

LncRNA AK085865 depletion ameliorates asthmatic airway inflammation by modulating macrophage polarization

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ABSTRACT

Accumulating evidence indicates that regulators of macrophages polarization may play a key role in the development of allergic asthma (AA). However, the exact role of long non-coding RNAs (lncRNAs) in regulating in macrophages polarization in the pathogenesis of dermatophagoides farinae protein 1 (Der f1)-induced AA is not fully understood. The purpose of this study was to determine the function of lncRNA AK085865 in regulating macrophages in AA. Here we report that lncRNA AK085865 served as a critical regulator of macrophages polarization and reduced the pathological progress of asthmatic airway inflammation. In response to the challenge of Der f1, AK085865^{-/-} mice displayed attenuated allergic airway inflammation, including decreased eosinophil in BALF and reduced production of IgE, which were associated with decreased mucous glands and goblet cell hyperplasia. In addition, Der f1-treated AK085865^{-/-} mice show fewer M2 macrophages when compared with WT asthmatic mice. After adopting bone marrow-derived macrophages (BMDM, M0) from WT mice, Der f1-treated AK085865^{-/-} mice also revealed a light inflammatory reactions. We further observed that the percentage of type II innate immune lymphoid cells (ILC2s) decreased in AK085865^{-/-} asthmatic mice. Moreover, M2 macrophages helped promote the differentiation of ILC2s, probably through the exosomal pathway secreted by M2 macrophages. Taken together, these findings reveal that AK085865 depletion can ameliorate asthmatic airway inflammation by modulating macrophage polarization and M2 macrophages can promote the differentiation of innate lymphoid cells progenitor (ILCP) into ILC2s.

1. Introduction

Asthma is a chronic inflammatory airway disease characterized by infiltration of eosinophils, neutrophils, mast cells, and T cells into the airway submucosa. It is the 14th most important chronic disease in the world based on prevalence, extent, and duration of disability, affecting 334 million individuals of all ages. Therefore, it is of great practical significance to explore the potential pathogenesis of asthma and to develop effective treatment strategies.

Investigation of CD4 T-helper cells has traditionally been a key focus of asthma research. However, AA is the result of both innate and

adaptive immune system interactions that complement each other and promote the occurrence of airway inflammation [1]. Targeted asthma research in recent years has increasingly highlighted the roles of innate immunity cell populations including macrophages and Type II innate lymphoid cells (ILC2s) in airway tissues.

Macrophages regulate airway hyperresponsiveness (AHR) through cell proliferation, epithelial integrity and immune response, thus modifying the genetic susceptibility to AA [2]. Adoptive transfer M2 macrophages aggravate airway inflammation in AA and promote pulmonary inflammation [3,4], suggesting that M2 macrophages polarization plays an important role in the pathogenesis of AA. Thus,

Abbreviations: AA, allergic asthma; AHR, airway hyperresponsiveness; APC, allophycocyanin; Arg-1, arginase 1; BALF, bronchoalveolar lavage fluid; BFA, Brefeldin A; BMDM, bone marrow-derived macrophage; Der f1, Dermatophagoides farinae protein; Fizz-1, Resistin-like-α; FITC, fluoresceine isothiocyanate; IFN, interferon; lncRNA, long non-coding RNA; ILC, innate lymphoid cell; ILCP, innate lymphoid cells progenitor; LPS, lipopolysaccharide; M1, classically activated macrophage; M2, alternatively activated macrophage; PE, Phycoerythrin; PE-Cy7, Phycoerythrin-Cy7; PMA, Phorbol 12-myristate 13-acetate; TNF, tumor necrosis factor; YM-1, chitinase 3-like 3

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molecular intervention has important therapeutic potential for regulating macrophages and other allergic inflammatory diseases. In addition, ILC2s have become an important area of interest in AA. Th2 cytokines secreted by ILC2s can promote the polarization of M2 macrophages. Therefore, it is of great significance to pay attention to the changes of ILC2s.

lncRNAs are recently identified RNA elements that have roles in a diverse range of cellular functions including development, differentiation, cell fate, and disease pathogenesis [5]. Further, lncRNAs that have no protein-coding functions play important roles in macrophage activation and differentiation [6–11] via the regulation of gene expression through different mechanisms including epigenetics, transcription, and post-transcription [12]. For example, Meng *et al.* observed that the lncRNA Mirt2 is a potential regulator of macrophage polarization that is associated with the ubiquitination of Lys63 (K63) in TRAF6, which inhibits the activation of NF- κ B and MAPK pathways, thereby limiting proinflammatory cytokines [13]. Sun *et al.* observed that the lncRNA GAS5 inhibits the transcription of interferon regulatory factor 4 (IRF4) by binding to Polycomb Repressive Complex 2 (PRC2), thereby inhibiting the polarization of macrophages or microglia to M2, and thus aggravating the progression of experimental autoimmune encephalomyelitis (EAE) [14]. Consequently, lncRNAs likely play an important role in macrophage polarization.

The aim of this study was to explore the role of the lncRNA AK085865 in the pathogenesis of AA. lncRNA AK085865 was highly expressed in asthmatic mice and promoted the polarization of macrophages to M2. Furthermore, mice deficient in AK085865 were protected from Der f1-induced allergic airway inflammation as manifested by attenuated airway inflammation, indicated that AK085865 plays an important role in asthma. Importantly, these data provide a new theoretical and experimental basis to understand the pathogenesis of AA and advance its biological control.

2. Materials and methods

2.1. Mice

C57BL/6 (H-2d) female mice (aged 6–8 weeks, 20–25 g) were purchased from the Experimental Animal Center of Qinglongshan (Nanjing, P. R. China) and housed in pathogen-free mouse colonies. lncRNA AK085865 knockout (KO) mice on a C57BL/6 background were generated by the Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China), and were housed in pathogen-free mouse colonies. The CRISPR/Cas9 technique was used to edit AK085865. In brief, the *in vitro*-synthesized gRNA and Cas9 mRNA were injected into zygotes of C57BL/6 mice via the microinjection technique to generate F0 generation mice. Heterozygous F1 mice were obtained by backcrossing F0 generation mice with C57BL/6 mice. Production of AK085865 KO (AK085865^{-/-}) mice by hybridization of heterozygous F1 mice. All animal experiments were performed according to the guidelines for the Care and Use of Laboratory Animals (Ministry of Health, PR China, 1998) and the guidelines of the Laboratory Animal Ethical Commission of Wannan Medical College. All experimental protocols were evaluated and approved by the Animal Ethical Committee of Wannan Medical College (Permit Number. 20130138).

2.2. Induction of allergic asthma

To induce allergic asthma, WT and AK085865^{-/-} mice were intraperitoneally sensitized with 100 μ g Der f1 extract that were absorbed onto 2 mg alum (Inject Alum; Thermo Scientific) at days 0, 7 and 14 of the experiment. Mice injected with an equal volume of PBS served as controls. Sensitized mice were then challenged with a Der f1 aerosol (50 μ g Der f1 extract delivered in 10 mL PBS) or PBS delivered via nebulizer for 15 min every day between days 21–28. Mice were sacrificed 24 h after the last challenges.

