



Interfering with alternatively activated macrophages by CSF-1R inhibition exerts therapeutic capacity on allergic airway inflammation

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ABSTRACT

Purpose: Allergic asthma is a chronic inflammatory disorder with airway hyperresponsiveness and tissue remodeling as the main pathological characteristics. The etiology of asthma is relatively complicated, involving genetic susceptibility, epigenetic regulation, environmental factors, and immune imbalance. Colony stimulating factor 1 receptor (CSF-1R), highly expressed in myeloid monocytes, plays an important role in regulating inflammation. However, the pathological role of CSF-1R and the therapeutic effects of CSF-1R inhibitor in allergic airway inflammation remain indistinct.

Methods: The house dust mite (HDM)-triggered allergic airway inflammation model was conducted to fully uncover the efficacies of CSF-1R inhibition, as illustrated by histopathological examinations, biochemical analysis, ELISA, RT-PCR, Western blotting assay, immunofluorescence, and flow cytometry. Furthermore, bone marrow-derived macrophages (BMDMs) were differentiated and polarized upon IL-4/IL-13 induction to clarify the underlying mechanisms of CSF-1R inhibition.

Results: Herein, we presented that the expression of CSF-1R was increased in HDM-induced experimental asthma and inhibition of CSF-1R displayed dramatic effects on the disease severity of asthma, referring to suppressing the secretion of allergic mediators, dysfunction of airway epithelium, and infiltration of inflammatory cells. Furthermore, CSF-1R inhibitor could markedly restrain the polarization and expression of transcriptional factors of alternatively activated macrophages (AAMs) in the presence of IL-4/IL-13 and reduce the recruitment of CSF-1R-dominant macrophages, both in acute and chronic allergic airway inflammation model.

Conclusion: Collectively, our findings demonstrated the molecular pathological mechanism of CSF-1R in allergic airway diseases and suggested that targeting CSF-1R might be an alternative intervention strategy on the homeostasis of airway immune microenvironment in asthma.

1. Introduction

Asthma manifests as a heterogenous inflammatory airway disease, mainly affecting adolescents and adults worldwide [1,2]. The clinical symptoms of asthma include cough, rhinorrhea, dyspnea, wheeze, shortness of breath, and chest tightness [2,3]. Data from World Health Organization (WHO) in 2021 suggested that an estimated 262 million patients suffering from asthma [4]. With a higher prevalence and incidence in low- and middle-income countries, asthma has become a great threat to human health [1,5]. Numerous classical therapeutic drugs, including glucocorticoids, leukotriene receptor antagonists, and

bronchodilators, have been used in clinical for adequate control of respiratory symptoms [6–8]. Recently, biological agents such as anti-IgE antibody (Omalizumab), anti-IL-5 antibody (Mepolizumab), and anti-TSLP antibody (Tezepelumab) have also emerged to treat patients with severe, uncontrolled asthma [7–9]. Nevertheless, there remain certain adverse effects and poor treatment responses. Therefore, it is urgent to uncover novel intervention strategy for asthma treatment.

The etiology of asthma is relatively complicated and closely associated with the intersect of genetics and environment [7–9]. The genome is a strong contributor to asthma risk, making it heritable and polygenic [10]. In the last decade, multiple genome-wide association studies

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(GWAS) have uncovered many genetic variants related to asthma susceptibility [10,11]. Environmental factors significantly contribute to asthma through directly interacting with airway architecture or modifying immune responses by epigenetic mechanisms, including allergen exposures, infection, obesity, tobacco smoke, and more [10–14]. Asthma is heterogeneous in terms of phenotypes defined as observable characteristics and endotypes defined as distinct molecular mechanisms [15–17]. Despite complex clinical and pathophysiological features, aberrant immune responses in the inflamed airways are central to asthma development and exacerbation [18]. Based on the major immune-inflammatory pathway, asthma can be broadly divided into two main endotypes described as type 2 and non-type 2 asthma [17,19–21]. Type 2 endotype mainly comprises T helper (Th) 2 cells, type 2 innate lymphoid cells (ILC2s), eosinophils, IgE-secreting B cells, mast cells, and their major cytokines (e.g., IL-4, IL-5, IL-13) [15,19]. Accordingly, tissue-resident and newly-recruited macrophages can be polarized into alternatively activated macrophages (AAMs) under type 2 airway inflammation [22,23]. In contrast, non-type 2 endotype comprises Th1 and/or Th17 cells, neutrophils, type I interferons, interleukin-17, and interleukin-1 β [15].

Colony-stimulating factor-1 receptor (CSF-1R), also known as FMS kinase, is a type I single transmembrane protein transcribed by *CSF1R* gene [24]. CSF-1R is mainly expressed in myeloid cells, such as monocytes, macrophages, glial cells, and osteoclasts [25,26]. The endogenous ligands, CSF-1 and IL-34, activate CSF-1R and downstream signaling pathways, such as PI3K-AKT, JAK-STATs, and MAPKs, and modulate the proliferation, differentiation, migration, and activation of macrophages [27–29]. The excessive activation of CSF-1/CSF-1R signal axis can result in inflammation in bone and central nervous system [30]. Therefore, intervention of CSF-1R could exert protective effects on rheumatic arthritis, multiple sclerosis, and Alzheimer's disease [31–34]. Over the past decades, CSF-1R has been considered as a promising target for anti-tumor research by regulating tumor-associated macrophages (TAMs) and the CSF-1R selective inhibitors, such as PLX3397, JNJ-40346527, and BLZ-945, have been widely used in clinical application [35–37]. Among them, PLX3397 was the first orally available small molecule CSF-1R inhibitor approved by US FDA and widely used for diverse researches involving CSF-1R inhibition [38].

Human *CSF1R* gene is located on chromosome 5 (5q32), adjacent to genes related to asthma genetic susceptibility, such as *IL4*, *IL5* and *ADRB2*. Previously, it has been confirmed that *CSF1R* gene polymorphism is positively associated with asthma risk by increasing CSF-1R expression on monocytes and neutrophils [39,40]. Moreover, the upregulation of CSF-1 in the lung secreted from asthma patients and murine models aggravated the disease process under allergens challenge [39,40]. In our research, we identified that an increase in CSF-1R expression was observed in HDM-induced asthma. Moreover, we found that inhibition of CSF-1R exerted therapeutic effects on the severity of asthma mainly due to suppression of the polarization of AAMs, which suggested that targeting CSF-1R might serve as an alternative therapeutic strategy in asthma treatment.

2. Materials and methods

2.1. Reagents and antibodies

House dust mite extract (HDM, XPB70D3A25) was purchased from Greer Lab (Lenoir, NC). Recombinant murine M-CSF, IL-4, and IL-13 were purchased from PeproTech (Rocky Hill, NJ, USA). BCA assay kit, proteinase and phosphatase inhibitor were obtained from Thermo Fisher Scientific (Pittsburgh, PA, USA). Mouse IL-4, TNF- α , and IL-6 ELISA kits were purchased from BD Pharmingen (San Diego, CA, USA), IL-5, IL-23, and IgE ELISA kits from Thermo Fisher Scientific, and IL-13 ELISA kit from R&D Systems (Minneapolis, MN, USA). Anti-CSF-1R, anti-IRF4, anti-STAT6, anti-p-STAT6, anti-Akt, and anti-p-Akt were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-ZO-1 antibody was

obtained from Proteintech (Chicago, IL, USA). Anti-Claudin-1 antibody was purchased from Abcam (Cambridge, MA, USA). The specific CSF-1R inhibitor, PLX3397, was obtained from MedChem Express (Monmouth Junction, NJ, USA). EasySepTM Mouse PE positive Selection Kit II was purchased from StemCell Technologies (Vancouver, BC, Canada). FITC-dextran was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Mice

Female 6–8-week-old C57BL/6 mice were purchased from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. All mice were housed under specific pathogen-free (SPF) conditions with a 12-h light/dark cycle at Shanghai Institute of Materia Medica. The mice were allowed *ad libitum* access to food and water and habituated in the animal facility for 1 week before experiments started. All animal experiments were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals, and were approved by the Bioethics Committee of Shanghai Institute of Materia Medica.

