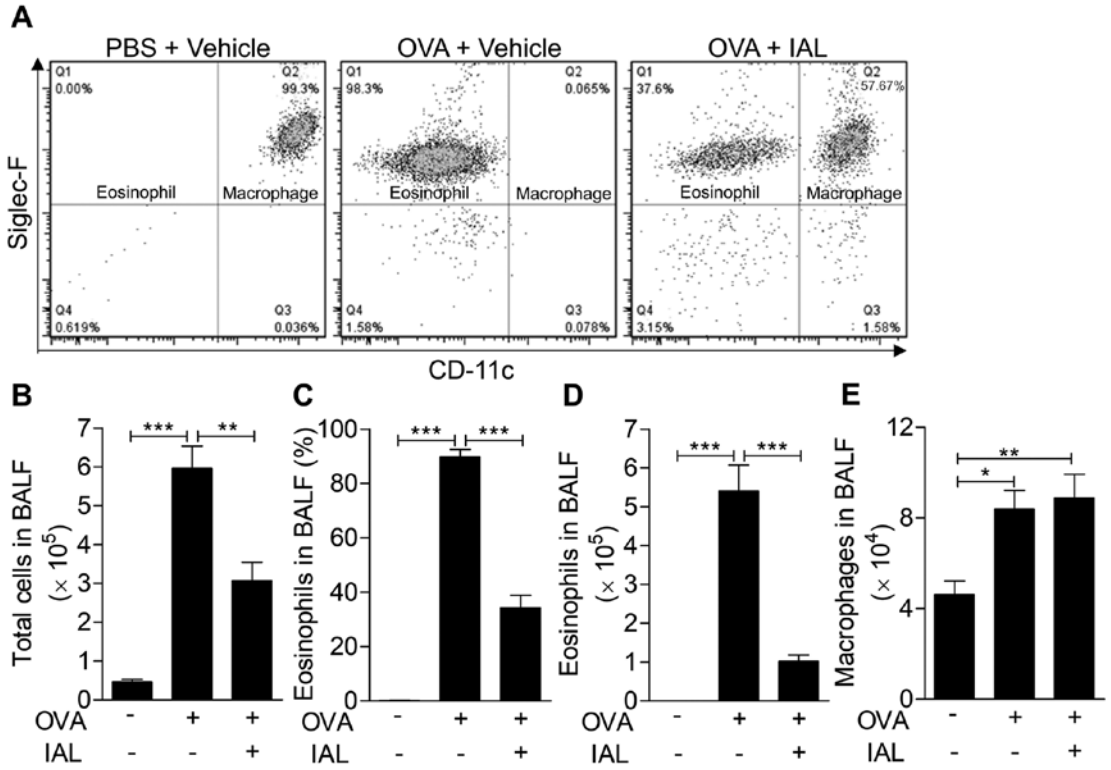

Figure 2. IAL reduces lung tissue permeability and the infiltration of inflammatory cells. (A) Infiltrating eosinophils and macrophages in BALF stained with phycoerythrin-conjugated anti-Siglec-F antibodies and allophycocyanin-conjugated anti-CD11c antibodies. (B) The number of total infiltrated cells, (C) the ratio of eosinophils, (D) the counts of eosinophils and (E) the counts of macrophages in BALF were detected. Values represent means ± standard error of the mean, n=5 mice, *P<0.05, **P<0.01, ***P<0.001. BALF, bronchoalveolar lavage fluid.

established as shown in Fig. 1, inflammatory cell infiltration was induced in OVA-induced asthmatic inflammation. However, IAL pretreatment significantly reduced cell infiltration (Fig. 1B), indicating that IAL relieved OVA-induced asthmatic inflammation. In addition, treatment with IAL did not result in an increase in serum OVA-specific IgE, whereas OVA treatment significantly increased the expression of OVA-specific IgE (Fig. 1C). Furthermore, IAL significantly reduced the protein concentration in BALF caused by OVA treatment (Fig. 1D). These results indicated that IAL had an inhibitory effect on asthmatic inflammation.

