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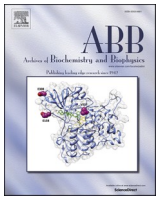

PACAP/PAC1R activation promotes group 2 innate lymphoid cells-dependent allergic rhinitis via ERK pathway

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ABSTRACT

Allergic rhinitis (AR) is a T helper type 2 (Th2)-mediated inflammatory disease. It has been reported that Group 2 innate lymphoid cells (ILC2s) may contribute to the pathogenesis of AR. While Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) has demonstrated anti-inflammatory properties in allergic contact dermatitis, its regulatory effects on ILC2s remain unclear. This study aimed to investigate the regulatory role of PACAP in ICL2 proliferation under allergic inflammation. In an ovalbumin (OVA)-induced AR mouse model, we observed significant elevations in both PACAP levels and ILC2 populations. Both *in vivo and in vitro* experiments confirmed that PACAP effectively promoted the expansion of IL-5^+ and IL-13^+ ILC2s subsets and enhances ILC2 proliferation. PACAP receptor PAC1R knockdown or PAC1R receptor antagonist PA-8 markedly suppressed ILC2s proliferation and cytokine production. Furthermore, *in vivo* experiments demonstrated that PACAP inhibition reduced ILC2 proliferation, thereby alleviating nasal mucosal inflammatory responses, confirming that PACAP exacerbates allergic inflammation through PAC1R-dependent activation of ILC2s. Mechanistic studies revealed that PACAP/PAC1R signaling activated the ERK pathway, as evidenced by upregulated p-ERK expression and increased IL-5/IL-13 secretion in ILC2s. These effects were effectively reversed by ERK inhibitor PD98059. Importantly, both PAC1R knockdown and ERK inhibition significantly decreased p-ERK expression and ILC2s proliferation, while ameliorating AR pathological features. Our findings revealed that PACAP/PAC1R activation promoted ILC2s proliferation and allergic inflammation through ERK pathway, which provides novel insights into the regulation of ILC2s and potential therapeutic targets in allergic rhinitis.

1. Introduction

Allergic rhinitis (AR) is characterized by sneezing, nasal congestion, nasal itching and rhinorrhea (nasal discharge) and is caused by immunoglobulin E (IgE)-mediated reactions to inhaled allergens [1]. Allergic rhinitis affects up to 78 % of people with asthma, and asthma occurs in 38 % of people with allergic rhinitis. Allergic rhinitis occurs in 5 %–15 % of people in the United States [2]. AR impairs quality of life, affects social life, school and work, and is associated with substantial economic costs [3]. Intranasal corticosteroids and second-generation antihistamines are the mainstay of treatment. Allergen immunotherapy is an effective immune-modulating treatment method that can be used as a supplement or alternative to pharmacologic therapy [4]. A deeper understanding of the underlying mechanisms of AR may provide novel insights to develop promising strategies for AR treatment.

AR is a T helper type 2 (Th2)-mediated inflammatory disease [5,6]. Th2 cells, which produce increased amounts of the cytokines interleukin-5 (IL-5) and IL-13, promote allergic disorders [7]. Innate lymphoid cells (ILCs) are a group of lymphocytes that are devoid of antigen-specific receptors and are mainly found in tissues. There are three subtypes ILC1, 2, and 3 [8]. ILC2s were identified as a novel lymphocyte subset lacking antigen receptors and respond to injuries through rapid interleukin (IL)-5 and IL-13 secretion [9,10]. Additionally, ILC2s are activated by mast cell lipid inflammatory mediators, such as cysteinyl leukotrienes and prostaglandin D2 [11]. Furthermore, MHCII-expressing ILC2s potentiate Th2 responses [12]. ILC2s and Th2 cells are the main sources of type II cytokines stimulate mucus production and airway hyperreactivity by recruiting effector immune cells such as basophils, mast cells, and eosinophils [13]. ICL2s were increased in patients with allergic rhinitis, and they can be rapid recruited to the upper airways of allergic patients [14,15]. And suppressing ILC2s can alleviates allergic rhinitis [16,17].

