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E3 ligase RNF128 restricts *A. alternata*-induced ILC2 activation and type 2 immune response in the murine lung

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Allergic airway inflammation is a universal airway disease induced by inhaling allergens. Published data show that RNF128, an E3 ligase, promotes Th2 activation in the OVA-induced asthma model. Recent advances have shown that group 2 innate lymphoid cells (ILC2s) produce the cytokines IL-5 and IL-13 to mediate type 2 immune response. However, whether RNF128 regulates ILC2-dependent allergic lung inflammation remains unclear. In this study, we observed greater expression of the E3 ligase RNF128 in ILC2s than in other immune cells. RNF128 deficiency caused a selective increase in the number of peripheral mature ILC2s, and mice with RNF128 deficiency were more susceptible to *Alternaria alternata (A. alternata)* -induced allergic lung inflammation. Furthermore, RNF128 deficiency increased recruitment of eosinophils and levels of IL-5 and IL-13 in the bronchoalveolar lavage fluid. RNF128 effectively inhibited the expansion of ILC2s and the number of IL-5- and IL-13-producing ILC2s. Specially, RNF128 deficiency promoted the expression of the interleukin-33 (IL-33) receptor ST2 in *A. alternata*-induced allergic lung inflammation. Above all, our study demonstrated that RNF128 played a key role in *A. alternata*-induced ILC2 activation and type 2 immune response, suggesting that RNF128 may be an effective therapeutic target for allergic lung inflammation initiated by ILC2s.

Keywords E3 ligase RNF128, Group 2 innate lymphoid cells, Allergic lung inflammation

Allergic lung inflammation (such as the inflammatory disorder known as asthma) is one of the most common respiratory diseases worldwide and mainly involves mucus production and the infiltration of lymphocytes, especially eosinophils, into the lungs after allergen stimulation[1]. During the last two to three decades, the incidence of allergic lung inflammation has been steadily increased[2]. In general, most of cases can be controlled with current therapeutic regimens; however, many cases are still poorly controlled[3,4]. Therefore, an understanding of the cellular and molecular mechanisms of pathogenesis could provide new therapies for this disease.

Cytokines such as IL-5 and IL-13 mediate the type 2 immune response which is involved in many allergic diseases such as allergic lung inflammation. Group 2 innate lymphoid cells (ILC2s) were defined in 2010 as a potent source of type 2 cytokines during the type 2 immune response[5–7]. Previous studies have suggested that T helper type 2 (Th2) cells are the primary source of type 2 cytokines, whereas more recent studies have shown that ILC2s can also produce type 2 cytokines such as IL-13 to induce allergic inflammation, even in the absence of Th2 adaptive immunity[8,9]. When epithelial cells are damaged or stressed, they produce alarmin cytokines[9–11], which activate ILC2s to rapidly produce high levels of IL-5 and IL-13, thus initiating allergic tissue inflammation at mucosal surfaces[5,6,12]. The cytokine IL-33 is the most potent factor for ILC2 activation. IL-33 binding to the IL-33 receptor (ST2) results in downstream signaling pathway activation and promotes the production of the cytokines IL-5 and IL-13[13]. IL-5 mainly recruits eosinophils, whereas IL-13 mediates goblet cell secretion of mucus. In addition to IL-33, IL-25 and TSLP are also involved in the activation of ILC2s[14]. In addition, our previous study has revealed that neurotransmitters[15] can regulate lung ILC2 function. ILC2s also receive signals from metabolites[16–18]. Although considerable advances have been made in defining different cell signals,

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including endogenous molecules and exogenous substances that trigger ILC2 responses, the mechanisms that control ILC2 activation and type 2 inflammation remain poorly understood.

Research has shown that the E3 ubiquitin ligase RNF128, which is also known as Grail, is localized in the endosomal compartment and plays important roles in controlling immune cell activation, proliferation and infiltration[19–22]. Most of these studies have focused on the function of RNF128 in T helper cells. Th2 cells are types of T helper cells. A previous study has suggested that RNF128 is a crucial regulator of Th2 activation and that RNF128 interacts with STAT6 and promotes STAT6 ubiquitination and degradation, thus inhibiting the downstream expression of GATA3 and cytokines[19]. ILC2s and Th2 cells share common features, such as the production of IL-5 and IL-13; moreover, they are predominantly regulated by the master transcription factor GATA3[23]. However, the function of RNF128 in ILC2s during the progression of allergic lung inflammation has not been elucidated. This study aimed to investigate the role of RNF128 in ILC2 responses and allergic lung inflammation via the use of RNF128 knockout (KO) mice.