2.3. HDM-induced allergic asthma model

Allergic airway inflammation model was established as previously described [41]. In brief, mice were anaesthetized with isoflurane (Baxter, Guayama, PR, USA) and sensitized by intranasal administration of 10 μ g HDM in 50 μ l sterile phosphate-buffered saline (PBS) on days 0, 1, and 2. From day 8 to day 12, mice were challenged intranasally with 10 μ g HDM in 50 μ l PBS. Control mice were sensitized and challenged with PBS. For the experiments with CSF-1R inhibitor, PLX3397 (40 mg/kg) was dissolved in 0.5% carboxymethylcellulose sodium Sigma-Aldrich (St. Louis, MO, USA), with 0.25% Tween 80 and orally administered daily during the challenge phase. 48 h after the last challenge, mice were sacrificed and the serum, bronchoalveolar lavage (BAL) fluid, mediastinal lymph nodes and lungs were collected for further analysis. For chronic HDM challenge, mice were anaesthetized with isoflurane and subjected to 10 μ g HDM five times a week via intranasal instillation lasting for 3 weeks. The CSF-1R inhibitor, PLX3397, was orally administered once daily. Mice were then rested for 48 h before samples collection.

2.4. Lung histopathological examination

Lungs were fixed in 10% PBS-buffered formalin, embedded in paraffin, and cut into 5- μ m sections. Hematoxylin and eosin (H&E) staining was performed for examining inflammatory cells infiltration and Periodic acid-Schiff (PAS) for visualizing goblet cells and mucus production. Histopathological features were observed under a light microscope and scored blindly by three investigators based on the standards previously published [42].

2.5. Bronchoalveolar lavage fluid collection

18G sterile needles were inserted into the trachea after tracheostomy, and the bronchoalveolar lavage fluid (BALF) was collected by lavage with 0.8 mL of cold PBS at a total of three times. The differential cell count was analyzed with flow cytometer using BD LSRFortessa instruments (BD Biosciences, San Jose, CA, USA).

2.6. Airway epithelial barrier permeability

To evaluate pulmonary epithelial permeability, mice were anaesthetized and intratracheally instilled with FITC-dextran solution (3–5 kDa, 600 mg/kg). 2 h later, the blood samples were obtained and centrifuged at 12000 rpm for 10 min at 4 $^{\circ}$ C. The fluorescence intensity of FITC-dextran in serum was then measured at 488 nm excitation and 525 nm emission with a microplate reader (Molecular Devices, San Jose, CA, USA).

2.7. Single cell preparation and flow cytometry analysis

Mediastinal lymph nodes were harvested and grinded, and then filtered through a 70- μ m strainer to obtain single cell suspensions. Lung single cell suspensions were prepared as previously described with minor modifications [41]. Briefly, the lungs were washed with PBS and then cut into small pieces, and incubated in digestion buffer containing 1 mg/ml collagenase IV and 0.04 mg/ml DNase I for 1 h at 37 °C. The digested tissues were then filtered through a 100- μ m strainer and red blood cells were depleted using ammonium chloride solution (Beyotime Biotechnology, Shanghai, China). Total lung cell suspensions were collected by centrifugation and filtering through a 70- μ m strainer.

For FACS analysis, the single cells were washed with PBS and then stained with fixable viability dye eFluor™ 780 (eBioscience, San Diego, CA, USA) for 30 min at 4 °C for identification of viable cells. To stain surface marker, the cells were blocked with anti-CD16/CD32 mAb (Thermo Fisher Scientific, MA, USA) and then stained with appropriate antibody-fluorophore conjugates in 4 °C for 30 min. For intracellular staining, the cells were fixed and permeabilized with Foxp3 Staining Buffer set (Thermo Fisher Scientific, MA, USA), followed by staining with PE-conjugated anti-IL-17A and PerCP-Cy5.5-conjugated anti-IL-4. All antibodies used in this research were purchased from BD Biosciences or Thermo Fisher Scientific. The data was collected using BD LSRFortessa and analyzed with Flow Jo software (Tree star, Ashland, OR, USA). All immune cells were gated on CD45⁺ live cells.

2.8. Lung macrophages isolation and polarization

Lung single cell suspension were prepared as described above from normal female C57BL/6 mice. Macrophages were isolated using Easy-Sep™ Mouse PE positive Selection Kit II (StemCell, Vancouver, BC, Canada) according to the manufacturer's instructions. Briefly, FcR blocker and PE-conjugated anti-mouse F4/80 antibody were added to lung single cell suspension at the indicated concentration. The cell sample was then mixed and incubated for 15 min at room temperature, followed by selection cocktail and RapidSpheres™ addition and incubation for indicated times, respectively. After topping up with PBS containing 2% fetal bovine serum and 1 mM EDTA, the tube with cell sample was then placed into the magnet for 10 min and the supernatant was discarded for a total of three times to collect cells. The purity of the final isolated fractions was consistently > 85% as indicated by flow cytometry analysis. The isolated F4/80⁺ lung cells were polarized into M2-like macrophages with 10 ng/ml IL-4 and 10 ng/ml IL-13 for 24 h. At the same time, the CSF-1R inhibitor, PLX3397, was incubated at 10 μ M. Cells were then collected for RT-PCR analysis.

2.9. Bone marrow-derived macrophages culture and treatment

Bone marrow-derived macrophages (BMDMs) were prepared as previously described [43]. Briefly, single cell suspension were obtained from the tibia and femur bones of normal female C57BL/6 J mice and cultured for 7 days in complete Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FBS and 10 ng/ml of mouse colony-stimulating factor (M-CSF). 5 mM EDTA in PBS buffer was used to detach macrophages. The purity of BMDMs (CD11b⁺F4/80⁺) was consistently > 98% defined by flow cytometry analysis. For CSF-1R inhibition, differentiated BMDMs were pretreated with 10 μ M and 5 μ M CSF-1R inhibitor for 1 h and then stimulated with 10 ng/ml IL-4 and 10 ng/ml IL-13 for indicated times. Cells were then collected for RT-PCR, western blot, immunofluorescence, and flow cytometry analysis.

2.10. siRNA transfection

To knock down CSF-1R expression, siRNA targeting CSF-1R (RiboBio, Guangzhou, China) was used according to the manufacturer's instructions. 50 nM siRNA was mixed gently with Lipofectamine®

RNAiMAX Reagent (Thermo Fisher Scientific) in serum-free opti-MEM medium. The mixture was then added onto BMDMs cultured in 12-well plates. Cells were collected and assayed for CSF-1R expression after 48 h of transfection. The siRNA oligo sequences were listed as following: S1, 5'-CTACTCAACTTCTCCGAA-3', S2, 5'-GGCAATAGCTACACCTTCA-3', and S3, 5'-GTACTTCTTAATGGCACAA-3'.

2.11. β -hexosamine measurement

The level of β -hexosamine in serum was determined by incubating with equal volume of substrate P-nitrophenyl-N-acetyl-D-glucosamide (2 mM in 0.1 M citrate, pH 4.5) in 96-well plates at 37 °C for 1 h. The enzyme reaction was then terminated by the addition of 2 volumes of 0.1 M bicarbonate-carbonate buffer (pH 10.4), and the absorbance at 405 nm was obtained using a microplate reader (Molecular Devices).

2.12. Enzyme-linked immunosorbent assay (ELISA)

Lungs from mice were homogenized with cold PBS and the concentration of total protein was determined by BCA protein assay kit (Thermo Fisher Scientific). Cytokines in lung homogenates were determined by using ELISA kits according to the manufacturer's instructions. Serum levels of total IgE were quantified using ELISA kit. For measuring HDM-specific IgE, high affinity-binding 96-well plates were coated with 5 μ g/ml HDM overnight at 4 °C and the rest of the procedure were performed according to IgE ELISA kit protocol.

2.13. RNA isolation and real-time quantitative PCR

Total RNA was extracted from lung tissues and BMDM cells by using RNA simple total RNA kit (Tiangen, Beijing, China) and then reversely transcribed to obtain cDNA with Hifair™ II 1st Strand cDNA Synthesis SuperMix for qPCR (Yeasen, Shanghai, China). Real-time PCR were then performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster city, CA, USA). Relative expression of target genes was normalized to β -actin using double delta CT ($\Delta\Delta$ CT) model.

2.14. Western blot analysis

Lung tissues and cells were lysed with SDS buffer containing proteinase and phosphatase inhibitor and the protein concentrations were uniformed with the BCA protein assay kit. Equal amounts of total protein were separated by 10% SDS-PAGE and subsequently transferred to nitrocellulose membranes (Bio-Rad, Hercules, A, USA). After blocking with super T20 at room temperature for 1 h, the membranes were incubated at 4 °C overnight with primary antibodies. HRP-conjugated anti-rabbit IgG (Bio-Rad, Richmond, CA, USA) or HRP-conjugated monoclonal mouse anti-GAPDH (Kangcheng, Shanghai, China) were then added at room temperature for 1 h, followed by signal detection using SuperSigna West Femto Maximum Sensitivity Substrate under ChemiDoc™ MP Imaging System (Bio-Rad). All images collected from Western blotting analysis were quantified by Image-Pro Plus software (Silver Springs, MD, USA).