IAL reduces the permeability of lung tissue and the infiltration of inflammatory cells. In the process of asthma, lung tissues are destroyed by the infiltration of inflammatory mediators and inflammatory cells, especially eosinophils, which are significantly increased in BALF. Therefore, the number of eosinophils in BALF indicates the severity of asthma (22). Thus, the accumulation of eosinophils in BALF was measured to determine the therapeutic effect of IAL on inflammatory cell infiltration. As shown in Fig. 2, the proportion of eosinophils increased significantly following OVA induction, while it was significantly reduced following IAL treatment, from 98.3 to 37.6% (Fig. 2A). In addition, the number of total cells in the BALF significantly increased following OVA induction. Following IAL treatment, the total number of cells in the BALF was significantly lower (Fig. 2B). Consistent with this, the proportion of eosinophils and the number of eosinophils in BALF were significantly decreased following IAL treatment (Fig. 2C and D). Furthermore, the absolute number of macrophages in BALF was detected and it was found that the total macrophage number in BALF was enhanced following challenge with OVA. However, IAL treatment did not have an effect on the OVA-induced number of macrophages (Fig. 2E). These results indicated that IAL inhibited OVA-induced eosinophil accumulation.

IAL inhibits OVA-induced generation of type 2 cytokines. Asthma is generally associated with enhanced type 2 immune responses, including increased production of IL-4, IL-5, IL-13, CCL17 and CCL22 (23-25). Therefore, the generation of IL-4, IL-5, IL-13, CCL17 and CCL22 in BALF was detected. The results demonstrated that compared to the control group, the expression of IL-4, IL-5, IL-13, CCL17 and CCL22 was significantly increased following OVA treatment and was significantly reduced by IAL treatment (Fig. 3). These results indicated that IAL ameliorated OVA-induced asthmatic inflammation and Th2 cytokine production.

IAL inhibits alternatively activated macrophages in vitro. It is well established that alternatively activated M2 macrophages, induced by IL-4, serve an important role in asthmatic inflammation (24). Arg-1 and Ym-1 are expressed by alternatively activated macrophages during asthmatic inflammation (26) and CCL17 and CCL22 are the main chemokines involved in eosinophil recruitment (7). To determine the effect of IAL on macrophage M2 polarization, BMDMs were pretreated with IAL (0, 3, 10, or 30 μM) for 30 min and stimulated with IL-4 (10 ng/ml) for 24 h. The mRNA and protein expression levels of CCL17 and CCL22 were detected. Although IL-4