Herein, we were the first to discover that RNF128 was more highly expressed in lung ILC2s than in other immune cells. RNF128 deficiency aggravated allergic lung inflammation and promoted the mature ILC2 development and function. In addition, RNF128 deficiency promoted ST2 expression on lung ILC2s after *A. alternata* challenge. Overall, our study revealed that RNF128 is essential for lung ILC2 responses and offers a possible strategy for controlling allergic diseases.

Results

Loss of RNF128 promotes mature ILC2 development

We first assessed the expression of RNF128 in lung ILC2s and other cells via quantitative real-time PCR and found that RNF128 was more highly expressed in ILC2s than in other cell populations (Fig. 1A), thus indicating that RNF128 may regulate ILC2 development and function. To understand whether RNF128 plays important roles in ILC2 development and function, we deleted exon2 of RNF128 in mice to study its function in vivo (Fig. S1A-C). We first analyzed ILC2s in different organs via gating with various surface markers. As reported in other articles, ILC2s in the bone marrow (which are known as immature ILC2s) are in the early stage of development, whereas peripheral ILC2s (which are known as mature ILC2s) are in the late stage of development[24]. Compared with their wild-type counterparts, RNF128-deficient ($RNF128^{-/-}$) mice exhibited normal frequencies and numbers of immature ILC2s in the bone marrow (Fig. 1B and C). Interestingly, both the frequency and number of ILC2s in peripheral nonlymphoid tissues, such as the lungs (Fig. 1D and E) and small intestines (SI) were increased (Fig. 1F and G). Together, these data indicated that RNF128 was a selective and potent regulator of the development of mature ILC2s.

RNF128 deficiency promotes *A. alternata* - induced allergic lung inflammation

Allergic lung inflammation is a universal airway disease driven by the hyperresponsiveness to inhaled allergens. *A. alternata*, which is a clinically relevant allergen, is also a major fungus associated with ILC2-mediated asthma[18,25]. *A. alternata*-induced allergic lung inflammation is mainly characterized by the secretion of type 2 cytokines, the aggregation of eosinophils and lung tissue damage. We treated mice with *A. alternata* or PBS for 4 days (Fig. 2A) and detected eosinophils via flow cytometry (Fig. S2A). Compared with wild-type mice, $RNF128^{-/-}$ mice exhibited significantly more eosinophils (Fig. 2B and C) in the lung and the type 2 cytokines (IL-5 and IL-13; Fig. 2F) in the bronchoalveolar lavage fluid (BALF) after *A. alternata* challenge. Consistent with lung tissue results, eosinophils in BALF were also significantly increased (Fig. 2E). However, lung macrophages showed no significant changes (Fig. 2D). The aggravated lung tissue damage and mucus production in $RNF128^{-/-}$ mice were further confirmed via hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS) staining of lung tissues (Fig. 2G). These results demonstrated that loss of RNF128 increased *A. alternata*-induced allergic lung inflammation.

RNF128 deficiency promotes ILC2 activation in response to *A. alternata* challenge

In *A. alternata*-induced allergic lung inflammation, ILC2s are the main secretors of type 2 cytokines. Therefore, we will focus on exploring the function of RNF128 in ILC2s. We treated mice with *A. alternata* or PBS for 4 days and then detected ILC2s via flow cytometry (Fig. S2B). We found that both the frequency and number of ILC2s in the mouse lung were greater in $RNF128^{-/-}$ mice after *A. alternata* treatment (Fig. 3A). In addition, we observed a significant increase in the frequencies and total numbers of IL-5- and IL-13-producing lung ILC2s in *A. alternata*-treated $RNF128^{-/-}$ mice compared with those in wild-type mice (Fig. 3B and C). Taken together, RNF128 deficiency potently promoted ILC2 activation induced by *A. alternata*.

RNF128 deficiency alters ST2 expression in lung ILC2s after *A. alternata* challenge

ILC2s express several receptors for cell development, homeostasis, and effector functions, including the IL-33 receptor ST2. The epithelial cell-derived cytokine IL-33 is the most powerful cytokine that is involved in the activation of ILC2s during allergic lung inflammation. When epithelial cells receive antigen stimulation, they rapidly produce a large amount of IL-33, which activates ILC2s by binding to the cell surface receptor ST2. Considering that RNF128 deficiency affected ILC2 function, we speculated that RNF128 might regulate ST2 expression. Our data showed that *A. alternata* administration resulted in an increase in the ST2 expression of lung ILC2s from $RNF128^{-/-}$ mice, whereas the responsiveness of lung ILC2s in wild-type mice was decreased (Fig. 3D). These observations collectively suggest that RNF128 affected the function of ILC2s associated with ST2 expression.