2.15. Immunofluorescent staining

Paraffin-embedded lung slices were deparaffinized with xylene and hydrated through graded ethanol to water, followed by unmasking epitope in 0.01 mol/L citrate buffer solution. For cell samples, cells on glass slides were fixed with fixing solution at room temperature for 10 min. The lung or cell samples were then blocked with 5% BSA and stained with anti-ZO-1 (Proteintech), anti-IRF4 (Cell Signaling Technology), FITC-conjugated CD11b (Abcam, Cambridge, MA, USA), FITC-conjugated F4/80 (eBioscience), and APC-conjugated CD206 (BD Biosciences) overnight at 4 °C. Unconjugated fluoresceins were further conjugated with FITC anti-rabbit secondary antibodies (Proteintech)

and the nuclei were counterstained with DAPI (Abcam). Images were subsequently captured using Leica TCS SPS microscope (Wetzlar, Germany).

2.16. Statistical analysis

All data were presented as Mean $\pm$ SEM. The one-way ANOVA followed by Dunnett's multiple comparison test was performed to determine significant difference involving multiple groups with GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) statistical software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Intervention of CSF-1R restrained the polarization of AAMs *in vitro*.

To probe the role of CSF-1R signaling in pro-asthmatic alternatively activated macrophages (AAMs), murine BMDMs were differentiated *in vitro* and transfected with small interfering RNAs targeting CSF-1R. We observed that efficient knockdown of CSF-1R resulted in the decreased expression of CD206 and CD301 on IL-4/IL-13-polarized M2-like macrophages (Fig. 1A-C). Consistently, the CSF-1R inhibitor, PLX3397, also remarkably inhibited the expression of CD206 and CD274 in BMDMs upon exposure of IL-4 and IL-13 (Fig. 1D), which could be further confirmed in a concentration-dependent manner by immunofluorescent staining of CD206 (Fig. 1E). Moreover, the level of *Retnla* (Fig. 1F), the well-defined transcriptional factors for AAMs, were also significantly suppressed by the CSF-1R inhibitor treatment. Correspondingly, the isolated lung F4/80⁺ cells were polarized into M2-like macrophages with IL-4 and IL-13, as indicated by increased expression of *Mrc1*, *Arg1*, *chil3*, and *Retnla* (Fig. 1G), while CSF-1R inhibitor could significantly suppress the polarization of lung macrophages. Collectively, the results above revealed that interfering with CSF-1R could restrain the polarization and activation of AAMs.

3.2. Inhibition of CSF-1R exerted therapeutic effects on HDM-induced allergic airway inflammation.

Previously, increasing evidence have demonstrated that AAMs played an important role in the initiation and progression of allergic asthma [23,44]. Given the effects of CSF-1R intervention on the activation of AAMs, we proposed the hypothesis that inhibition of CSF-1R might display therapeutic efficacies on asthma. Herein, we introduced the HDM-induced allergic airway inflammation model and the CSF-1R specific inhibitor, PLX3397, was orally administrated at the dosage of 40 mg/kg (Fig. 2A). Compared to the vehicle controls, CSF-1R inhibitor could largely attenuate the infiltration of inflammatory cells and mucus secretion as proven by H&E and PAS staining of lung sections (Fig. 2B-C). It is well-known that the production of IgE by plasma B cells and subsequent activation of mast cells were distinctly increased in allergic airway disorders [45]. As shown in Fig. 2D, the serum total IgE, HDM-specific IgE, and β -hexosaminidase were elevated after HDM challenge and were remarkably decreased upon the treatment of CSF-1R inhibitor. Accordingly, the CSF-1R inhibitor could reduce the population of CD19⁺ B cells in peripheral blood, as well as the percentage of germinal center B cells (B220⁺GL-7⁺) and plasma cells (B220^{int}CD138⁺) in mediastinal lymph nodes (Fig. 2E-F). These results suggested for the first time that inhibition of CSF-1R could reverse allergic inflammation in HDM-induced experimental asthma.

3.3. Inhibition of CSF-1R maintained the barrier function of pulmonary epithelium

The airway epithelial integrity serves as the first line of defense against exogenous microorganisms and allergens, which contributes to the homeostasis in lung immune microenvironment [46]. As expected,

disrupted epithelial barrier function was found in HDM-induced allergic inflammation, as evidenced by the loss of intercellular junctions (Fig. 2G). Nevertheless, oral administration of the CSF-1R inhibitor could dramatically rectify the decreased expression of ZO-1 and claudin-1 and maintain a normal epithelial integrity (Fig. 2G). Furthermore, the immunofluorescence confirmed that inhibition of CSF-1R could promote the local reservation of ZO-1 on the epithelial cells (Fig. 2H).

3.4. Inhibition of CSF-1R decreased the population of CSF-1R-predominant monocytes/macrophages in asthma

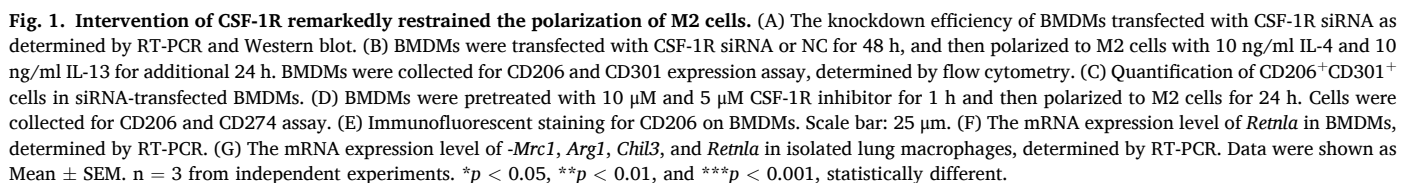
Owing to the injured airway epithelium, direct exposure of allergens functioned as the critical triggers for further abnormal activation and recruitment of inflammatory cells [47]. In order to elucidate the disordered immune responses mediated by CSF-1R in the allergic airway inflammation, we analyzed the inflammatory cells in the inflamed tissues of HDM-induced experimental asthma. In line with the dysfunction of airway epithelial barrier, increased populations of monocytes, and macrophages were observed in bronchoalveolar lavage fluid (BALF) after HDM induction, which could be restored by CSF-1R inhibitor (Fig. 3A). Moreover, CSF-1R inhibition could decreased the infiltration of CD11b⁺ myeloid cells (Fig. 3B) in lungs upon HDM challenge. Further confirmation by flow cytometric analyses showed that treatment with CSF-1R inhibitor substantially reduced the percentage of CD11b⁺F4/80⁺ macrophages and CD11b⁺CX3CR1⁺ (Fig. 3C) monocytes in lung tissues (Fig. 3C). The increased CD11b⁺Gr-1⁺ neutrophils and CD11b⁺Ly6C⁺ monocytes (Fig. 3D) were also observed in lungs after HDM challenge, however, it seemed that CSF-1R inhibition showed little effects on the infiltration of these cell populations. In line with the blockage of monocytes and macrophages in lung tissues, CSF-1R inhibitor reduced the excessive percentage of CD11b⁺ myeloid cells (Fig. 3E) and CD11b⁺F4/80⁺ macrophages (Fig. 3F) in mediastinal lymph nodes, compared with vehicle control mice.

3.5. CSF-1R inhibitor suppressed the expression of CSF-1R on macrophages in HDM-triggered allergic inflammation

To further determine the role of CSF-1R-doninant monocytes/macrophages in allergic asthma, we next examined CSF-1R expression in lung tissues. The results demonstrated that the protein level of CSF-1R protein, including membrane receptor and cytoplasmic domain (Fig. 4A), was distinctly increased under HDM challenge, and CSF-1R inhibitor could reduce the excessive expression of all fragments of CSF-1R protein (Fig. 4A). This finding was further confirmed by flow cytometry analyses, showing that CSF-1R inhibitor reversed the increased population of CD11b⁺CSF-1R⁺ cells in lung tissues (Fig. 4B), and mediastinal lymph nodes (Fig. 4C). In addition, the enhanced accumulation and colocalization of CSF-1R with F4/80 were observed in lung sections of HDM-treated vehicle mice (Fig. 4D), which could be restored by CSF-1R inhibition.