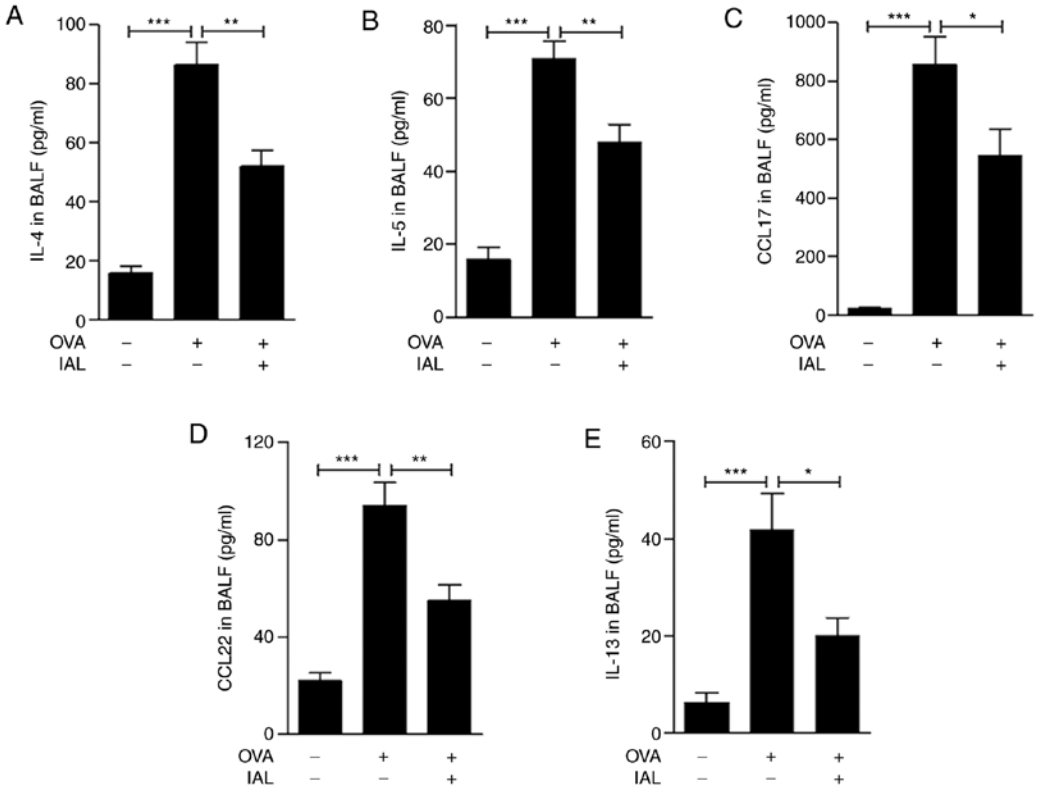

Figure 3. IAL inhibits OVA-induced type 2 cytokine production. The expression of the cytokines (A) IL-4 and (B) IL-5, and the chemokines (C) CCL17, (D) CCL22 and (E) IL-13 in BALF were measured by ELISA. Values represent means ± standard error of the mean, n=5 mice in each group, *P<0.05, **P<0.01, ***P<0.001. IAL, isoalantolactone; OVA, ovalbumin; BALF, bronchoalveolar lavage fluid.

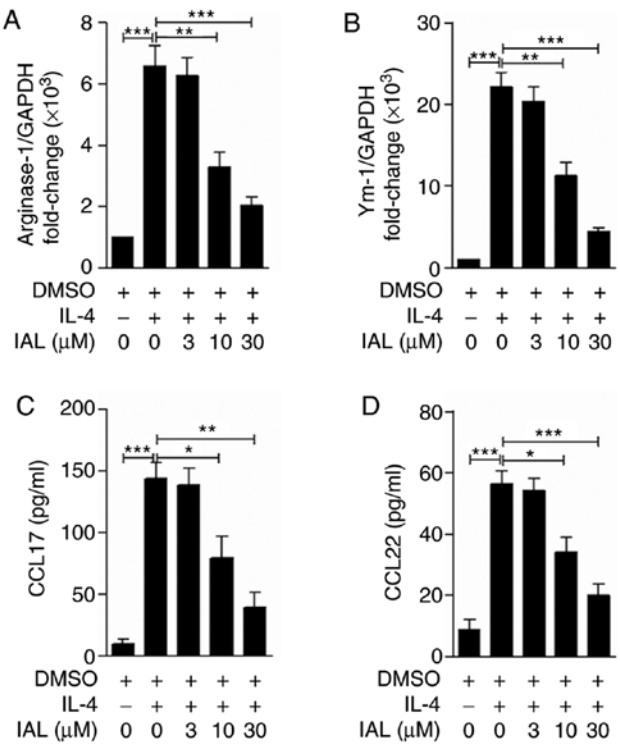

Figure 4. IAL inhibits polarization of alternatively activated M2 macrophages *in vitro*. BMDMs were pre-treated with IAL (0, 3, 10 and 30 μM) for 30 min and were stimulated with IL-4 (10 ng/ml) for 24 h. The mRNA level of (A) arginase-1 and (B) Ym-1 were quantified with reverse transcription-quantitative PCR. The expression of (C) CCL17 and (D) CCL22 was measured with ELISA. Values represent means ± standard error of the mean, n=3, *P<0.05, **P<0.01, ***P<0.001. IAL, isoalantolactone; BMDMs, bone marrow-derived macrophages.

treatment induced the expression of Arg-1, Ym-1, CCL17 and CCL22 in BMDMs, they were significantly decreased in a dose-dependent manner following treatment with IAL (Fig. 4). These results suggested that IAL inhibited IL-4-induced M2 macrophages.