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Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) is an endogenous neuropeptide known to inhibit inflammation and promote neuronal survival [18]. PACAP involved in a diverse array of physiological and pathological processes through activating the G protein-coupled receptors: PACAP type I receptor (PAC1R) [19]. Apart from in neuronal system, PACAP is an important endogenous mediator in human upper airways, with a potential role as a regulator of vascular smooth muscle, secretion, plasma extravasation, neutrophil recruitment and cytokine activity [20]. PACAP exerts anti-inflammatory effects in allergic contact dermatitis [21,22]. PACAP can reverse the vanadate-induced airway hyperresponsiveness by counteracting the proinflammatory and prooxidative effects of the metal [23]. However, the regulatory effects of PACAP on ILC2s proliferation under allergic inflammation remain unclear.

The objective of this study was to investigate the association between PACAP/PAC1R and the proliferation of ICL2 in AR using ovalbumin (OVA)-exposed mice. We aim to explore the potential role of PACAP/PAC1R in affecting the proliferation of ICL2s polarization via ERK pathway in AR, which may provide deeper insights into allergic inflammation.

2. Materials and methods

2.1. Cell culture

ILC2 cells were purchased from iCell Bioscience Inc. It was grown in 89 % RPMI 1640 (Gibco, USA), with 10 % FBS (Gibco) and 1 % Penicillin-Streptomycin medium (Sigma-Aldrich, USA). Cells were maintained at 37 °C in a humidified 5 % CO_2 incubation.

2.2. Reagents

Recombinant Human IL-33 (CP060179) was purchased from Proscript. PACAP (A1439-0.1 mg) was purchased from Sigma. ERK inhibitor PD98059 (HY-12028), IL-33 (HY–P73210), PAC1R antagonist PA-8 (HY-133529) and PACAP antagonist PACAP 1–27 (HY–P0176) were purchased from MCE.

2.3. Animals

Female BALB/C mice aged 8 weeks were obtained from purchased from the Charles River Co. Ltd. The mice were placed in controlled environments (12-h light/dark cycle; 20–26 °C; 40–60 % humidity) and had free access to bacteria-free water and food. All animal housing and experiments were conducted in accordance with the ethical guidelines formulated by the Animal Experimental Committee of The Affiliated Hospital of Guizhou Medical University.

2.4. The establishment of the AR model

The allergic rhinitis (AR) model induced by OVA was established based on previously described methods [24,25]. Briefly, mice were first intraperitoneally injected with 20 μg ovalbumin (OVA) (Sigma, A5503) plus 2 mg aluminium hydroxide adjuvant (Bio dragon, KX0210054) in 400 μL normal saline 2 times a week for 2 weeks. Then, the mice were treated daily with 20 μL OVA solution (containg 10 μg OVA) via the nostrils for 7 days. Twenty-four hours after the last nasal stimulation, the number of times each mouse scratched its nose or sneezed was recorded every 5 min.

2.5. Cell transfection

ILC2s were planted on 6-well plates for 24 h. The original culture medium was removed, washed twice with PBS, and each well of the six-well plate with cultured cells was replaced with 2 mL fresh culture medium. Different 2.5 μg plasmid sh PAC1R and 4 μL lipofectamine 8000 (Beyotime, C0533)in the experimental group were prepared with the corresponding volume of medium into 125 μL diluent. The amount of 125 μL lipofectamine-DNA mixture was uniformly added to the entire well and then gently mixed. There were 3 replicates in each group. Empty vector transfected cells were used as negative control. The sh-PAC1R sequences are used as in Table 1.

2.6. RNA extraction and quantitative real-time PCR analysis(qRT-PCR)

Total RNA was extracted using TRIzol reagent (Service bio, G3013) according to the manufacturer's instructions. The purified RNA was reverse-transcribed to form cDNA using PrimeScript™ RT reagent Kit (Takara, RR037Q). Quantitative real-time PCR (qRT-PCR) was performed with TB Green® Premix Ex Taq™ II (Takara, RR820Q). The relative expression level of RNA was normalized to GAPDH expression and was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are used as in Table 2.