2.3. Measurements of total IgE and Der f1-specific IgG

Sera were collected from mice and total IgE was quantified using an ELISA kit (R&D Systems). Der f1-specific IgG was measured as previously described [15]. Briefly, 96 well plates were coated with a 5 mg/mL solution of Der f1 and incubated with diluted sera. Samples were then incubated with alkaline phosphatase-conjugated anti-mouse IgG1/IgG2a (BD Pharmingen), followed by addition of the p-nitrophenyl phosphate substrate (Sigma-Aldrich) and absorbances were measured at 450 nm.

2.4. Measurement of airway AHR

Mice were anesthetized with pentobarbital (Sigma-Aldrich) at a dosage of 25 mg/kg body weight. Lung function was determined in tracheotomized and mechanically ventilated mice via direct measurement of airway resistance in response to increasing methacholine (Sigma-Aldrich) doses using the FinePointe RC system (Buxco Research Systems).

2.5. Lung histology and airway mucus expression

Asthmatic mice in each group were perfused via their hearts with ice-cold PBS and paraformaldehyde while under deep anesthesia. The lungs were subsequently removed and placed in fresh 4% neutral-buffered paraformaldehyde for 24 h at room temperature. After embedding the tissues in paraffin, they were sliced into 5 μ m sections and subjected to haematoxylin and eosin (H&E) staining in addition to periodic acid-Schiff (PAS) staining. Blind measurements of inflammatory infiltration of the lungs was evaluated microscopically (Eclipse 80i; Nikon, Tokyo, Japan) by two examiners using the following scoring criteria [16]: 0, normal; 1, a few cells; 2, a ring of inflammatory cells one cell layer deep; 3, a ring of inflammatory cells two to four cells deep; 4, a ring of inflammatory cells more than four cells deep. Airway mucus secretion was scored on a scale of 0–4, with 0 being negative and 1–4 positive for PAS-staining bronchi. The extent of mucus production in positive bronchi was scored as follows [17]: 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, > 75%.

2.6. Preparation of bronchoalveolar lavage fluid (BALF)

BALF was collected by cannulating tracheas and lavaging lungs with 0.8 mL of sterile PBS three times using previously described methods [18]. Approximately 1.5 mL of BALF was recovered from each mouse. BALF was centrifuged at 1200 rpm for 5 min, and the supernatants were stored at -80°C prior to cytokine measurements. Total cell suspensions were resuspended in PBS for further analysis.

2.7. Cytokine evaluation via ELISA

The levels of cytokines including IFN- γ , IL-4, IL-5, IL-13, IL-10, and IL-17A, were quantified in homogenized lung tissues and BALF using cytokine ELISA kits (MultiSciences (Lianke) Biotech Company, Ltd, Hangzhou) according to the manufacturer's instructions.

2.8. Flow cytometry analysis

Bronchoalveolar lavages were performed with three instillations of 0.8 mL PBS. red blood cells were lysed with ACK lysis buffer for 5 min at RT. Whole lungs were then minced and incubated in Dulbecco modified Eagle medium with 0.1% DNase I (Worthington Biochemicals, Lakewood, NJ) and 1.6 mg/mL of collagenase IV (Worthington Biochemicals) for 40 min at 37°C . Cells collected from BALF and lungs were incubated with the FcR Blocking Reagent (Miltenyi Biotec, Germany) followed by incubation with different antibodies. Analysis of intracellular cytokines was conducted with the eBioscience™

Intracellular Fixation & Permeabilization Buffer Set reagents (Thermo Fisher) after pre-culture for 5 h at 37 °C with Roswell Park Memorial Institute (RPMI) 1640 media that was supplemented with PMA/ionomycin and BFA/monensin mixture (Multi Sciences, Hangzhou, China). The cells were then harvested after blocking with FcR for 20 min at 4 °C, and incubated with different antibodies. Cells were analyzed on a Beckman FC500 flow cytometer and data were analyzed using the FlowJo software package.

2.9. Quantitative PCR

Total RNA was extracted from cells using the TRIzol reagent (Invitrogen) and the extracted RNA was used to synthesize cDNA using a first strand cDNA synthesis kit (Applied Biosystems). qPCR was then performed with the cDNA as template on a Lightcycler 480 system (Roche), using the primers shown in Table S1. The resulting cDNA was further amplified using a universal reverse primer and different specific forward primers with the following PCR procedure: pre-denaturation at 95 °C for 2 min, followed by 40 cycles of 94 °C for 10 s, 58 °C for 15 s, and 72 °C for 20 s, followed by a melting curve analysis. All reactions were conducted in triplicate. The mRNA expression levels were normalized to GAPDH, respectively, and calculated using the $2^{-\Delta\Delta Ct}$ method.

2.10. Adoptive transfer of bone marrow-derived macrophages

To evaluate the adoptive transfer of macrophages, we first prepared mouse bone marrow-derived macrophages (BMDM). Femoral and tibial bone marrow were isolated from WT mice. Mouse bone marrow cells from femurs and tibias were flushed with Mouse Fibroblast Line (L929) conditioned medium containing 20% of the L929 cell culture supernatant after three days of culture, 20% FBS, 1% penicillin/streptomycin, and 2 mM glutamine. The cells were then cultured in a six well plate. After seven days, non-adherent cells were removed and cultured in complete RPMI 1640 medium containing 10% calf serum, 1% penicillin/streptomycin, and 2 mM glutamine. After a day, the adherent cells represented bone marrow-derived macrophages (M0).

BMDM (M0, 5×10^5 cells/mouse) were then intratracheally (i.t.) injected into the Der f1 sensitized WT and AK085865^{-/-} mice one day before the atomization challenge at day 20. Recipient mice were sacrificed on days 29 and analyzed for allergic asthma inflammation. The protocol schematic for the adoptive transfer of macrophages is shown in Fig. 3E.

2.11. ILCP sorting

ILCP cells were defined as lineage⁻ $\alpha 4\beta 7^+$ IL-7R⁺ cells, with lineage negative cells contained within lineage cocktail antibodies (anti-CD3e, anti-CD19, anti-CD11c, anti-CD11b, anti-F4/80, anti-Fc ϵ RI α , and anti-CD49b). To achieve ILCP sorting, lineage negative cells were first sorted with a lineage cell depletion kit (Miltenyi Biotec Inc., CA, USA) from femoral and tibia bone marrow, following the manufacturer's protocol. Sorted lineage negative cells were then stained with PE labeled anti mouse $\alpha 4\beta 7^+$ and APC labeled anti mouse IL-7R (Thermo Fisher, USA) for 30 min at 4 °C. The labeled cells were finally sorted on a Beckman MoFlo XDP instrument.