IAL downregulates the phosphorylation of STAT6 and the expression of PPAR-γ and KLF4 in BMDMs. To determine how IAL inhibits alternatively activated macrophages, BMDMs were pre-treated with IAL (0, 3, 10, or 30 μM) and BMDMs challenged with IL-4. p-STAT6 and PPAR-γ and KLF4 were detected by western blotting. As shown in Fig. 5, the phosphorylation of STAT6 and the expression of PPAR-γ and KLF4 were induced by IL-4 treatment, while IAL inhibited their expression in a dose-dependent manner (Fig. 5). Taken together, these results suggested that IAL inhibited IL-4-mediated activation of STAT6/PPAR-γ/KLF4.

Discussion

Asthma is a heterogeneous inflammatory pulmonary disease that lacks effective medicines and causes serious issues to patients throughout life (27). Components of traditional medicines have been widely identified as promising drugs for treating various diseases, including asthma (28-30). In line with this, the effect of IAL on inflammatory diseases, such as acute lung injury and sepsis, has been reported (19,20). However, the effect of IAL on asthma and the underlying mechanism remain unclear. The present study investigated the anti-inflammatory effect of IAL on OVA-induced asthma

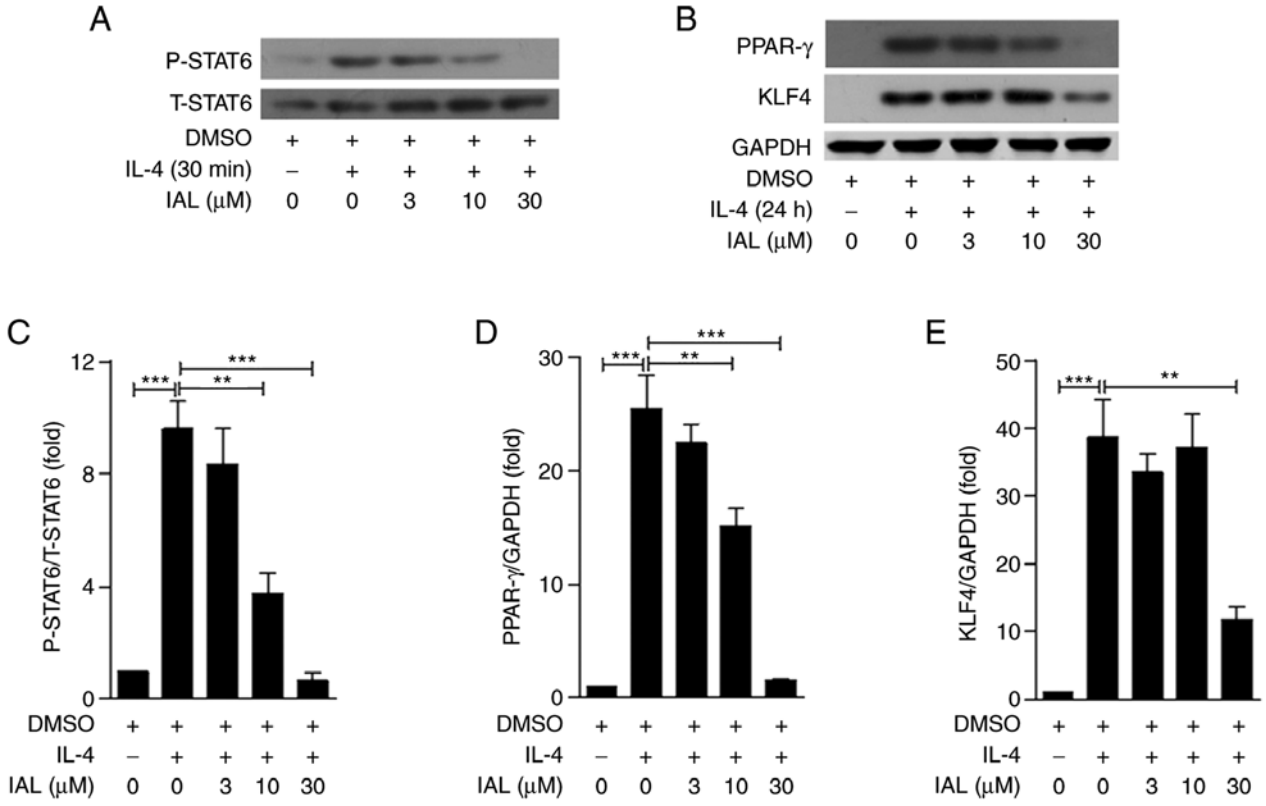

Figure 5. IAL downregulates the phosphorylation of STAT6 and the expression of PPAR-γ and KLF4 in BMDMs. Following preincubating with IAL (0, 3, 10, or 30 μM) for 30 min, BMDMs were challenged with IL-4 (10 ng/ml) for 30 min or 24 h. (A) The expression of total and phosphorylated STAT6 in BMDMs following challenge with IL-4 for 30 min and (B) the expression of PPAR-γ and KLF4 in BMDMs following challenge with IL-4 for 24 h were measured by using western blotting. Quantitative analyses of (C) P-STAT6, (D) PPAR-γ and (E) KLF4 were normalized to T-STAT6 and GAPDH. Values represent means ± standard error of the mean, n=3; **P<0.01, ***P<0.001. IAL, isoalantolactone; BMDMs, bone marrow-derived macrophages; PPAR-γ, peroxisome proliferator-activated receptor γ; KLF4, Krüppel-like factor 4; p, phosphorylated; t, total.

by inhibiting M2 macrophages. The results demonstrated that IAL has an inhibitory effect on the STAT6/PPAR-γ/KLF4 pathway and can therefore be considered as a potential therapeutic agent for the treatment of asthma.