2.7. Western blot

RIPA lysis buffer was used to extract protein from kidney tissue or cultured cells, and a BCA protein detection kit was used to measure the concentration. Approximately 25 mg of protein was boiled for 5 min at 100 °C, separated by different SDS-PAGE, and transferred to PVDF membranes. The PVDF membranes were then blocked with 5 % skim milk for 2 h at room temperature and incubated with primary antibodies PACAP (Aff biotech, DF14838), ERK1/2 (Abways, CY 5487), p-ERK1/2 (Abways, CY 5044), PAC1R (Aff biotech, DF7197), IL-5 (Aff biotech, AF5123), IL-13 (Aff biotech, DF6813), ST2 (Protein tech, 60112-1-Ig), VPAC1 (BOSTER, BA1462-2), VPAC2 (BOSTER, A03768-1) and β-tublin (Abways, AB0039) overnight at 4 °C. Then, the membranes were washed three times with TBST and incubated with the appropriate horseradish peroxidase-labeled secondary antibodies. An enhanced chemiluminescence reagent was used to view the reaction. Standardization was performed using β-tublin.

2.8. Immunofluorescence assay

Nasal mucosa tissue was post-fixed in 4 % paraformaldehyde overnight at room temperature and cut into 5-μm sections. The sections were blocked with 1 % BSA. For PACAP and PAC1R staining, sections were incubated overnight at 4 °C with a primary antibody against PACAP (Genetex, GTX106794) and PAC1R (Thermo Fisher, PA5-96229). Immunoreactivity was visualized using Cy3-conjugated Goat anti-Rabbit IgG and stained with 4′,6-diamidino-2-phenylindole (DAPI) for 3 min and imaged using a laser scanning confocal microscope (Leica, Germany). Sections were analyzed and quantified using Image J to measure the number of positive cells, with six sections of each sample used to calculate the average.

The ILC2s were seeded on glass coverslips in 48-well plates. After various treatments, the cells were fixed in 4 % paraformaldehyde at room temperature for 20 min and then washed thrice in PBST. After permeabilization with 0.1 % Triton X-100/PBS for 15min, the cells were washed with PBS, blocked in PBS with 5 % BSA at room temperature for 1h, and then incubated with PACAP (Thermo Fisher, PA5-96229), PAC1R (Alomone, AVR-003-0.2 mL) primary antibodies at 4 °C overnight. After being washed with PBS, the cells were incubated Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (Abcam, ab150077) and stained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min and imaged using

Table 1
Sequence of shRNA.

Gene	Species	Sequence
sh-PAC1R-1	Mouse	CUUCCCUGAGAGGAGAUAUUU
sh-PAC1R-2	Mouse	CCCUGUAGUUGGCUCUAUAAU

Table 2
Primers used in qRT-PCR.

Gene	Species	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
PAC1R	Mouse	CTGCGTGCAGAAATGCTACTG	AGCCGTAGAGTAATGGTGGATAG
IL-5	Mouse	TCAGGGGCTAGACATACTGAAG	CCAAGGAACTCTTGCAGGTAAT
IL-13	Mouse	CAGCCTCCCCGATACCAAAAT	GCGAAACAGTTGCTTTGTGTAG
ST2	Mouse	AGAAGCCCCAACTTGAATAAGAC	TCTGATCCACGTACTGTCGAG
VPAC1	Mouse	CAGACTGAGTTCTACGATGCAG	TGAACAGGCTCAAGATAGCCAT
VPAC2	Mouse	AGGCCATTTATACCTTGGGCT	GCAGTAGACCTGAGCTGGAGTA
GAPDH	Human/Mouse/Rat	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

a microscope (Keyence, BZ-X800). Sections were analyzed and quantified using Image J to measure the number of positive cells.

2.9. Cell sorting

For ILC2 sorting, peripheral blood mononuclear cells were isolated from the mouse blood sample withe the lymphocyte isolation liquid. After counting, 10^7 PBMC cells were taken and centrifuged in a 1.5 mL EP tube at 300 rpm for 10 min. Cells were suspended with a 78 μL 1 × Binding Buffer. 20 μL Fc receptor blocking solution (Bio legend, 422301) and 2 μL CD294 (Bio legend, 165102) was applied to the cells. 10^8 Cells were suspended with a 500 μL 1 × Binding Buffer. In CD294 positive cells, 2.5 μL Lineage (Biolegend, 133301) and 2.5 μL APC Anti-CD161 (Abcam, ab269325) were added, mixed, and incubated at room temperature for 30 min. Add 200 μL 2 % FACS for resuspension. 150 μL cell suspension were analysis by flow cytometry.

To detect IL-5 positive cells in ILC2, 2 μL IL-5 antibody (Thermo fisher, 12-7052-82) and 2 μL APC anti-human CD161 antibody (Abcam, ab269325) were added.