2.12. Coculture of ILCP and macrophages in vitro

OP9-DL1 cells were purchased from the Zeye Biotechnology Company (Shanghai). The cells were maintained in complete MEM- α supplemented with 20% FBS, 1% penicillin/streptomycin, 0.1% 2-mercaptoethanol and non-essential amino acids (GIBCO) with at least 6 h of incubation to allow adherence. 1×10^4 Mitomycin-C (200–300 μ L/well at a 50 μ g/mL final concentration) was added to each well and then incubated for 25 min at 37 °C with a 5% CO₂ atmosphere. Then,

1 mL media was used to wash the Mitomycin-C out three times. The OP9-DL1 feeder layer was then ready to plate-sort ILCP.

Sorted ILCP populations (2×10^4 cells/well) were seeded onto OP9-DL1 monolayers and cultured for 18 days in complete HDMEM containing 10% FCS and 1% penicillin/streptomycin that was supplemented with 25 ng/mL rmIL-7 (Biolegend) in addition to 25 ng/mL rmSCF (Biolegend). Flow cytometric analysis was then used to sort progeny cells. After 24 h, M2 macrophages (5×10^4 cells/well) were added to the upper chamber of the transwell and cultured for 18 days, with exchange of fresh polarized macrophages and OP9-DL1 cells every three days. After 18 days, cells in the lower chamber of the transwell were harvested for flow cytometric analysis.

2.13. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). A two-tailed unpaired Student's t-test was used to determine differences across groups. All data were presented as the mean $\pm$ SEM. A P-value level of < 0.05 was considered significant.

3. Results

3.1. Asthmatic mice exhibit aberrant lncRNA AK085865 expression

We previously screened lncRNA AK085865 expression with microarray analysis and confirmed that it regulates the polarization of macrophages to M2. Because macrophages are key mediators of asthma pathogenesis, we first evaluated AK085865 expression in the BAL cells and lung homogenates of mice with allergic asthma that was induced by Der f1 in female C57BL/6 mice. The workflow for the induction of asthma is shown in Fig. 1A and resulted in the construction of a successful model (Fig. 1B). AK085865 was expressed at low levels in the BAL cells and lung homogenates from PBS-induced mice, whereas high AK085865 expression was observed in asthmatic mice (Fig. 1C). Collectively, these data suggest that lncRNA AK085865 is involved in the development of allergic asthma.

3.2. AK085865^{-/-} mice show impaired development of Der f1-induced allergy airway inflammation

We next evaluated whether AK085865 plays a role in mediating Der f1 allergen-induced allergic inflammation. When compared against PBS-challenged mice, asthma pathologies developed in Der f1-challenged WT and AK085865^{-/-} mice, as indicated by histological results showing a significant recruitment of inflammatory cells to lungs with dense peribronchial infiltrates and goblet cell hyperplasia (Fig. 2A and B). Der f1-treated AK085865^{-/-} mice displayed alleviated peribronchial inflammation and goblet cell hyperplasia compared to WT mice. In response to the Der f1 challenge, the total IgE in the plasma of WT mice increased to 914.37 ± 95.8 ng/mL, while that of AK085865^{-/-} mice was much lower at 508.46 ± 53.25 ng/mL (Fig. 2C). At the same time, the level of Der f1-specific IgG1 was also reduced, but IgG2a level was unaltered (Fig. 2D).

To further evaluate the effects of AK085865 deficiency on asthma pathogenesis, we investigated AHR by measuring the lung resistance (Rn) response to increasing concentrations of acetylcholine in mechanically ventilated mice. As expected, Der f1-challenged WT mice developed AHR, as demonstrated by increased Rn values induced by acetylcholine compared to PBS-treated control mice. Notably, Der f1-challenged KO mice exhibited a marked reduction in Rn values in response to acetylcholine compared to WT mice (Fig. 2E).

Furthermore, the abundances of eosinophils (CD45⁺ Siglec F⁺ CD11c⁻) and alveolar macrophages (CD45⁺ Siglec F⁺ CD11c⁺) in Der f1-treated AK085865^{-/-} mice were reduced in BALF compared to that of WT mice (Fig. 2F). Interestingly, the abundances of neutrophils did

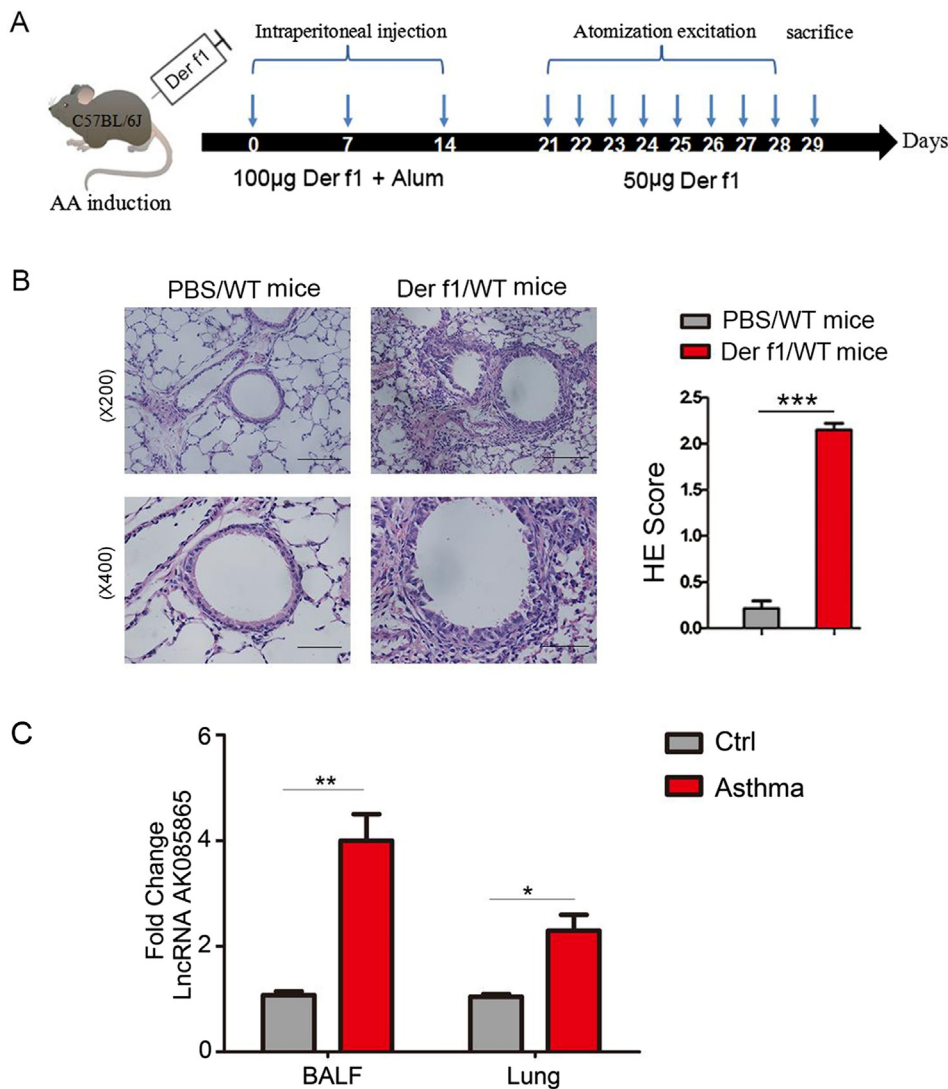


Fig. 1. Asthmatic mice exhibit aberrant lncRNA AK085865 expression. (A) The schematic timeline shows that mice were sensitized with Der f1 on days 0, 7 and 14 and challenged with Der f1 from days 21 to days 28. On days 29, mice were sacrificed for analysis. (B) Histopathology was performed based on H&E staining to determine the asthmatic inflammation in PBS- or Der f1-treated WT mice (original magnification 400 $\times$, scale bar: 200 μ m). (C) AK085865 expression was detected in the BALF and lung. The mRNA expression levels were normalized to GAPDH, respectively, and calculated using the $2^{-\Delta\Delta C_t}$ method. Results represent mean $\pm$ SEM. n = 5 mice for each group. *P, < 0.05, ***P, < 0.001.