3.6. Inhibition of CSF-1R rectified the Th2-biased pulmonary inflammation in asthma

Allergic asthma is widely considered as a Th2-dominant allergic disorder marked by accumulation of Th2 lymphocytes and increased secretion of IL-4, IL-5, and IL-13 [22]. The mRNA analysis of lung tissues revealed that CSF-1R inhibitor suppressed the gene expression level of multiple chemokines and chemokine receptors (Fig. 5A), referring to the macrophages and T lymphocytes recruitment. To directly uncover the effects of CSF-1R inhibition on regulating Th2-mediated responses, we measured the levels of classic Th2 cytokines in lung tissues from HDM-induced allergic airway inflammation. Notably, CSF-1R inhibitor decreased the population of CD4⁺IL-4⁺ and CD4⁺IL-17⁺ cells in lung tissues, compared to vehicle mice (Fig. 5B-C). Correspondingly, the increased gene expression level of cytokines, *Il4* and *Il13* (Fig. 5D), and



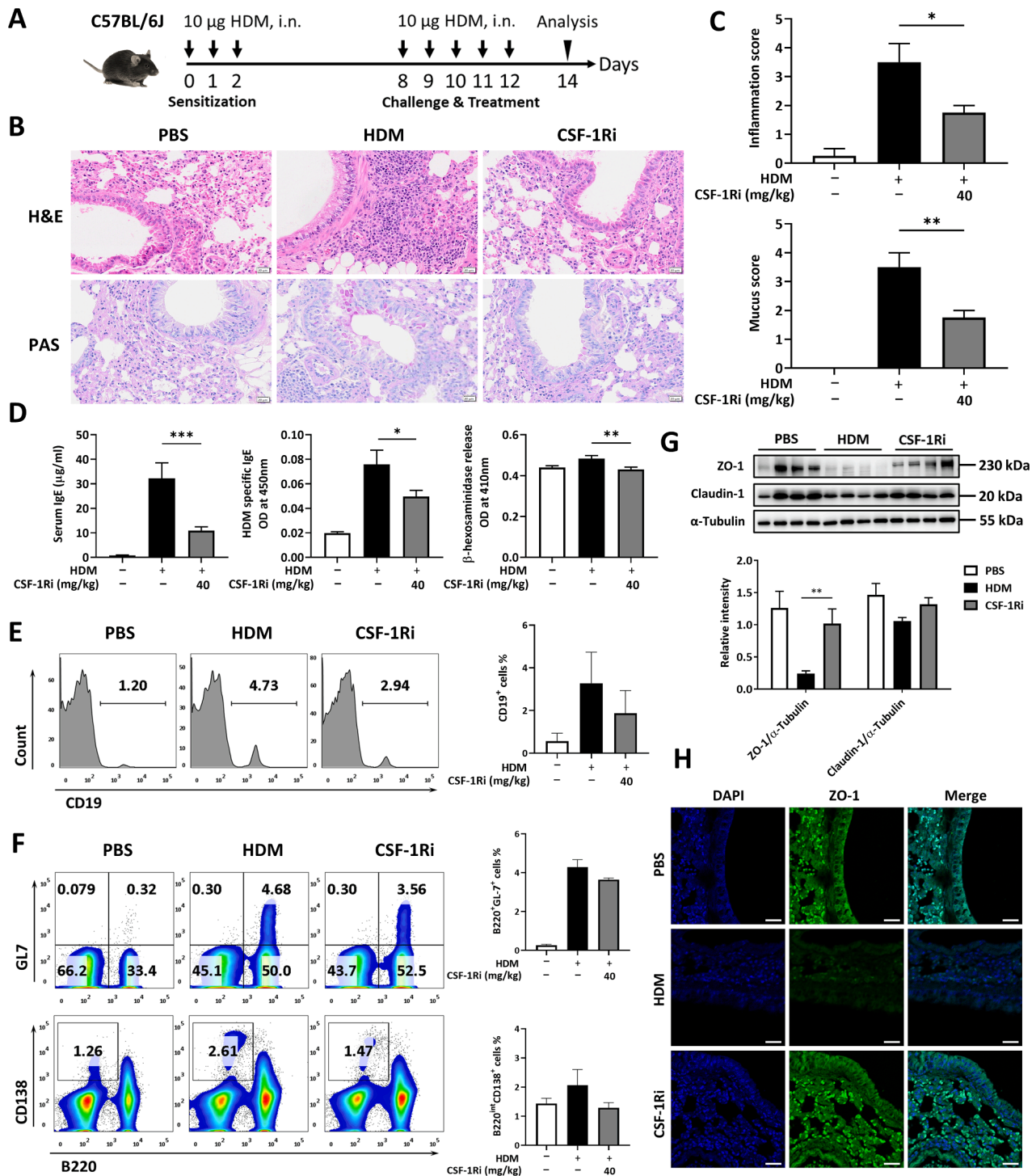


Fig. 2. CSF-1R inhibitor ameliorated the allergic inflammation upon HDM induction. (A) The flow diagram of HDM-induced experimental asthma as described in **Methods**. (B) The representative images of H&E and PAS staining of lung tissues. (C) The inflammation score and mucus score of lung sections based on H&E and PAS staining. (D) The serum IgE, HDM-specific IgE, and β-hexosaminidase level, assayed by ELISA. (E) The population of CD19⁺ B cells in peripheral blood. (F) The population of B220⁺GL7⁺ and B220^{int}CD138⁺ cells in lymph nodes. (G) Western blot and quantification analysis of ZO-1 and claudin-1 in lung tissues. (H) Immunofluorescent staining for ZO-1 in lung sections. Scale bar: 30 µm. Data were shown as Mean ± SEM. n = 9–11 mice per group. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001, statistically different.

protein level of cytokines, IL-4, IL-5, and IL-13 (Fig. 5E), were blunted by CSF-1R inhibitor. Moreover, CSF-1R inhibition could reduce the production of inflammatory genes *Il6*, *Il9*, *Il25*, and *Mcp1* (Fig. 5D), and suppress the secretion of pro-inflammatory cytokines, IL-17, TNF-α, and IL-6 (Fig. 5E). These results suggested that CSF-1R inhibition attenuated allergic inflammation in HDM-induced experimental asthma.

3.7. Inhibition of CSF-1R suppressed the population and signaling transduction of AAMs in asthma

In the presence of abnormal activation of Th2 cells and increased level of IL-4 and IL-13, macrophages could be polarized to AAMs in the progression of allergic inflammation [24]. In keeping with the findings