To detect IL-13 positive cells in ILC2s, 2 μL IL-13 antibody (Thermo fisher, 12-7133-82) and 2 μL APC anti-human CD161 antibody (Abcam, ab269325) were added.

To detect the proliferation of ILC2s, 2 μL Ki-67 antibody (Thermo fisher, 11-5698-82) and 2 μL和2 μL APC anti-human CD161 antibody (Abcam, ab269325) were added.

2.10. Cell sorting for PAC1R

5×10^5 ILC2s were suspended with cooled 1 × PBS and centrifuged at 1500 rpm for 5 min twice. Dilute the cells in distilled water with 4 × Binding Buffer. Take 195 μL cell suspension into a labeled EP tube, add 2 μL PAC1R antibody (Alomone, AVR-003-0.2 mL) mix well, and incubate at room temperature for 30 min. Add 190 μL 1 × Binding Buffer for resuspension and 10 μL 20 μg/mL Propidium Iodide and mix well for analysis.

2.11. Hematoxylin and eosin staining (H&E staining)

The tumor section was cut conventionally and were stained with hematoxylin (Service bio, G1004) and eosin (Service bio, G1001). The stained sections were dehydrated with ethanol and transparently dried with xylene, which were then placed under a microscope (100 ×) and photographed.

2.12. ELISA

The PACAP, total IgE levels, specific IgE, IL-5 and IL-13 were measured using a Mouse PACAP ELISA Kit (Fine Test, EM1259), Mouse Immunoglobulin E ELISA Kit (Cusabio, CSB-E07983 m), Mouse OVA sIgE ELISA Kit (Fine Test, EM1254), Mouse IL-5 ELISA Kit (Elabscience, E-EL-M0722) and Mouse IL-13 ELISA Kit (Elabscience, E-EL-M0727) respectively. The results were read with an enzyme-labeled instrument (Thermo Fisher Scientific, Multiskan FC).

2.13. Statistical analysis

Each assay was performed for 3 times. All data were expressed as mean ± standard deviation and GraphPad Prism 8.0 (La Jolla, CA, USA) software was used for statistical processing. One-way ANOVA was used for comparisons between multiple groups, and *t*-test was used for comparisons between any 2 groups. $P < 0.05$ was considered as statistically significant difference.

3. Results

3.1. Serum PACAP is positively correlated with ILC2s in allergic rhinitis

To elucidate the potential mechanism of the allergic rhinitis (AR), an allergic rhinitis model was generated (Fig. 1A). The rhinorrhea, sneezing, rubbing and symptom scores of model group were higher than the control group (Fig. 1B). HE staining showed that the thickness of the submucous tissue was higher than that in the control group (Fig. 1C). AR is a kind of immune disease mediated by IgE [26]. We found that AR mice exhibited more amount OVA-specific IgE compared with Control mice (Fig. 1D). Moreover, AR mice had a greater number of ILC2s (Fig. 1E). Subsequently, we evaluated the absolute amount of PACAP in the two groups. We found that the concentration of PACAP were elevated in the AR model induced by OVA (Fig. 1F). The serum PACAP protein expression was positively correlated with the frequency of ILC2s and IgE in mouse (Fig. 1G). Thus, these results indicate that serum PACAP is positively correlated with ILC2s in Allergic rhinitis.

3.2. PACAP induces the production of IL-5 producing and IL-13 producing ILC2s

ILC2 are GATA3(+) and secrete IL5 and IL13 [17,27]. Above results showed that AR mice had a greater number of ILC2s. Next, we checked the amount of IL5 and IL13. Subsequently, we validated the cytokine IL5 and IL13 were increased in AR mice. (Fig. 2A). Then, the addition of 1 μg/μL IL-33 and 15.7 ng/mL PACAP increased the concentration of IL-5 and IL-13, of which the DMSO have no effects (Fig. 2A) (Fig. S1). Moreover, the 1 μg/μL IL-33 and 15.7 ng/mL PACAP further increased the proportion of IL-5 positive and IL-13 positive ILC2s (Fig. 2B). Thus, these results indicate that PACAP induces the production of IL-5 producing and IL-13 producing ILC2s.