not significantly differ between them. Reduced alveolar macrophage abundances were also observed in the lungs of AK085865^{-/-} mice. Although eosinophils and neutrophils were also observed in lungs, their numbers did not differ between WT and KO mice in response to Der f1 challenges (Fig. 2G).

Proinflammatory cytokines (IL-4, IL-5, IL-13, IFN- γ and IL-17A) and inhibitory cytokine (IL-10) were also quantified in the supernatant of BALF and lung homogenates. The concentrations of IL-4, IL-5, IL-13 and IFN- γ decreased by 19%, 33%, 20%, and 40%, respectively, in the BALF supernatants from KO mice relative to those of WT mice (Fig. 2H). In contrast, IL-10 concentrations increased in KO mice, while the expression level of IL-17A did not change significantly. Concomitantly, the levels of IL-4, IL-13, IFN- γ , and IL-17A of AK085865^{-/-} were lower in the lung homogenates compared to WT mice (Fig. 2I). Notably, AK085865 was knocked out without changing the expression levels of IL-5 and IL-10 in the lungs. Collectively, these data suggest that the deletion of AK085865 can protect against Der f1 allergen-triggered airway inflammation.

3.3. AK085865^{-/-} mice display impaired polarization of M2 macrophages during allergic airway inflammation

Given the key role of macrophages in mediating the pathogenesis of asthma, we investigated the macrophages of AK085865^{-/-} mice in asthma development. Flow cytometry revealed a significantly lower

number of M2 macrophages in the BALF of Der f1-challenged AK085865^{-/-} mice, as manifested by CD206 expression ($12.25 \pm 0.71\%$ versus $20.56 \pm 0.80\%$, $p < 0.01$; Fig. 3A). Similar observations were made in the lung tissues, with decreased frequencies of M2 macrophages in KO mice by 36% compared with WT mice ($4.60 \pm 0.68\%$ versus $7.26 \pm 0.93\%$, $p < 0.01$; Fig. 3B). Notably, M1 macrophages were lower in the BALF of AK085865^{-/-} mice, but no differences were observed in lungs.

Real-Time PCR analysis of BALF samples from Der f1-challenged KO mice revealed a 0.4-fold decrease in the expression of the M2 marker, Arginase-1, when compared with WT mice (Fig. 3C). Similar results were observed for one additional M2 markers, FIZZ-1, although differences in YM-1 expression were not significant between WT and KO mice. AK085865 deficiency also affected the production of the M1 marker, as evinced by differences in iNOS levels among treatment groups. The expression of the two M2 markers, Arg-1 and Fizz-1, and the M1 marker, TNF- α , were lower in the lungs of KO mice compared to those of WT mice (Fig. 3D). Together, these data suggest that the loss of AK085865 confers protection in mice from Der f1-induced allergic airway inflammation by inhibiting the polarization of M2 macrophages in airways.