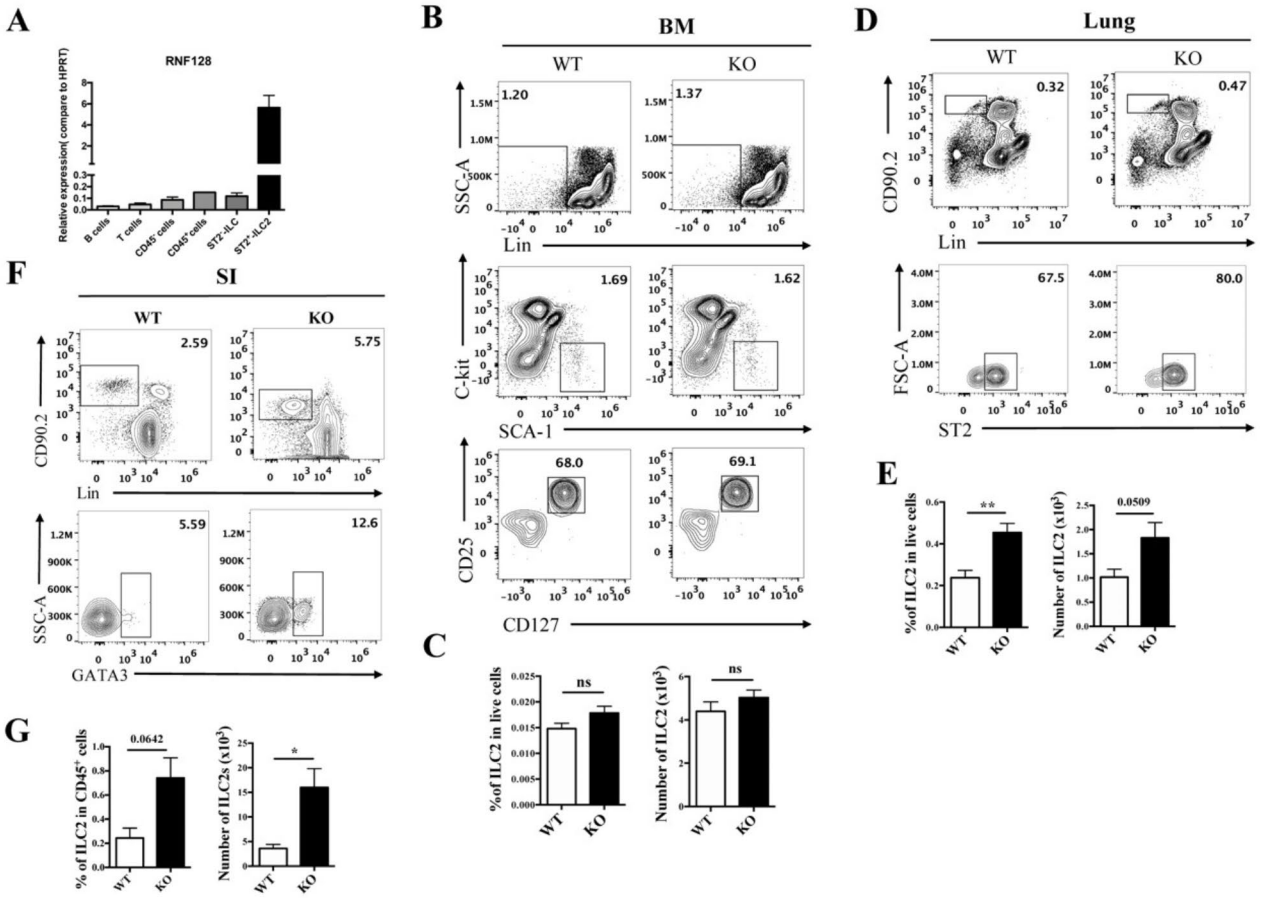

Fig. 1. RNF128 deficiency promoted mature ILC2 development. (**A**) Relative expression of RNF128 in different cell types sorted from naïve mice. (**B**) Gating strategy and flow cytometric analysis of ILC2s from the bone marrow (BM) of wild-type (WT) and RNF128$^{-/-}$ (KO) mice[15,35]. BM ILC2s were identified as lineage negative (Lin$^-$) C-kit$^-$SCA-1$^+$CD25$^+$CD127$^+$. (**C**) Quantification of the percentage and number of BM ILC2s ($n=4$ or 5 per group). (**D**) Gating strategy and flow cytometric analysis of ILC2s from the lungs of WT and RNF128$^{-/-}$ mice. Lung ILC2s was identified as Lin$^-$CD90.2$^+$ST2$^+$ cells. (E) Quantification of the percentage and number of lung ILC2s ($n=4$ or 5 per group). (F) Gating strategy and flow cytometric analysis of ILC2s from the Small intestine (SI) lamina propria of WT and RNF128$^{-/-}$ mice. SI ILC2s were identified as CD45$^+$Lin$^-$CD90.2$^+$GATA3$^+$ cells. (G) Quantification the percentage and number of SI ILC2s ($n=4$ or 5 per group). The bars and error bars show the means ± SEMs; *, $P<0.05$; **, $P<0.01$ according to unpaired Student's t-tests. ns, not significant.

Discussion

Once they are triggered by alarmins from epithelial cells, ILC2s release copious amounts of inflammatory factors in a very short period of time, thus mediating the type 2 immune response and playing an important role in allergic inflammatory diseases[26]. In this study, we detected greater expression of the E3 ligase RNF128 in lung ILC2s than in other