3.3. PACAP induces the production of ILC2s via its specific receptor PAC1R

Following the euthanasia of the mice, PACAP and its specific receptor PAC1R in the ILC2s was detected by flow cytometry and immunofluorescence. As illustrated in Fig. 3A–B, PACAP and its specific

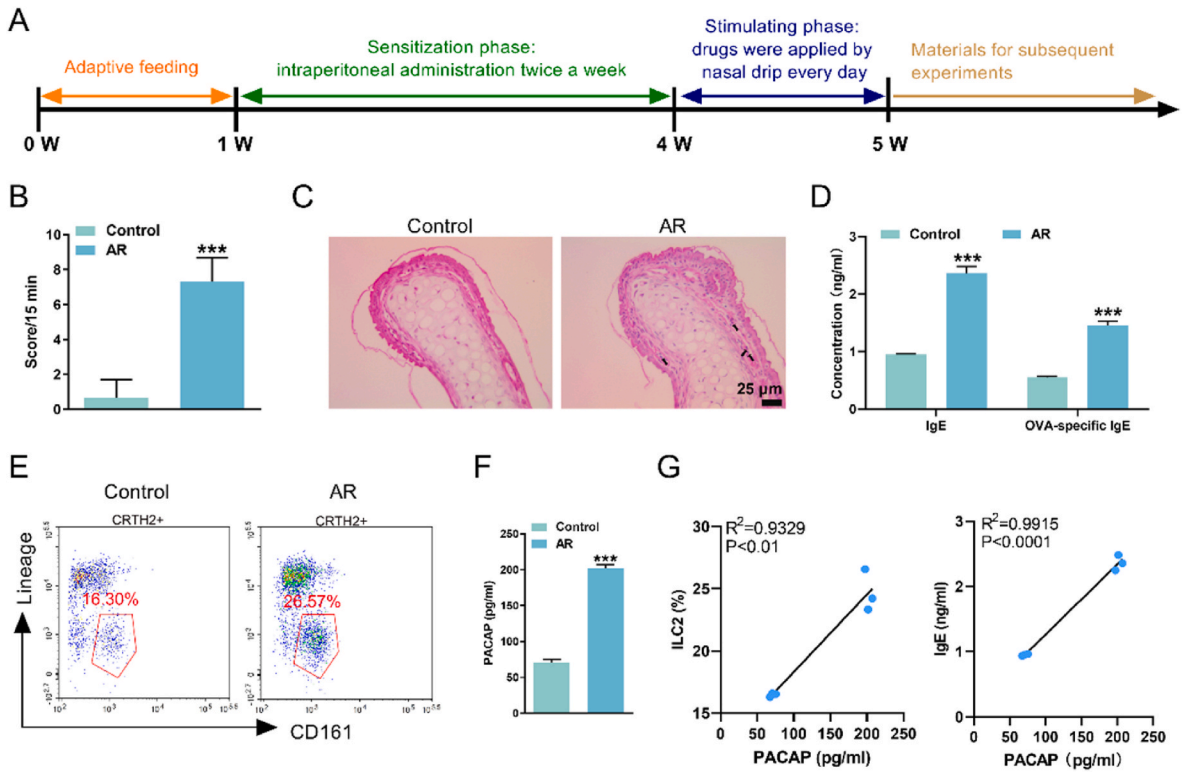


Fig. 1. Serum PACAP is positively correlated with ILC2s in Allergic rhinitis. (A) A schematic view of the treatment plan. (B) The sneezing, rubbing, symptom score of Control and AR mice in 15 min (n = 6, F = 1.750). (C) Representative images of H&E staining. (D) Serum level of IgE and OVA-specific IgE were detected using ELISA (n = 3, F_{IgE} = 65.75, $F_{OVA\text{-}spescific\ IgE}$ = 18.69). (E) The sorting ILC2s by flow cytometry from healthy and AR mouse (n = 3). (F) Nasal mucosal tissue levels of PACAP were detected by ELISA in Control and AR mice (n = 3, F = 1.711). (G) Positive correlation between PACAP protein expression and the amounts of ILC2s and IgE (n = 3, F_{ILC2} = 55.57, F_{IgE} = 467.8). ***$p < 0.001$ vs Control.