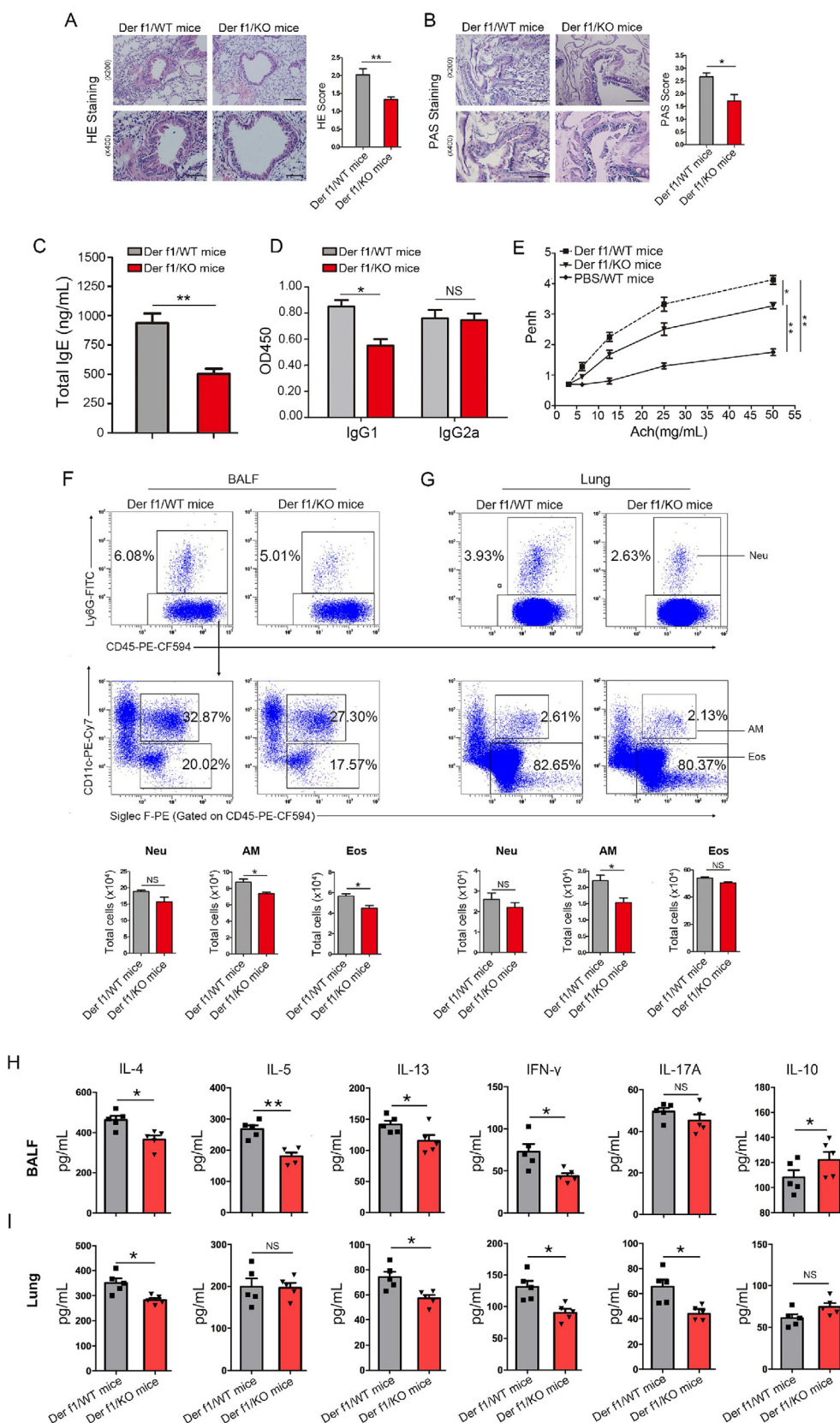
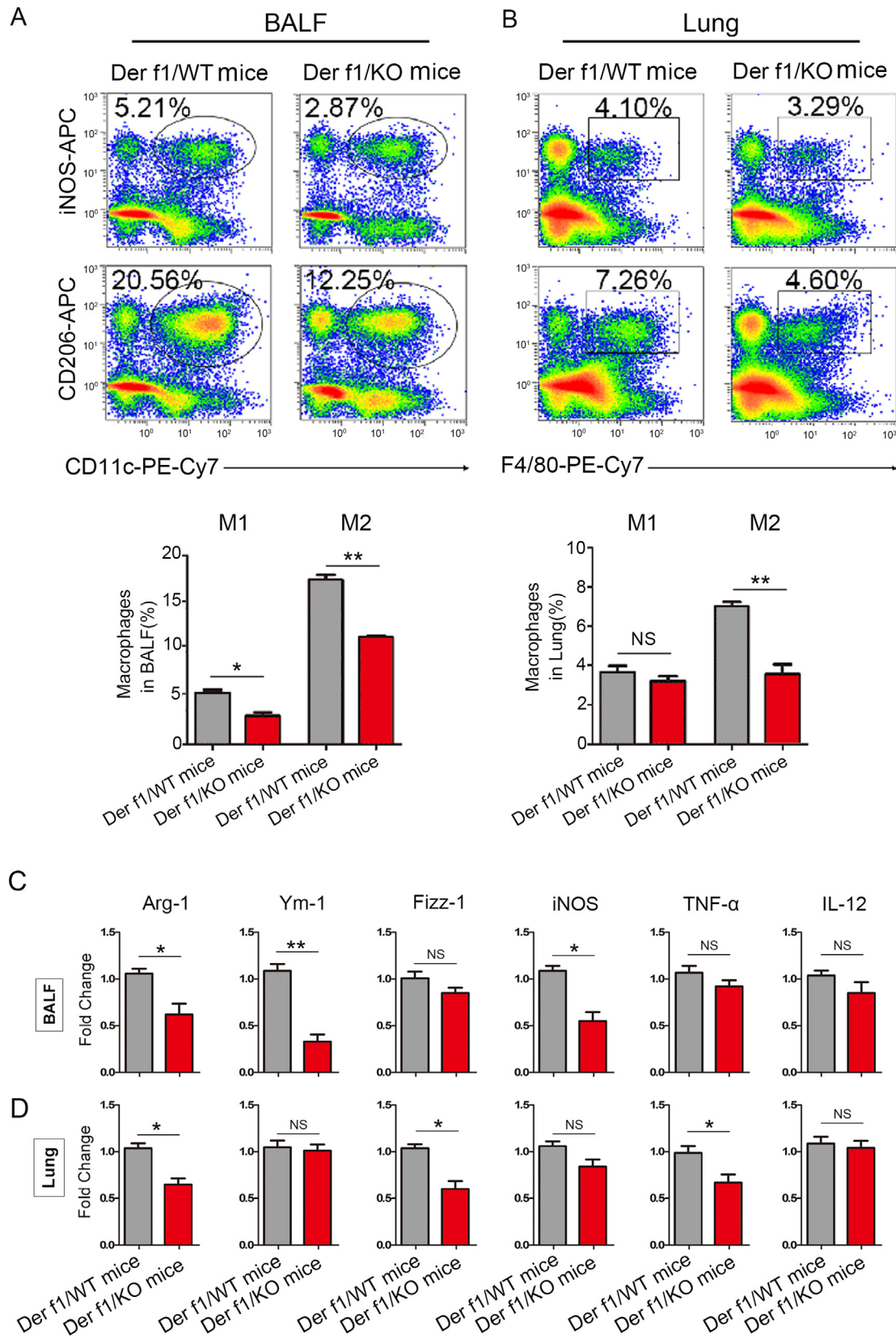


Fig. 2. AK085865^{-/-} mice show an impaired development of Der f1-induced allergic airway inflammation. (A and B) Histopathology was performed based on H&E and PAS staining to determine the asthmatic inflammation in Der f1-treated WT or KO mice (original magnification 400 $\times$, scale bar: 200 μ m). (C) Total IgE level in mouse plasma was detected by ELISA. (D) Allergen-specific IgGs were detected by ELISA. (E) Lung resistance in response to increasing doses of methacholine. (F) Cell distribution in BALF of Der f1-challenged mice. Gating strategy for BALF neutrophils (Neu: CD45⁺ Ly6G⁺), eosinophils (Eos: CD45⁺ Ly6G⁻ Siglec-F⁺ CD11c⁻), alveolar macrophages (AM: CD45⁺ Ly6G⁻ Siglec-F⁺ CD11c⁺), and DC cells (DC: CD45⁺ Ly6G⁻ Siglec-F⁻ CD11c⁺). (G) Cell distribution in Lung of Der f1-challenged mice. (H) Quantification of IFN- γ , IL-4, IL-5, IL-13, IL-17A and IL-10 by ELISA in BALF. (I) Quantification of IFN- γ , IL-4, IL-5, IL-13, IL-17A and IL-10 by ELISA in lung. Results represent mean $\pm$ SEM. n = 5 mice for each group. *, p < 0.05; **, p < 0.01, NS, no significance.

3.4. LncRNA AK085865 deletion in macrophages is the major cause of decreased susceptibility of AA induced by Der f1

To investigate the critical role of AK085865 deletion in macrophages in the development of AA, we compared disease severity in Der

f1-challenged WT or KO mice after the adoptive transfer of bone marrow-derived macrophages (BMDM) of WT mice. The workflow to conduct the adoptive transfer of WT M0 is shown in Fig. 3E. M0 macrophages (5×10^5 cells/mouse) were intratracheally (i.t.) injected into Der f1 sensitized mice one day before the atomization challenge on days



(caption on next page)

20. The recipient mice were sacrificed on days 29 and examined for allergic asthma inflammation. As expected, AK085865^{-/-} mice adoptively transferred with WT M0 macrophages had less severe inflammatory responses after Der f1 challenge compared with WT mice

adoptively transferred with WT M0 macrophages, as determined by using histologic analysis of lung sections, numbers of inflammatory cells and cytokines in BALF and lung (Fig. 4). Collectively, our data support that AK085865 deletion in macrophages is the major cause of