immune cells, thus indicating that RNF128 may regulate lung ILC2 development and function. We used knockout mice and revealed that RNF128 plays an important role in promoting the effector function of ILC2s associated with ST2 expression. Although RNF128 deficiency did not affect immature ILC2s in the bone marrow, the number and percentage of ILC2s in peripheral lung tissues were increased. Overall, these data indicated that RNF128 was a selective and potent regulator of the development of mature ILC2s. RNF128 deficiency aggravated the airway inflammation and promoted IL-5 and IL-13 production in lung ILC2s induced by *A. alternata* administration. RNF128-deficient ILC2s exhibited increased expression of ST2 after *A. alternata* challenge. From these observations, we identified the E3 ligase RNF128 could be a novel target for the therapeutic intervention of ILC2-mediated inflammatory diseases.

Previous studies have revealed that most ILC2s (95%) in the bone marrow are recognized as being ILC2 precursors (ILC2Ps) or immature ILC2s[27,28]. A recent study suggested that ILC2s undergo a two-stage maturation process. At the early stage, these cells remain in the bone marrow or maybe other organs with general ILC2 properties. At the late stage, ILC2s respond to peripheral microenvironmental cues, after which they further express maturation markers and differentiate; subsequently, they rapidly secrete type 2 cytokines upon allergen stimulation. Researchers have reported that the E3 ligase VHL regulates peripheral ILC2 maturation (but not