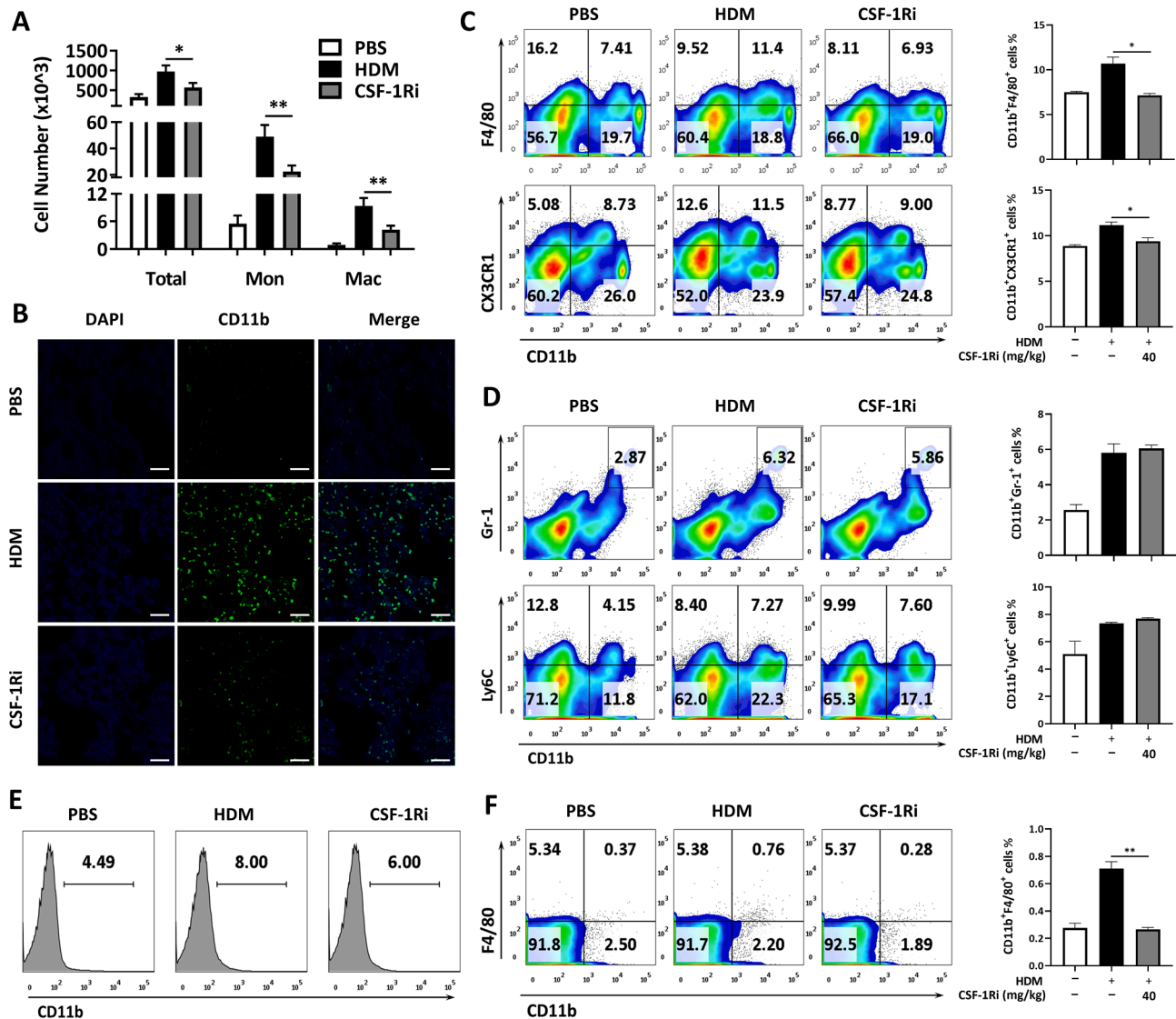


Fig. 3. CSF-1R inhibitor reduced the recruitment of monocytes/macrophages in HDM-triggered allergic inflammation. (A) The cell number of immune cells in BALF from HDM-induced asthma. (B) Immunofluorescent staining for CD11b in lung sections. Scale bar: 30 μ m. (C) The population of CD11b⁺F4/80⁺ and CD11b⁺CX3CR1⁺ cells in lung tissues. (D) The population of CD11b⁺Gr-1⁺ and CD11b⁺Ly6C⁺ cells in lung tissues. (E) The population of CD11b⁺ cells in mediastinal lymph nodes. (F) The population of CD11b⁺F4/80⁺ cells in mediastinal lymph nodes. Data were shown as Mean $\pm$ SEM. n = 9–11 mice per group. *p < 0.05, **p < 0.01, and ***p < 0.001, statistically different.

in Fig. 1, we further investigated the effects of CSF-1R inhibitor on the polarization of AAMs in HDM-induced allergic inflammation model. As shown in Fig. 6A, the immunofluorescent staining indicated that CD206⁺F4/80⁺ cells in the lung tissues were obviously increased in response to HDM challenge, while CSF-1R inhibitor substantially blunted the over-expression and colocalization of CD206 and F4/80. Further flow cytometry analyses confirmed that CSF-1R inhibitor remarkably decreased the population of CD206⁺F4/80⁺, CD301⁺F4/80⁺, and CD274⁺F4/80⁺ cells in lung tissues (Fig. 6B–C). Moreover, CSF-1R inhibitor dramatically inhibited the expression of transcriptional factors of AAMs, including *Arg1*, *Retnla*, and *chil3* (Fig. 6D). Additionally, STAT6-IRF4 signaling contributes the differentiation of AAMs and subsequent Th2 immune responses [22,24]. As expected, the expression levels of IRF4 and STAT6 by western blotting assay were reduced in CSF-1R inhibitor-treated HDM-challenged mice (Fig. 6E), compared to the vehicle mice. Likewise, the increased colocalization of IRF4 (Fig. 6F) with nuclei was observed in IL-4/IL-13-primed BMDMs, while not in CSF-1R inhibitor-treated BMDMs.

3.8. Inhibition of CSF-1R suppressed the polarization of pulmonary AAMs and type 2 biased inflammation under chronic HDM challenge

In order to further confirm that CSF-1R inhibition could exert therapeutic effects on type 2 allergic airway inflammation by interfering with macrophage infiltration and polarization, HDM-induced chronic allergic airway inflammation model was subsequently conducted in the present research (Fig. 7A). In line with the protective effects of CSF-1R inhibition on airway epithelial barrier, CSF-1R inhibitor significantly reduced the exudation of FITC-dextran as indicated by decreased serum fluorescence intensity compared to that in HDM-treated vehicle mice (Fig. 7B). Moreover, inhibition of CSF-1R also suppressed the level of total IgE in serum (Fig. 7C). Upon chronic HDM challenge, the infiltration of CD11b⁺F4/80⁺ macrophages into the lung lesions were increased, whereas CSF-1R inhibition significantly restored such pathological manifestations, largely related to the suppression of CSF-1R expression on CD11b⁺ cells and macrophages (Fig D–E). Consistently, CSF-1R inhibition displayed the capacity to suppress the polarization of AAMs in lung inflamed tissues under long-term exposure of HDM, as

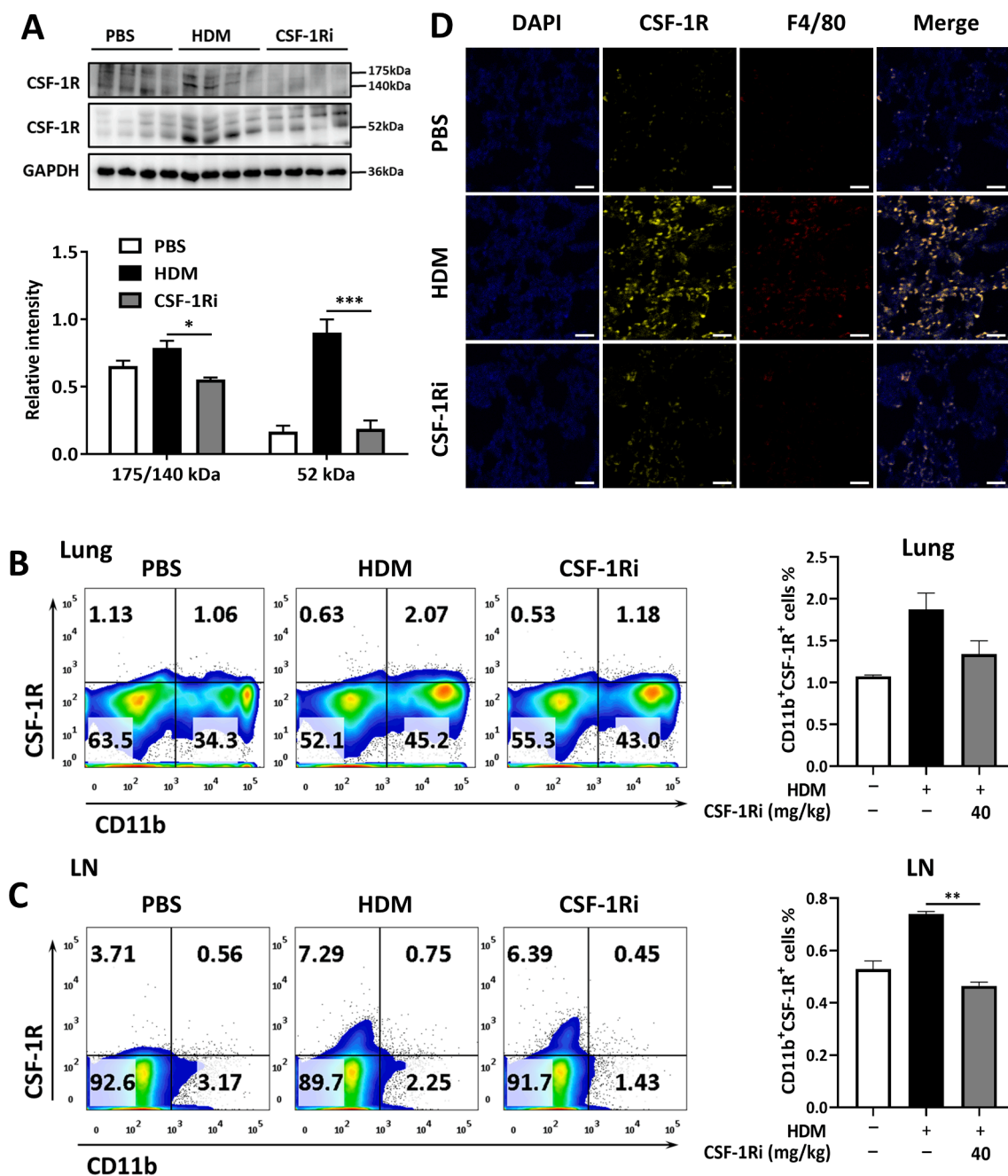


Fig. 4. CSF-1R inhibitor suppressed the expression of CSF-1R on monocytes/macrophages in HDM-triggered allergic inflammation. (A) Western blot analysis of CSF-1R expression in lung homogenates. (B) The population of CD11b⁺CSF-1R⁺ in lung tissues. (C) The population of CD11b⁺CSF-1R⁺ in mediastinal lymph nodes. (D) Immunofluorescent staining for F4/80 and CSF-1R in lung sections. Scale bar: 30 μ m. n = 9–11 mice per group.

evidenced by the decreased expression of CD206, CD301, and CD274 on macrophages (Fig. 7F–G). Further investigations showed that CSF-1R inhibition could decrease the secretion level of IL-4, IL-13, IL-17, and IL-2 (Fig. 7H). Collectively, these findings indicated that CSF-1R inhibition could attenuate the polarization of AAMs under type 2 allergic inflammation.