Fig. 3. LncRNA AK085865 deficiency protects mice from developing asthma by attenuating M2 programming in macrophages. (A) Flow cytometry analysis of CD206 and iNOS in macrophages following Der f1 challenges in BALF. (B) Flow cytometry analysis of CD206 and iNOS in macrophages following Der f1 challenges in lung homogenates. (C) Total RNAs were isolated from BALF, and expression levels for M1 related genes and M2 related genes were quantified using real-time PCR. The mRNA expression levels were normalized to GAPDH, respectively, and calculated using the $2^{-\Delta\Delta Ct}$ method. (D) Total RNAs were isolated from lung homogenates, and expression levels for M1 related genes and M2 related genes were quantified using real-time PCR. The mRNA expression levels were normalized to GAPDH, respectively, and calculated using the $2^{-\Delta\Delta Ct}$ method. (E) The protocol of adoptive transfer of bone marrow-derived macrophages induced peritoneal macrophages into KO mice. Donor bone marrow-derived macrophages from WT mice were intratracheally (i.t.) instilled into KO mice on days 20 after asthma was induced. Then the mice received transferred macrophages were intranasally (i.n.) challenged with Der f1 from days 21 to 28. The allergic inflammation was detected on days 29. (F) Histopathology was performed based on H&E staining to determine the asthmatic inflammation in Der f1-treated KO mice (original magnification 400 $\times$, scale bar: 200 μ m). Data are shown as means $\pm$ SEM of 2 independent experiments. n = 5–6 mice for each group. *P < 0.05, **P < 0.01, and ***P < 0.0001, NS, no significance.

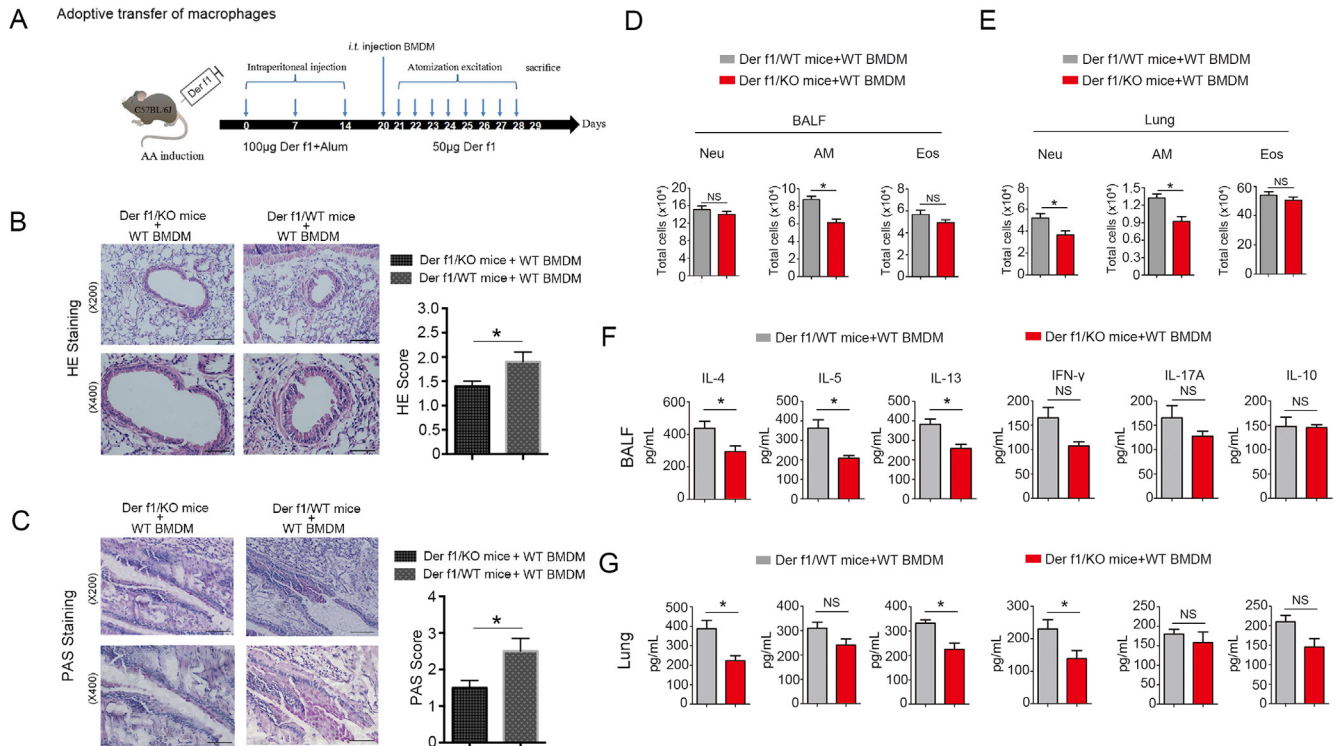


Fig. 4. LncRNA AK085865 is a key factor in the development of AA. (A) The protocol of adoptive transfer of bone marrow-derived macrophages induced peritoneal macrophages into KO mice. Donor bone marrow-derived macrophages from WT mice were intratracheally (i.t.) instilled into KO mice on days 20 after asthma was induced. Then the mice received transferred macrophages were intranasally (i.n.) challenged with Der f1 from days 21 to 28. The allergic inflammation was detected on days 29. (B) Histopathology was performed based on H&E staining to determine the asthmatic inflammation in Der f1-treated KO mice (original magnification 400 $\times$, scale bar: 200 μ m). (C) Histopathology was performed based on PAS staining to determine the asthmatic inflammation in Der f1-treated KO mice (original magnification 400 $\times$, scale bar: 200 μ m). (D) Cell distribution in BALF of Der f1-challenged mice. (E) Cell distribution in Lung of Der f1-challenged mice. (F) Quantification of IFN- γ , IL-4, IL-5, IL-13, IL-17A and IL-10 by ELISA in BALF. (G) Quantification of IFN- γ , IL-4, IL-5, IL-13, IL-17A and IL-10 by ELISA in Lung. Data are shown as means $\pm$ SEM of 2 independent experiments. n = 5–6 mice for each group. *P < 0.05, NS, no significance.

decreased susceptibility of AA induced by Der f1, which depends on the attenuation of M2 polarization in macrophages.

3.5. Reduction of M2 macrophages leads to a decrease in the number of ILC2s in AK085865^{-/-} mice

Studies have reported a relationship between macrophages and ILCs [19]. Therefore, the population distribution and composition of ILCs in the BALF and lungs were evaluated by flow cytometry. Since a variety of cellular molecules are expressed on the surface of ILC2s (ST2, Gata3, CD90, CD25, CD117, Sca-1, ICOS, and KLRG1), we measured their expression levels in AA. Increased percentages of CD90, CD117, ST2 and Gata3 were observed in the BALF from asthmatic mice when compared with healthy controls. In contrast, the expression levels of Sca-1, KLRG1, ICOS, and CD25 were all very low in the two groups of mice (Fig. 5A and B). Similar results were observed in lung tissues, wherein the expression of CD90 of the ILC2s populations in lung tissue was lower than in healthy controls, while KLRG1 expression was higher (Fig. 5C). Consequently, expression of the CD45⁺ lineage⁻ CD117⁺

ST2⁺ was used to define ILC2s based on the above results and previously published reports.