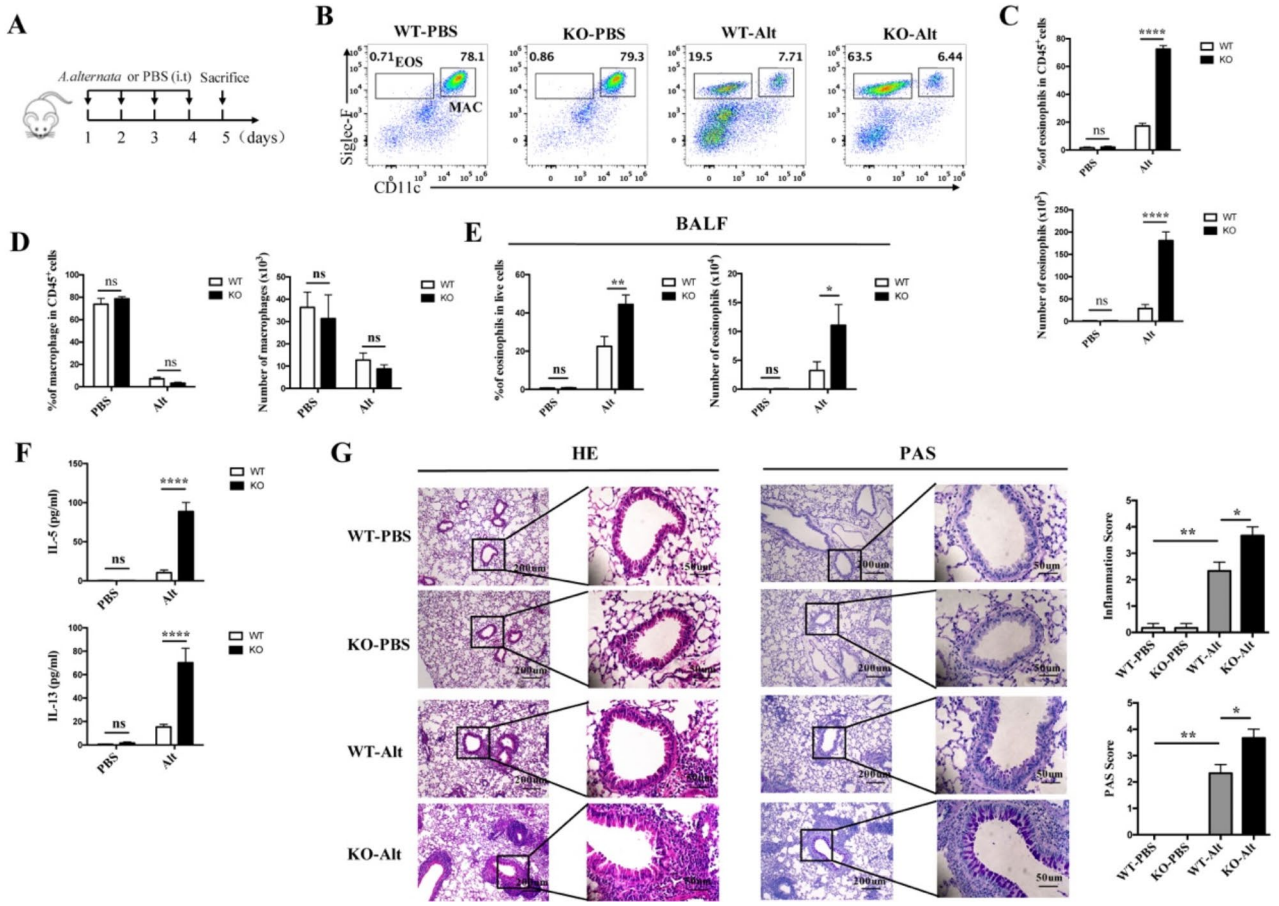

Fig. 2. RNF128 deficiency promoted *A. alternata -induced* lung inflammation. The mice were intratracheally challenged with *A. alternata* or PBS for 4 consecutive days and sacrificed on Day 5 ($n = 4$ or 5 per group). (**A**) Schematic of *A. alternata* challenge. (B) Representative flow cytometry analysis of the total eosinophils (live $CD45^{+}CD11c^{-/lo}SiglecF^{+}$) and macrophages in the lung. EOS: eosinophils. MAC: macrophages. (**C**) Quantification of the percentage and number of eosinophils in (**B**). (**D**) Quantification of the percentage and number of macrophages in (**B**). (**E**) Quantification of the percentage and number of eosinophils in the BALF. (**F**) The levels of IL-5 and IL-13 in the BALF were measured via ELISA. (**G**) Representative H&E and PAS staining of lung sections (bars, 200 μm, 50 μm). Data were analyzed by two-way ANOVA with Bonferroni posttest. The bars and error bars show the means ± SEMs; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; ns, not significant.

bone marrow ILC2 maturation) via glycolysis inhibition and the induction of ST2[24]. In agreement with these findings, we found that RNF128 selectively regulated ILC2s in peripheral non-lymphoid tissues such as the lungs and SI. Interestingly, there was no difference observed in ST2 expression between naïve WT and KO mice. We inferred that in naïve mice, RNF128 may not regulate mature ILC2 development via the regulation of ST2 expression. According to the results of previous studies, ILC2s express Il-13R and IL-33R, and both receptors are upregulated after *A. alternata* or *N. brasiliensis* challenge. IL-13 and IL-33 bind to their receptors and regulate down-stream ST2 expression[29,30]. In naïve mice, there are low levels of IL-33 and IL-13 in the lung tissue microenvironment. When mice were exposed to *A. alternata*, epithelial cells released a large amount of IL-33 and lung ILC2s were activated, released an amount of IL-13, which promoted ST2 expression. Therefore, only after *A. alternata* exposure, the ILC2s of $RNF128^{-/-}$ mice increased the level of ST2 expression compared with those of WT mice.