4. Discussion

CSF-1R played a critical role in maintaining the differentiation, ligand-dependent activation, and cytokines production from hematopoietic precursor cells, especially mononuclear phagocytes, such as macrophages and monocytes [24,48]. Previously, abnormal expression and signaling transduction of CSF-1R and its ligand, CSF-1, were found

in several diseases, including cancers, multiple sclerosis, Alzheimer's disease, and rheumatic arthritis [31,32,36,49,50]. Recently it was reported that the secretion of CSF-1 was markedly increased in the bronchoalveolar lavage fluids of patients with mild asthma after allergen challenge. In experimental allergic asthma model, airway epithelial cells derived CSF-1 could enhance the migration of conventional DC2 to the draining lymph node by upregulating the chemokine receptor CCR7 expression [39,51]. Moreover, the mutations of CSF-1R have been identified in tumor-associated macrophages (TAMs) and targeting CSF-1R has been a promising therapeutic target for cancer treatment [29,52]. In response to IL-34 and CSF-1, activation of ERK1/2 and JNK pathway by CSF-1R recognition promotes to the formation of membrane ruffles and cancer cell migration and invasion, which has been identified in breast, ovarian, prostate, and endometrial cancers [24,48,53]. Over the

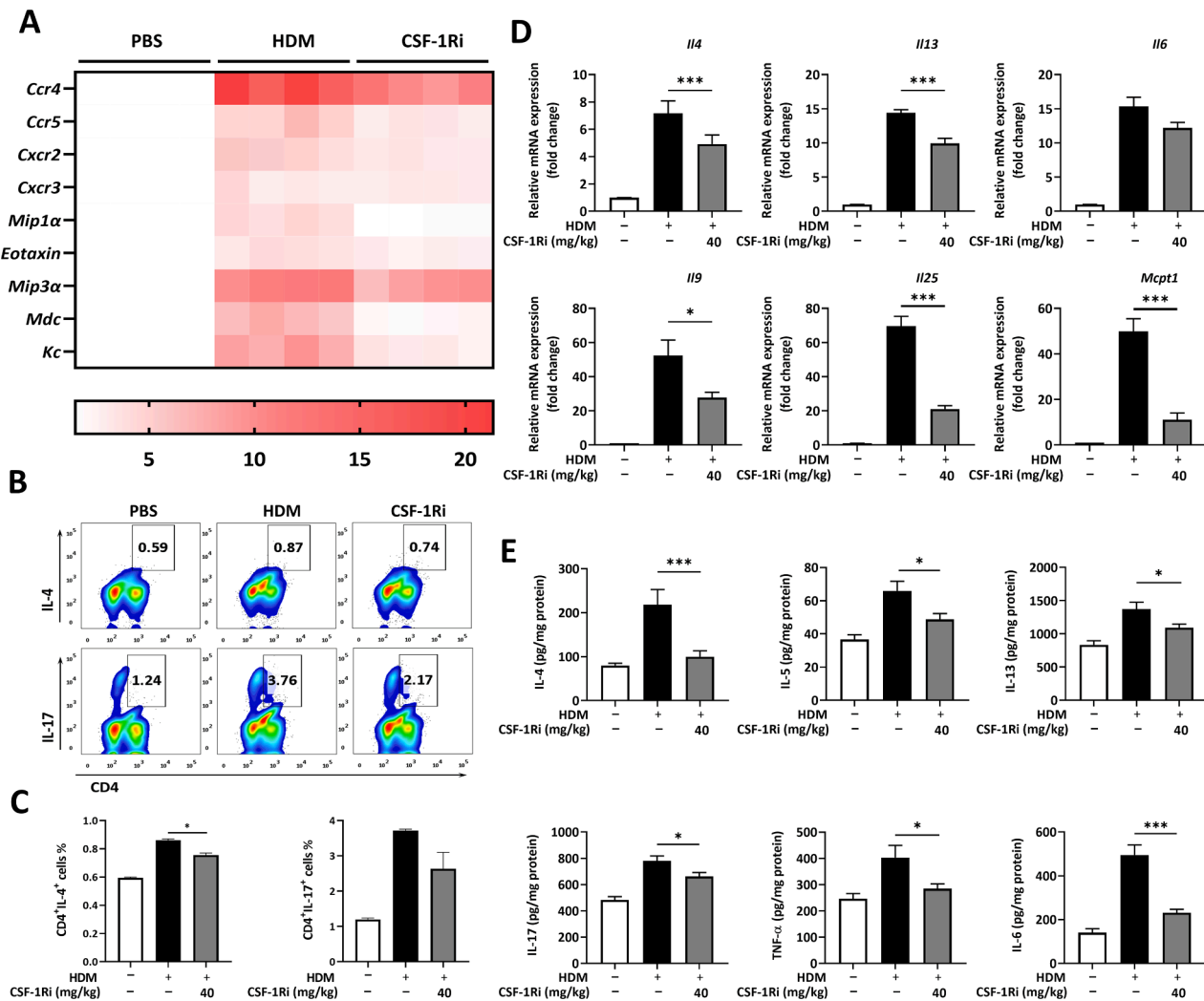


Fig. 5. CSF-1R inhibitor rectified the Th2-biased pulmonary inflammatory responses in HDM-induced allergic inflammation. (A) The mRNA expression of chemokines and chemokine receptors in lung homogenates by RT-PCR. (B) The population of CD4⁺IL-4⁺ and CD4⁺IL-17⁺ cells in lung tissues by flow cytometry analysis. (C) Quantification analysis of CD4⁺IL-4⁺ and CD4⁺IL-17⁺ cells in lung tissues. (D) The mRNA expression of cytokines in lung homogenates by RT-PCR. (E) The secretion of cytokines in lung homogenates by ELISA. Data were shown as Mean $\pm$ SEM. $n = 9-11$ mice per group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, statistically different.

past decades, CSF-1R inhibitors have been designed, synthesized, and evaluated for the treatment of cancer and inflammatory diseases [37]. Nevertheless, the pathological role of CSF-1R and the potential therapeutic effects of CSF-1R inhibitors in allergic airway inflammation remains ill-defined. Herein, we demonstrated that increased expression and colocalization of CSF-1R on macrophages were observed in HDM-induced experimental allergic asthma. Furthermore, the CSF-1R inhibitor, PLX3397, exerted dramatic efficacies on the disease severity and inflammatory responses in the inflamed lung tissues, which were mainly due to the modulation of alternatively activated macrophages (AAMs) (Fig. 8).

CSF-1R activation regulated both innate and adaptive immunity through modulating the development and signaling transduction of tissue-resident monocytes, such as osteoclasts in bone, Langerhans cells in skin, microglia in brain, and macrophages in colon [25,32]. In macrophage lineage, the downstream pathways of CSF-1R were closely associated with the process of survival, proliferation, differentiation, and chemotaxis, which have been well-defined in several tumors and inflammatory disorders [24]. Meanwhile, macrophage infiltration in the tumor microenvironment has been considered as a critical prognostic element in various cancer types [29]. Notably, CSF-1R inhibition could

lead to strong effects on circulating and tissue myeloid cells, including CCR2⁺ monocyte progenitor cells, c-Kit⁺ hematopoietic progenitor cells, CX3CR1⁺ BMDMs, and CD68⁺ cells [54]. Furthermore, the CSF-1R selective inhibitor or monoclonal antibody could suppress the proliferation and promote the cell death of CSF-1R-prodominant macrophages, which led to the dramatic reduction of CSF-1R⁺CD163⁺ macrophages, also known as the AAMs [52]. Consistently, in the present research, intervention of CSF-1R was found to be effective in restraining the polarization of AAMs both in BMDMs and HDM-injured lung tissues (Fig. 1), which contributed to the therapeutic effects of CSF-1R inhibitor in the treatment of asthma (Fig. 2 and Fig. 7).