The percentage of ILC2s expression was significantly lower in KO mice when compared with WT mice (Fig. 5D and E). It is worth noting that the percentage of ILC1s and ILC3s expression also increased in WT mice, but their levels were much lower than those of ILC2s (Supplementary figure 1). These results suggest that ILC2s is the main cell type involved in the Der f1-induced asthma model. Concomitantly, the levels of ILC1s and ILC3s expression were lower in KO mice. We then examined the secretion of Th2 cytokines by ILC2s in BALF and lungs. IL-5 and IL-13 production were markedly lower (Fig. 5F) in the BALF of KO mice. However, only the expression of IL-5 was lower in the lungs of KO mice (Fig. 5G). These data suggest that macrophage abundances might be associated with crosstalk between them and other immune cells, and especially ILCs. Specifically, a decrease in the number of M2 macrophages may result in a weakened immune response of ILC2, thereby reducing the development of asthmatic diseases.

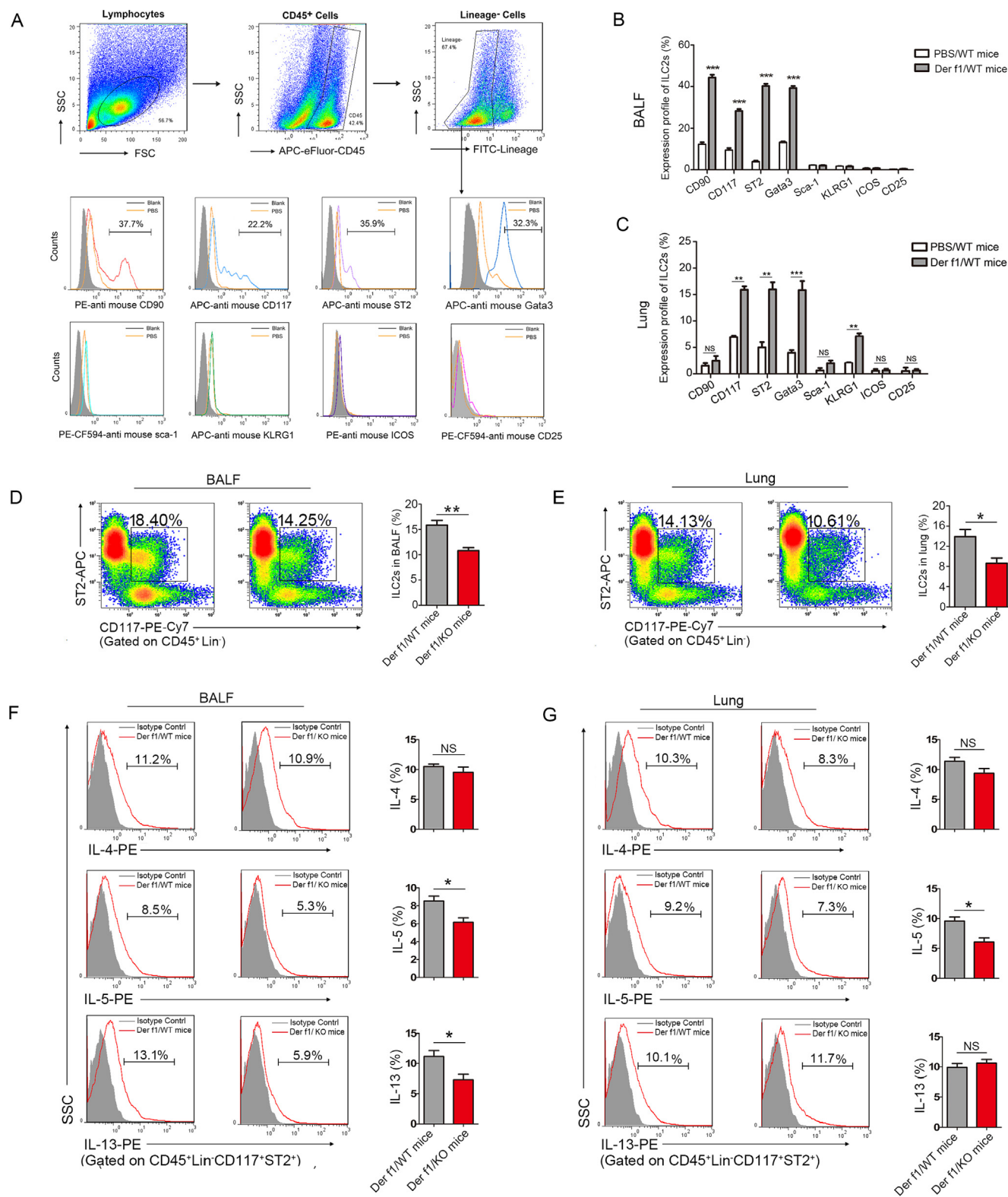


Fig. 5. Different subset of ILCs in BALF and lung. (A-B) Expression of profiles of ILC2s in the BALF of WT Der f1-induced mice. (C) Expression of profiles of ILC2s in the lung of WT Der f1-induced mice. (D) ILC2s in BALF; (E) ILC2s in lung; (F) Th2 cytokine of ILC2s in the BALF. (G) Th2 cytokine of ILC2s in the lung. Data are shown as means $\pm$ SEM of 2 independent experiments. n = 10 mice for each group. *P < 0.05, **P < 0.01, NS, no significance.

3.6. M2 Macrophages may help promote the differentiation of ILCP cells into ILC2 by secreting exosomes

Based on the above results, we supposed that specific types of macrophages affect the differentiation of ILCs. To test this, we first

compared the ability of M2 macrophages from WT and KO mice to promote the differentiation of ILP into ILC2s *in vitro*. As shown in the [Supplementary figure 2](#), there is no significant difference between them, indicating that they all can promote ILCP into ILC2s, which means that it is not the role of AK085865.