RNF128, which is known as an E3 ligase, promotes the K27-linked polyubiquitination of IL-3Rα and subsequently facilitates its degradation via the lysosomal pathway[22]. Furthermore, RNF128 has been found to regulate TLR4 degradation, which subsequently leads to the reduction of proinflammatory cytokines in the early stages of LPS-induced acute lung injury[21]. Recent research has shown that RNF128 attenuates colitis and colorectal tumorigenesis by inhibiting IL-6-STAT3 signaling. Mechanistically, RNF128 interacts with the IL-6 receptor and the membrane glycoprotein gp130 and promotes their K48-linked polyubiquitination[31]. In Th2 cells, RNF128 promotes STAT6 ubiquitination and degradation, thereby affecting its activation[19]. In this study, the data revealed that RNF128 inhibited ILC2 function, likely via inhibition of downstream ST2, IL5 and IL13. Based on our experimental data and existing literature reports, we speculate that in ILC2s, RNF128 may regulate ST2 via ubiquitination and degradation. This hypothesis requires further investigation.

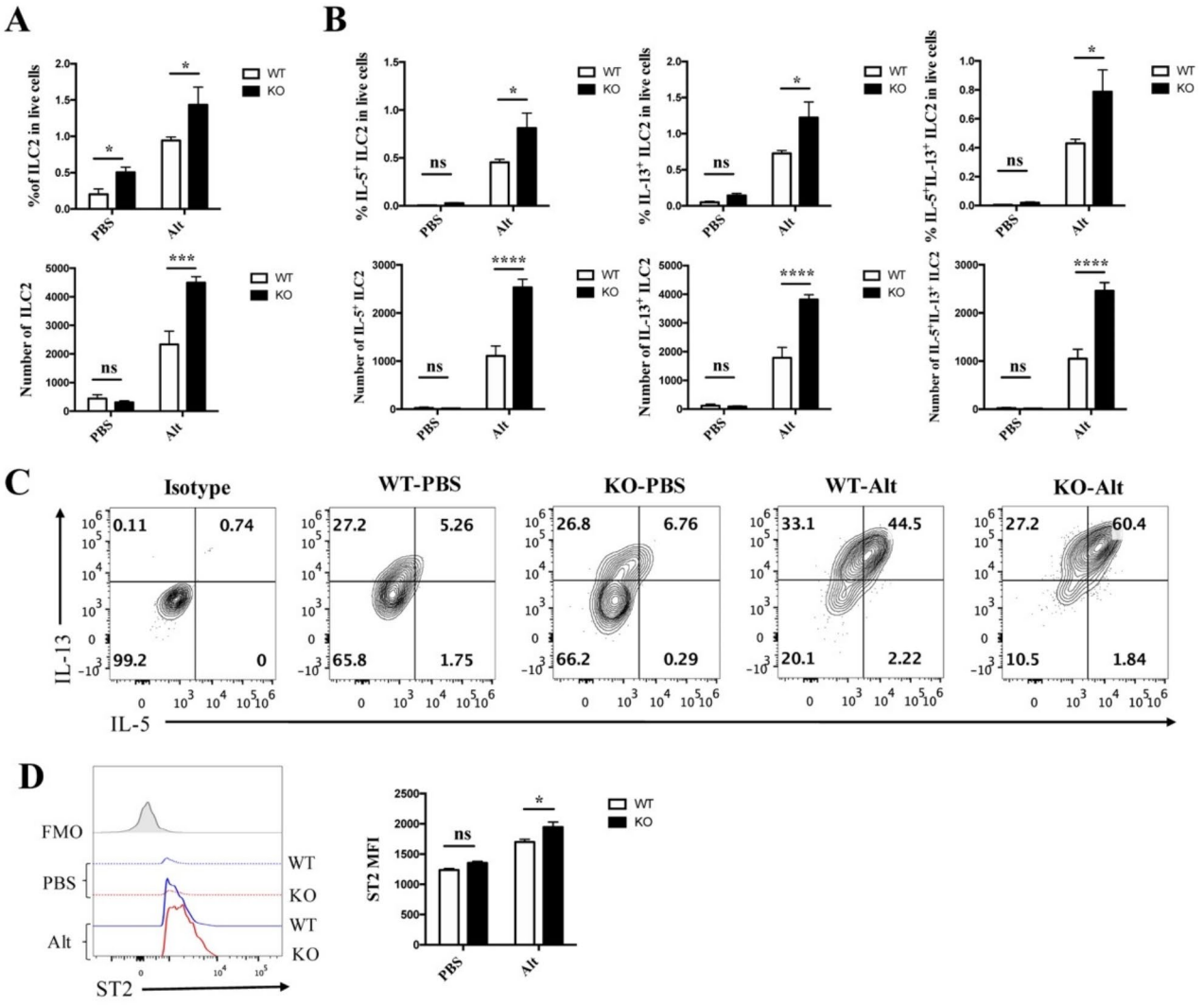

Fig. 3. RNF128 deficiency promoted ILC2 activation in response to *A. alternata* challenge. The mice were intratracheally challenged with *Alternaria* or PBS for 4 consecutive days and sacrificed on Day 5 ($n = 4$ or 5 per group). (**A**) Quantification of the percentage and number of ILC2s. (**B**) The percentage and number of IL-5$^+$, IL-13$^+$ and IL-5$^+$IL-13$^+$ cells among lung ILC2s after stimulation with PMA, ionomycin and brefeldin A (BFA) for 4 h. (**C**) Representative flow cytometry analysis of IL-5 and IL-13 production in lung ILC2s. (**D**) Flow cytometric analysis of ST2 mean fluorescence intensity in lung ILC2s. Data were analyzed by two-way ANOVA with Bonferroni posttest. The bars and error bars show the means ± SEMs; *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

In summary, our research revealed that the E3 ligase RNF128 restricted lung ILC2 effector functions and ST2 expression. An understanding of the mechanisms by which E3 ligases govern ILC2 homeostasis may provide potential drug targets for treating type 2 inflammatory diseases.