receptor PAC1R were highly expressed in the ILC2s (Fig. S2A). 1 sequence of sh-PAC1R showed the better knockdown effects, so we chose 1 sequence for the next study (Fig. S2B–C). qRT-PCR result showed that PAC1R mRNA expression was decreased after sh-PAC1R treatment (Fig. 3C). Knocking down PAC1R resulted in a substantial reduction in the number of ICL2, IL-5 positive ICL2s, IL-13 positive ICL2s cells and the proliferation of ICL2 in ILC2s (Fig. 3D) (Fig. S2D–F). Knocking down PAC1R also resulted in a substantial reduction the concentration of IL-5 and IL-13 in ILC2s (Fig. 3E). Moreover, IL-33 stimulated ILC2 cells were treated with PACAP or PACAP + PAC1R receptor antagonist (10 nmol/L PA-8). PACAP treatment could increase the number of ICL2, IL5 positive ICL2s, IL-13 positive ICL2s cells and the proliferation of ICL2. However, these increases would be weakened with PACAP and PA-8 treatment (Fig. 3F–H) (Fig. S2G–H). Thus, these results indicate that PACAP induces the production of ILC2s via its specific receptor PAC1R.

3.4. PACAP antagonist PACAP 1–27 alleviates allergic rhinitis by decreasing number of ICL2

As indicated in Fig. 4A–B, the levels of PACAP were increased in IL-33 and PACAP treated mice, but which were decreased after given PACAP antagonist PACAP 1–27. Flow cytometry showed the proliferation of ICL2s and the number of ICL2s were upregulated in IL-33 and PACAP treated mice, which was decreased by PACAP antagonist PACAP 1–27 (Fig. 4C–G). The levels of the Th2 inflammatory factors IL-5 and IL-13 showed the similar trend (Fig. 4H). The PCNA were increased in IL-33 and PACAP - induced control mice, but was reduced following PACAP 1–27 treatment (Fig. 4I). IL-33 and PACAP treated mice showed severe histopathological changes, such as infiltration of immune cells and increased epithelium thickness, compared with WT control mice; these changes were ameliorated by PACAP antagonist PACAP 1–27 (Fig. 4J). These data imply that inhibiting PACAP alleviates allergic rhinitis by decreasing number of ICL2s.

3.5. PACAP induces the production of ILC2s via ERK pathway

PACAP can induce ERK activation in HEK cells expressing PAC1R [28]. As depicted in Fig. 5A, sh-PAC1R significantly depressed p-ERK expression in ICL2s. ELISA revealed that IL-33 and PACAP increased the concentration of IL-5 and IL-33, while ERK inhibitor PD98059 reversed effect of IL-33 and PACAP (Fig. 5B). Flow cytometry revealed the amounts of IL-5 positive ILC2s and IL-13 positive ILC2s were significantly upregulated in IL-33 and PACAP treated mice, but ERK inhibitor PD98059 effectively reversed this upregulation (Fig. 5C–E). These data imply that PACAP induces the production of ILC2s via ERK pathway.

3.6. PACAP induces allergic rhinitis via ERK pathway in vivo

First, the mice were injected with sh-PAC1R lentivirus and the AR model was induced with OVA (Fig. 6A). To explore downstream signaling of the PACAP/PAC1R pathway, which was activated in the nasal cavity of OVA-lesioned mouse, we conducted a series of related detection analyses. Immunofluorescence detection analysis found that the OVA and PACAP significantly increased PAC1R expression in the mouse nasal mucosa, while PAC1R expression was downregulated after sh-PAC1R or PD98059 treatment (Fig. 6B). The mRNA and protein expressions of PAC1R, IL-5, IL-13, ST2, ERK1, ERK2, p-ERK were significantly upregulated in the OVA and PACAP treated mouse nasal mucosa, while sh-PAC1R or PD98059 effectively reversed this upregulation (Fig. 6C) (Fig. S3A–B). Moreover, the mRNA and protein expressions of PACAP receptor VPAC1 and VPAC2 showed the similar trend, which