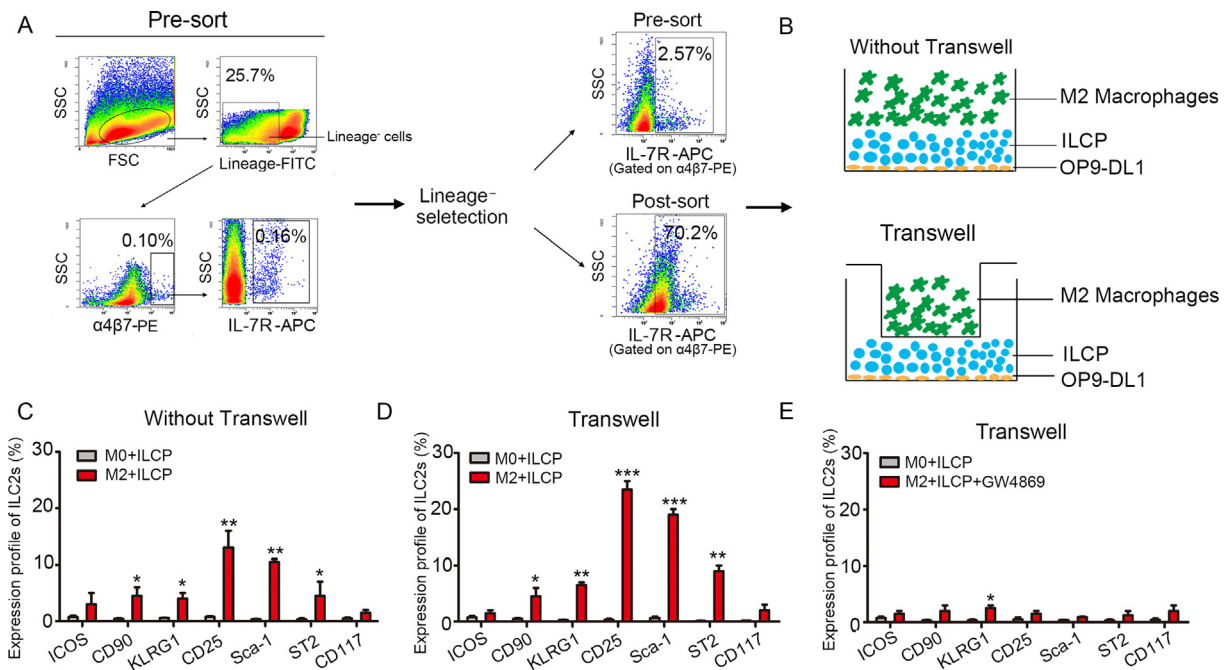


Fig. 6. Macrophage-ILCP interaction affects differentiation of ILCP. (A) Flow cytometry for sorting ILCP. (B) Schematic diagram of the co-culture procedure using the transwell/without transwell system. (C) After 18 days of co-culture without transwell, the level of change in ILC expression profile was detected by flow cytometry. (D) M2 macrophages (5×10^4 cells/well) were added to the upper chamber of the transwell and cultured for 18 days, with exchange of fresh polarized macrophages and OP9-DL1 cells every three days. After 18 days, cells in the lower chamber of the transwell were harvested for flow cytometric analysis. (E) Exosome inhibitor GW4869 was added in the co-culture system, the level of change in ILC2s expression profile was detected by flow cytometry. Data are shown as means $\pm$ SEM of 2 independent experiments. Three replicate wells for each coculture group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

We further investigated the relationship between ILCs and macrophages, as the changes in these two populations were most pronounced in patients with asthma. The expression of ILC2s-related profiles like CD25, Sca-1, and ST2 increased when M2 macrophages were co-cultured with ILCP (Fig. 6C). These results clearly indicated that related genes from macrophages probably induce ILCP differentiation. Then the transwell system was used to study whether soluble cytokines in macrophages influence ILCP differentiation. M2 macrophages were cultured in the upper chamber, while ILCP were added to the lower chamber. The lower chamber cells were collected after 18 days to analyze the expression level of ILC2s. The results were consistent with the co-culture results (Fig. 6D). Interestingly, ILC2 differentiation was inhibited after the addition of the exosome inhibitor GW4869 (Fig. 6E). Thus, it is possible that exosomes secreted by M2 macrophages could promote ILCP differentiation into ILC2s.

4. Discussion

It is increasingly clear that lncRNA regulate a variety of biological responses. Indeed, immunology studies have confirmed that lncRNAs play important roles in macrophage activation and differentiation [20–22], but little is known about the role of lncRNAs in AA. We previously analyzed the expression profile of lncRNAs in M1 and M2 macrophages (BMDM) with microarray assays. These analyses indicated that lncRNA AK085865 expression was most significantly associated with M2 macrophages, which are up-regulated during M1 to M2 polarization, but down-regulated during M2 to M1 polarization. Similarly, we have confirmed that AK085865 was highly expressed in airway of AA. Therefore, AK085865 was defined as a modulator of macrophage polarization during Der f1-induced AA.

Macrophages are key orchestrators of AA pathogenesis and promoters of inflammatory responses associated with lung injury, fibrosis, and goblet cell hyperplasia. Airway macrophage from asthmatic patients are exposed to type-2-associated cytokine micro-environments, including the presence of IL-4, IL-13, and IL-33, which further drives their

differentiation toward M2 macrophages and is implicated in the pathogenesis of asthma [23,24]. Research by our group confirmed that deleting AK085865 did reduce the percentage of M2 macrophages, accompanied by a decrease in the expression of M2 macrophage-related genes, such as Arg-1. In addition, adoptive infusion of M0 macrophages from WT mice to AA, Der f1 treated WT mice show more severe airway inflammation than KO mice. This shows that AK085865 deletion is a key factor for the protective effect of AA. These observations are consistent with previous reports and suggest that there is therapeutic potential in the regulation of macrophages in allergic inflammatory diseases via molecular intervention.

Depletion of lncRNA AK085865 reduced M2 macrophages in AA, with a concomitant decrease in the percentage of ILC2s in Der f1-treated AK085865^{-/-} mice, which attracted our attention. The number of ILC1s and ILC3s cells also decreased, although to a lesser extent than ILC2s cells. These results indicate that the regulation of M2 macrophages polarization may affect the proportions of ILC2s. Looking at the previous studies, we found that most of them are studying a single cell population, such as macrophages, eosinophils and CD4⁺ Th2 cells. Of course, there are many papers to investigate the relationship between macrophages and CD4 T cells, while the interactions between ILCs and macrophages have not been fully clarified. In our study, we found that M2 macrophages can promote the differentiation of ILC2s. What's more interesting is that in the transwell experiment, we added an exosome inhibitor, and the differentiation of ILC2s was affected to some extent, which may mean that the secretion of exosomes by M2 macrophages can promote the differentiation of ILCP into ILC2s. We will further explore the relationship between macrophage-derived exosomes and ILC2s.

5. Conclusion

In conclusion, these findings reveal that AK085865 depletion ameliorates asthmatic airway inflammation by modulating macrophage polarization. These data provide a new theoretical and experimental

basis for understanding the pathogenesis of allergic asthma and advancing the biological control of its treatment. Further, we also observed a relationship between macrophages and ILCs. Nevertheless, additional experiments are needed to fully evaluate the relationships between ILCs, macrophages, and other immune cells in airway systems.

6. Data availability

The Gene Expression Omnibus (GEO) database accession number for the lncRNA profile data of M1 and M2 macrophages reported in this paper is GSE125510.

CRediT authorship contribution statement

Weiya Pei: Writing - review & editing, Methodology, Formal analysis, Project administration. **Yingying Zhang:** Funding acquisition, Methodology, Resources, Supervision. **Xueqin Li:** Project administration, Methodology, Formal analysis. **Mengsha Luo:** Data curation, Validation. **Tianbing Chen:** Software. **Mengying Zhang:** Investigation, Validation. **Min Zhong:** Software. **Kun Lv:** Funding acquisition, Methodology, Resources, Supervision.

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Declaration of Competing Interest

The authors declare no conflicts of interest in this work.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.intimp.2020.106450>.

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