Materials and methods

Reagents and antibodies

Antibodies specific to mouse CD5 (53-7.3), CD45R (RA3-6B2), Gr-1 (RB6-8C5), NK1.1 (PK136), and TCR γ/δ (UC7-13D5), as well as a mouse erythroid cell marker (TER119) were purchased from BioLegend; antibodies specific to mouse CD3 (2C11), CD4 (RM4-5), CD11b (M1/70), CD11c (N418), CD127 (A7R34), CD45.2 (104), FcεR1 (MAR-1), IL-5 (TRFK5), IL-13 (eBio13A) were purchased from eBioscience. Antibodies specific to CD90.2 (53-2.1) was purchased from BD Biosciences. Antibody specific to IL-33R (DJ8) was purchased from MD Bioproducts. The cell viability dye FVS780 was purchased from BD Biosciences. Additionally, phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma-Aldrich; Brefeldin A and the Foxp3/Transcription Factor Staining Buffer Set were purchased from eBioscience. Collagenase type I was purchased from Invitrogen. Penicillin-streptomycin was purchased from Gibco. Percoll was purchased from GE Healthcare. ELISA kits for IL-5 and IL-13 were purchased from eBioscience.

Animals

RNF128-deficient mice were purchased from GemPharmatech (Nanjing, China) and the mice were generated on a C57BL/6 background via CRISPR/Cas9 technology. The sgRNA was transcribed in vitro. Cas9 and sgRNA were microinjected into the fertilized eggs of C57BL/6 mice. The fertilized eggs were subsequently transplanted to obtain positive F0 mice which were confirmed via PCR and sequencing. A stable F1 generation mouse model was obtained by mating positive F0 generation mice with C57BL/6 mice. The mice were maintained under specific pathogen-free conditions at the Animal Care Facility of Jiangxi University of Traditional Chinese Medicine (Nanchang, China) and aged to 8–10 wks. The animal care and use procedures complied with the guidelines of the Institutional Animal Care and Use Committee.

Lung inflammation mouse models

To establish *A. alternata*-induced acute type 2 airway inflammation, the mice were anaesthetized with isoflurane and then intratracheally administered *A. alternata* (4 μg per mouse in 40 μL of PBS) or PBS for 4 consecutive days[32]. BALF and lungs were harvested for analysis on Day 5.