Asthma represented as an obstinate and chronic airway diseases with a relative complicated pathogenesis, which might attribute to genetic and environmental factors, pulmonary and extra-pulmonary co-morbidities, smooth muscle construction, distinct inflammatory responses, and infection [2,9]. In addition, airway epithelial cells were the first line of defense and functioned as the natural barrier for pulmonary inflammatory triggers [47]. Given the experimental and human subjects studies, increasing evidence have suggested that the dysfunction of airway epithelial cells was defined as the initial responder to the inhaled allergens and crucial for the development of asthma [46,47]. Upon the

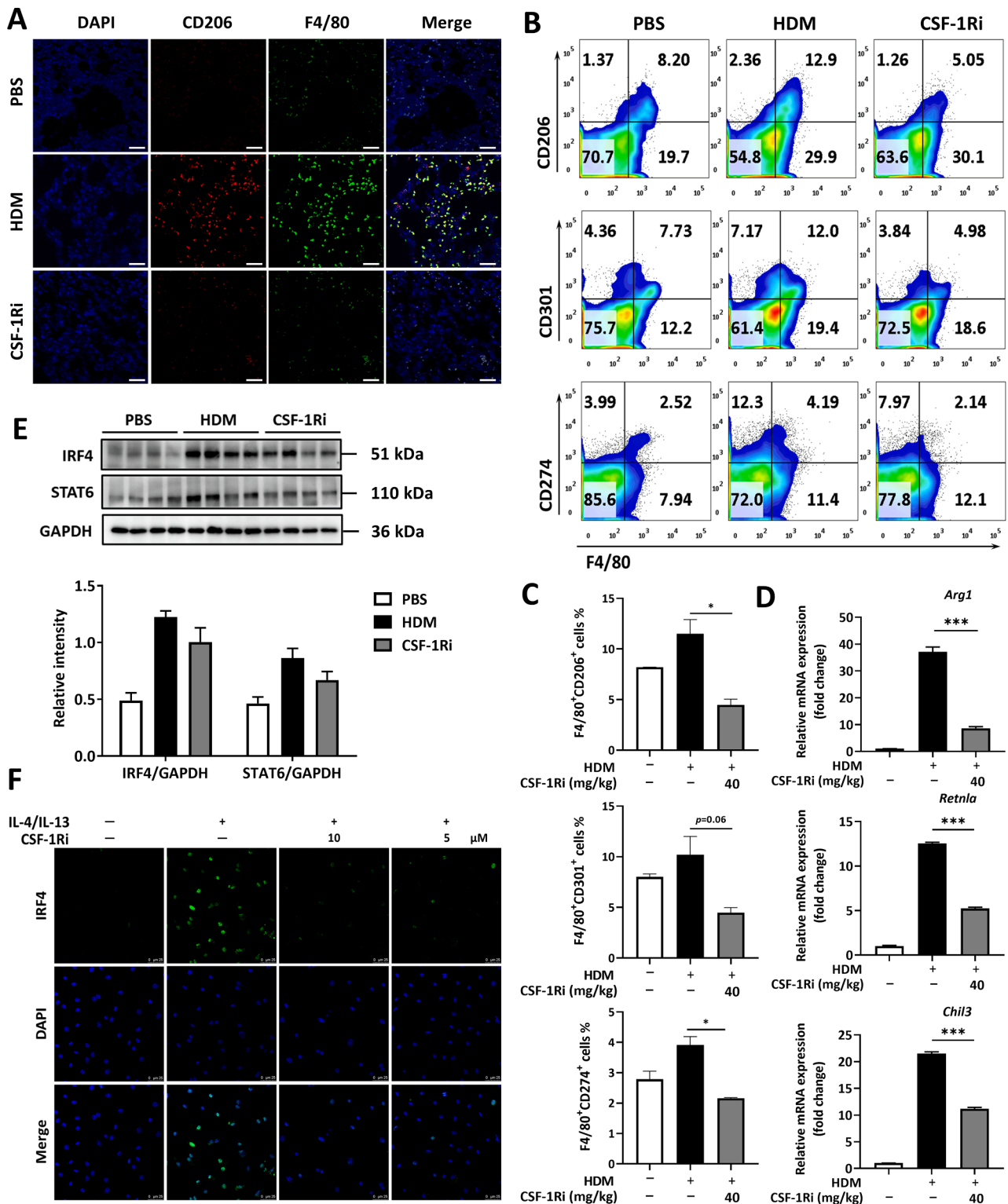


Fig. 6. CSF-1R inhibitor suppressed the polarization and transcriptional factors of M2 cells in HDM-induced allergic inflammation. (A) Immunofluorescent staining for CD206 and F4/80 in lung sections. Scale bar: 30 μ m. (B) The expression of CD206, CD301, and CD274 on F4/80⁺ cells by flow cytometry analysis. (C) Quantification analysis of the expression of CD206, CD301, and CD274 on F4/80⁺ cells in lung tissues. (D) The mRNA expression of *Arg1*, *Chil3*, and *Retnla* in lung homogenates by RT-PCR. (E) Western blot analysis of the expression of IRF4 and STAT6 in lung tissues. (F) Immunofluorescent staining for IRF4 in BMDMs. Scale bar: 30 μ m. Data were shown as Mean $\pm$ SEM. n = 9–11 mice per group. * p < 0.05, ** p < 0.01, and *** p < 0.001, statistically different.

exposure of allergens in susceptible populations, airway epithelial cells are the main source of CSF-1 and required for allergen-reactive IgE production, which further result in the Th2-biased adaptive immune responses [39,55]. Accordingly, targeting CSF-1R displayed protective

effects on the loss of tight junction of airway epithelial cells in experimental asthma (Fig. 2 and Fig. 7B) and decreased the level of cytokines from Th2 cells, including IL-4 and IL-13 (Fig. 5 and Fig. 7H).