Macrophages, the most abundant immune cells in the pulmonary microenvironment, serve a vital role in the pathology of asthmatic inflammation (31). Macrophages can be polarized to either classically activated (M1) phenotypes, induced by interferon (IFN)-γ and LPS, or M2 phenotypes, induced by interleukin (IL)-4 and IL-13 (32). Upon exposure to pathogens, such as peanuts, pollen, house dust mites and fungal spores, mononuclear monocytes are recruited to the lung and then activated to different subtypes by a variety of cytokines produced from injured epithelial cells and innate immune cells (32). Increased M2 macrophage polarization is considered a major phenomenon of asthmatic inflammation; increased M2 macrophages promote Th2 polarization of T cells and contribute to the aggravation of allergic asthma (27,28). Studies have shown that various endogenous proteins participate in the development of asthma by modulating M2 polarized macrophages. For instance, NLRP3 aggravates the pathological process of asthma by upregulating M2 macrophage polarization. The ATP/P2X7r axis also accelerates the development of asthma by promoting M2 macrophage polarization (33). Therefore, modulation of macrophage polarization is a promising method for treating asthma. Studies have investigated the inhibition of macrophage M2 polarization as a potential target for asthma treatment, such as treatment with mannose androgen and long non coding (lnc) RNA PTPRE-AS1 (34-36). It is worth noting that macrophage polarization is an important target of various natural products. For example, smiglaside A can effectively improve pulmonary inflammation by modulating macrophage polarization (37). *Peucedanum japonicum* extract attenuates allergic airway inflammation by inhibiting Th2 cell activation and macrophage activation (38). In addition to asthma, M2 polarized macrophages serve a critical role in several chronic diseases. For example, a recent study suggests that M2 macrophages enhance RT immunity in lung cancer (39). A previous study has also shown that M2 polarized BMDMs secrete exosomes containing miR-690 that, when administered to obese mice, improved glucose-insulin homeostasis (40). The present study suggested that IAL suppresses the M2 macrophage phenotype and reduces the expression of Arginase-1, Ym-1, CCL7 and CCL22, which together contributes to the alleviation of OVA-induced asthma *in vitro* and *in vivo*. Therefore, IAL is a promising compound for the treatment of asthmatic inflammation and a potential agent for alternatively activated macrophage-related diseases, such as tumors, obesity and diabetes.