Preparation of cell suspensions from lung tissue

We prepared cell suspensions from lung tissue according to our previous report[15]. BALF cells were collected from the lungs via two washes with 800 μl of PBS twice. For the isolation of lung cells, the mice were perfused with 10 ml of cold PBS through the right ventricle of the heart to remove blood from the tissues. The lungs were subsequently isolated and cut into small pieces. These small pieces were placed in RPMI-1640 containing 10% FCS, and digested with 0.5 mg/ml collagenase type I and 1% penicillin-streptomycin for 30 min at 37 °C in a shaker. Afterwards, the crude tissues were passed through 70 μm cell strainers. The total cells were suspended in 40% Percoll, which was added to 80% Percoll; afterwards, the gradient was centrifuged at 600 × g for 20 min to obtain single cells, and the isolated cells were washed with RPMI-1640 before further ILC2 analysis.

Flow cytometric analysis and cell sorting

For the staining of cell surface markers, we used the cell viability dye FVS780 to exclude dead cells. Afterwards, these single-cell suspensions were pre-treated with an anti-Fc receptor blocking antibody (anti-CD16/CD32) and then stained with a cocktail of fluorochrome-conjugated antibodies for various lineage markers, including CD3, CD5, CD45R, CD11b, CD11c, NK1.1, Gr-1, TER119, FcεR1, and TCR γ/δ, to identify Lin^- cells. For the staining of transcription factors, the cells were first stained with antibodies against surface antigens and then fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set according to the manufacturer's instructions. For intracellular cytokine staining, the cells were isolated and stimulated with 50 ng/ml PMA, 1 μg/ml ionomycin, and 1 μg/ml brefeldin A for 4 h. Afterwards, the cells were permeabilized and fixed with a Foxp3/Transcription Factor Staining Buffer Set and stained with IL-5, IL-13. Flow cytometry data were collected on a CytoFLEX flow cytometer (Beckman Coulter), and the data were analyzed with FlowJo V10 (FlowJo). For flow cytometric sorting, as described in our previous report[15], mouse lungs were isolated, and ILC2s were enriched with the EasySep™ Mouse ILC2 Enrichment Kit (STEMCELL). Afterwards, $ST2^+$ILC2s (live $CD45^+Lin^-CD90.2^+ST2^+$ cells) were sorted with an Aria SORP or Aria III-405 (BD Biosciences). The remaining cells were then stained with the specific fluorochrome-conjugated antibodies ($CD4^+$T cells: live $CD45^+CD4^+$; B cells: live $CD45^+B220^+$; $CD45^-$ cells: live $CD45^-$; $CD45^+$ cells: live $CD45^+$; $ST2^-$ILCs: live $CD45^+Lin^-CD90.2^+ST2^-$).

Cytokine assays

BALF from different lung inflammation model mice was collected. The levels of the cytokines IL-5 and IL-13 were measured via ELISA following the manufacturer's instructions.

Real-time quantitative PCR (RT-qPCR)

Total RNA from different types of cells sorted via flow cytometry was extracted via TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and RT-qPCR was performed via SYBR Green PCR Master Mix (Vazyme Biotech, China).

Histological analysis

The mice were euthanized by using CO_2. The lungs were fixed for at least 24 h with 4% paraformaldehyde and were embedded in paraffin. Sections of the fixed lung lobes were stained with PAS or H&E. Lung inflammation was determined via semiquantitative scoring as previously reported[32–34]: The inflammation score based on peribronchial and perivascular inflammation using the criteria : scoring 0 for normal, 1 for minimal cells, 2 for a single cell layer of inflammatory cells, 3 for two to four cell layers, and 4 for more than four cell layers of inflammatory cells. PAS scores were determined via semiquantitative analysis of 3 consecutive fields per slide, where 0 indicated less than 5% goblet cells of PAS-positive, 1 for 5–25%, 2 for 25–50%, 3 for 50–75%, and 4 for more than 75% staining. An increment of 0.5 was used when the inflammation fell between two levels.

Statistical analysis

Statistical significance was assessed via an unpaired two-tailed t test or two-way ANOVO with GraphPad Prism 6. The results are presented as the means ± SEMs, and P values < 0.05 were considered to indicate statistical significance.

Ethics statement

All of the animal experiments were approved by the ethics committee of Jiangxi University of Traditional Chinese Medicine (No.JZLLSC20240564). All of the methods were performed in accordance with relevant guidelines and regulations. All of the methods are reported in accordance with the ARRIVE guidelines.

Data availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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Author contributions
C.Y. and W.K. designed and performed the experiments and analyzed the data. F.G. prepared experimental materials and bred mice, G.M., L.J., H.T. and L.W. discussed the data, H.W., J.Z., and Y.L. provided technical support, C.Y. and W.K. generated figures and wrote the manuscript. C.Y. supervised the project and revised the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

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