Upon the destruction of epithelial cells, the exogenous allergens

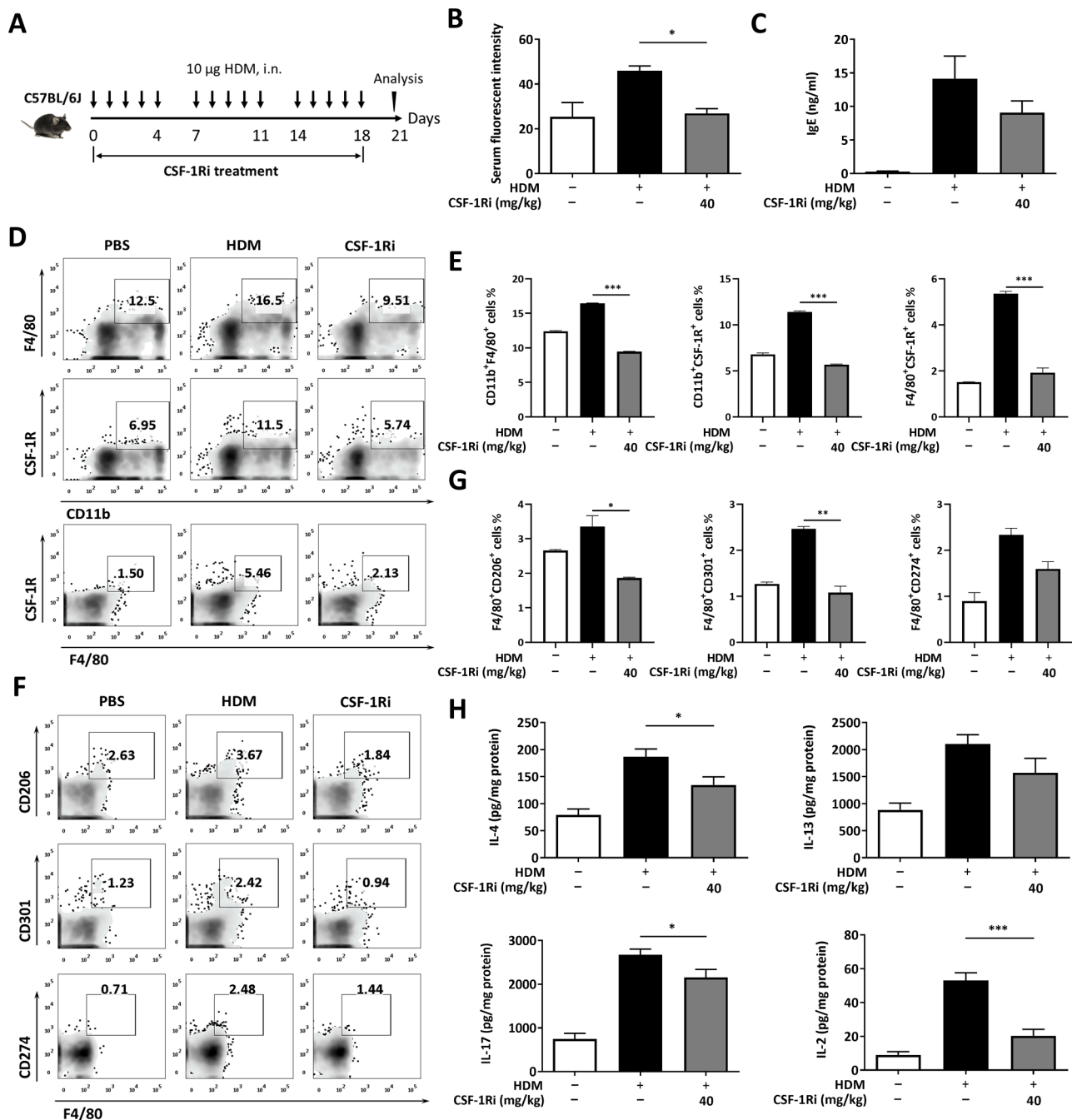


Fig. 7. CSF-1R inhibitor suppressed the polarization of M2 cells and Th2-biased allergic inflammation in HDM-induced chronic allergic inflammation. (A) The flow diagram of HDM-induced experimental chronic asthma as described in **Methods**. (B) Serum fluorescent intensity of FITC-dextran in HDM-induced asthma. (C) The serum level of IgE, analyzed by ELISA. (D) The population of CD11b⁺F4/80⁺, CD11b⁺CSF-1R⁺, and F4/80⁺CSF-1R⁺ cells by flow cytometry analysis. (E) Quantification analysis of CD11b⁺F4/80⁺, CD11b⁺CSF-1R⁺, and F4/80⁺CSF-1R⁺ cells in lung tissues. (F) The expression of CD206, CD301, and CD274 on F4/80⁺ cells by flow cytometry analysis. (G) Quantification analysis of the expression of CD206, CD301, and CD274 on F4/80⁺ cells in lung tissues. (H) The protein level of IL-4, IL-13, IL-17, and IL-2 in lung homogenates by ELISA. Data were shown as Mean $\pm$ SEM. $n = 8$ mice per group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, statistically different.

could be further recognized by inflammatory cells, macrophages and DCs, through binding the pattern recognition receptors [39,47]. Pathologically, chemokines and adhesive molecules, derived from epithelial cells and immune cells, could further recruit the immune cells from peripheral circulatory system and lymph nodes [56,57]. As illustrated in the present research, we indeed identified an increase of CSF-1R-dominant macrophages from lung tissues and mediastinal lymph nodes in HDM-triggered allergic airway inflammation (Fig. 3). Upon the

inhalation of HDM, the increased protein expression of CSF-1R was observed in the inflamed lung tissues, which might largely due to the infiltration of macrophages. Inspiringly, inhibition of CSF-1R possessed the capacity to reduce the population of macrophages and the mRNA expression of related chemokines in lung tissues (Figs. 3-5 and Fig. 7). Besides, in the presence of IL-4 and IL-13, macrophages could differentiate into the subtype of AAMs, on which CD206, CD301, along with the transcriptional factors were highly expressed [22,57]. In the present

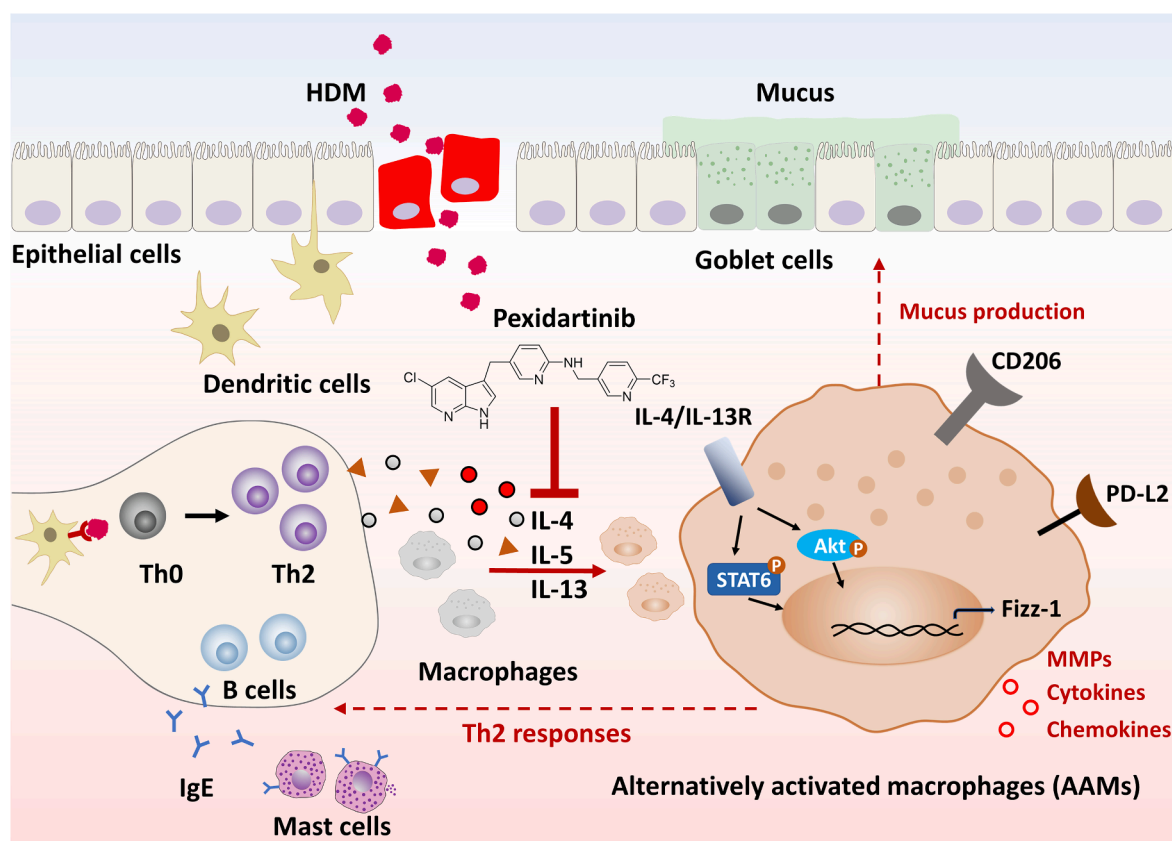


Fig. 8. The schematic diagram of targeting CSF-1R in attenuating the disease severity of allergic inflammation.

study, we indicated that CSF-1R inhibitor displayed potent effects on the polarization of AAMs in HDM-induced asthma and downregulate the expression of *Arg1*, *Retnla*, and *Chil3*, which were closely related to the regulation of IRF4-STAT6 signaling (Fig. 6). In line with our research, the modulatory effects of CSF-1R inhibition on the function of pulmonary macrophages were confirmed in radiation-induced pulmonary fibrosis [33]. Upregulation of *Arg1* from tissue-infiltrating macrophages were sufficient to induce the abnormal activation of myofibroblasts [33].

In summary, the present research collectively demonstrated that inhibition of CSF-1R exerted therapeutic effects on HDM-induced experimental asthma, as evidenced by suppressing the inflammatory responses, mucus secretion, IgE production, and destruction of airway epithelial barrier. Further investigations suggested that inhibition of CSF-1R could modulate the differentiation of AAMs *in vitro*, decrease the infiltration of CSF-1R-predominant macrophages, and subsequently inhibited the Th2-biased inflammatory responses. Briefly, this research suggested that CSF-1R represented as a drug target for allergic inflammation and targeting CSF-1R might be an alternative therapeutic strategy for asthma patients.

Author contributions

CG Xiang, H Li, and W Tang designed the research and wrote the manuscript; CG Xiang, H Li, C Fan, QK Lu, MT Liu, HM Lu, CL Feng, YW Wu, and B Wu performed the experiments. H Li and W Tang revised the manuscript. All the authors contributed to the interpretation of data and approved the final draft.

CRediT authorship contribution statement

Caigui Xiang: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft. **Chen Fan:** Data curation, Investigation, Methodology, Validation. **Qiukai Lu:** Data curation, Formal analysis, Investigation, Methodology, Validation. **Moting Liu:** Data curation, Formal analysis, Investigation, Methodology. **Huimin Lu:** Investigation, Methodology, Validation. **Chunlan Feng:** Investigation, Methodology, Validation. **Yanwei Wu:** Investigation, Methodology. **Bing Wu:** Investigation, Methodology. **Heng Li:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Resources, Supervision, Validation, Writing – review & editing. **Wei Tang:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The author declare that there is no conflict of interest.

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