The M2 phenotype of macrophages is regulated by various signaling pathways, including the IL-4-mediated signaling pathway, lncRNA-MM2P and VEGF signaling (41-43). An improved understanding of the molecular mechanisms underlying macrophage polarization is essential to understand the causal relationship between allergen exposure and the

development of allergic diseases such as asthma. JAK1/STAT6 signaling, the main downstream signal following IL-4 treatment, is regarded as a major therapeutic target for various compounds, such as kaempferol, emodin and chrysophanol (44-46). PPAR-γ, a subgroup of ligand-activated nuclear receptors, is induced by IL-4 and participates in the activation of M2 macrophages (47). The STAT6/PPAR-γ signaling pathway is also an important target for the HuoXueTongFu Formula (composed of six crude herbs: Chinese rhubarb, peach kernel, *Corydalis yanhusuo*, radish seed, Glauber's salt and safflower at a ratio of 5:5:5:5:5:3) to induce macrophage M2 polarization (48). KLF4 is a downstream protein of PPAR-γ (49). Through binding to the PPAR response element within the *KLF4* promoter, PPAR-γ induces the expression of KLF4 and promotes M2 macrophage polarization (50). Several studies have indicated that the KLF4 related pathway is closely associated with the regulation of macrophage polarization and is the target of various compounds, such as tanshinone IIA, iron and Epigallocatechin-3-Gallate (51-53). Collectively, the present study confirmed that the inhibition of IAL in macrophage activation is associated with the reduction of KLF4 expression by inhibiting the STAT6/PPAR-γ signaling pathway.

The target signaling pathways of IAL have been widely investigated in various diseases, such as pancreatic cancer, osteoporosis and other metabolic bone diseases. IAL attenuates the expression of p-STAT3 and STAT3, leading to apoptosis of prostate cancer cells (54). The p38 MAPK/NF-κB signaling pathway can also be inhibited by IAL treatment in MDA-MB-231 cells, as demonstrated by inhibition of migration and invasion (55). The anti-inflammatory effect of IAL on pulmonary disorders is modulated by the inhibition of NF-κB or ubiquitination of TRAF6 (19,20). The present study suggested that the alternative activation of macrophages regulated by IAL was related to the STAT6/PPAR-γ/KLF4 pathway, which provides further information on the multiple pharmacological functions of IAL.

The present study demonstrated that IAL has a potential therapeutic effect on OVA-induced asthmatic inflammation in mice via inhibition of M2 macrophage polarization and attenuation of the STAT6/PPAR-γ/KLF4 signaling pathway. These findings suggested that IAL is a candidate for the treatment of asthma and other diseases associated with macrophage polarization.

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Availability of data and materials

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

Authors' contributions

YSh designed the project and drafted the manuscript. YSo and XL performed the experiments and drafted the manuscript. FL and HZ participated in the data analysis and revised the manuscript. YSh and XL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental protocols of the present study were approved by the Institutional Animal Care and Use Committee of Shanghai Pudong Hospital (Shanghai, China) and procedures were approved by the Biological Research Ethics Committee of Shanghai Pudong Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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