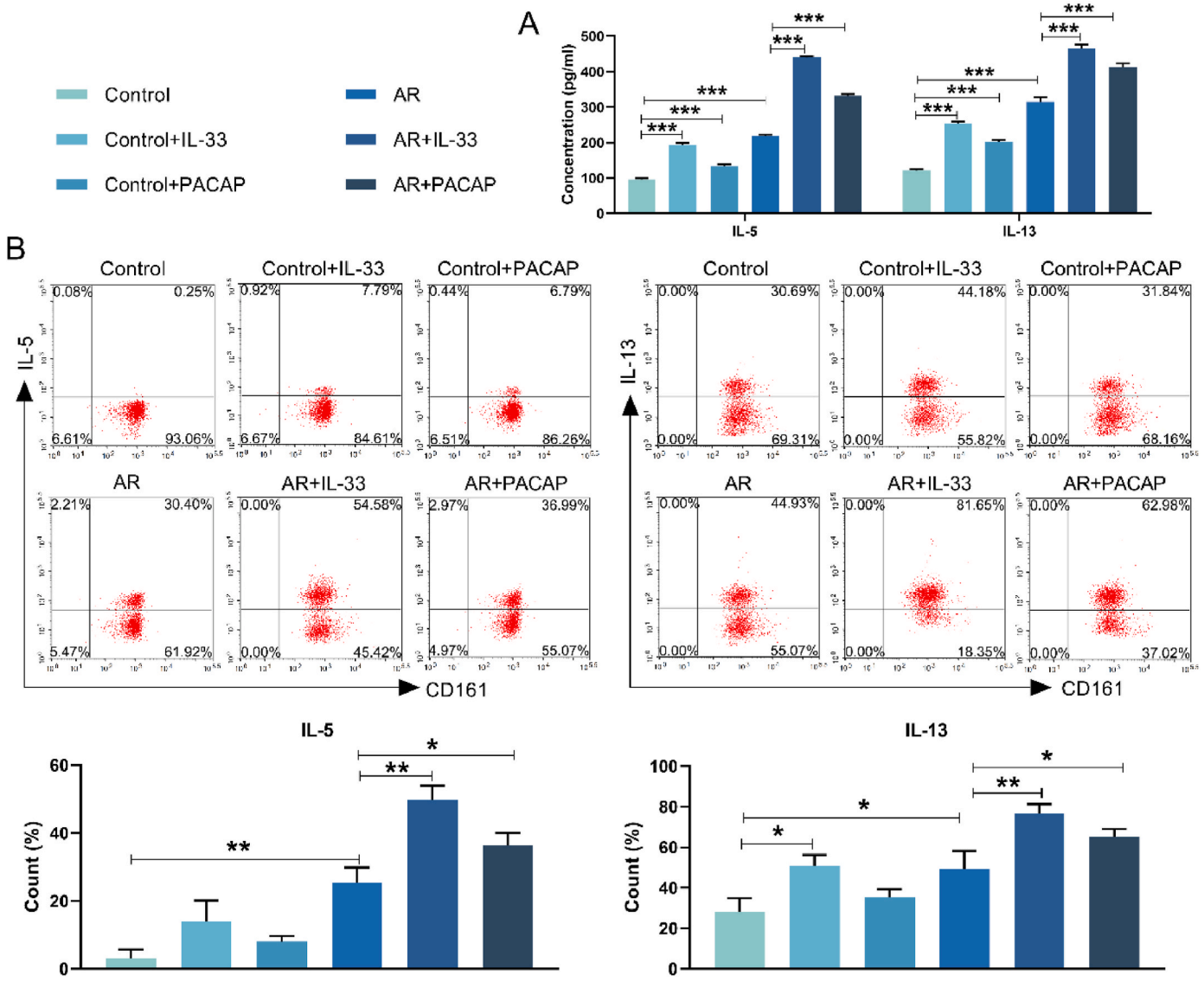


Fig. 2. PACAP induces the production of IL-5 positive and IL-13 positive ILC2s. AR mice model was established. 1 μg/μL IL-33 and 15.7 ng/mL PACAP was used to treat the PBMCs from the mice. (A) The concentration of IL-5 and IL-13 was analyzed by ELISA (n = 3, $F_{IL\text{-}5} = 0.2739$, $F_{IL\text{-}13} = 0.4791$). (B) Representative flow cytometric plots display IL-5 positive and IL-13 positive ILC2s (n = 3, $F_{IL\text{-}5} = 0.4244$, $F_{IL\text{-}13} = 0.1534$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs Control or AR.

indicated that PAC1R knockdown could reduce VPAC1 and VPAC2 expression (Fig. S3C–D). sh-PAC1R or PD98059 decreased the number of ICL2, IL5 positive ICL2s, IL-13 positive ICL2s cells and the proliferation of ICL2 in the nasal mucosa of AR mice (Fig. 6D) (Fig. S3E–I). sh-PAC1R or PD98059 also decreased the concentration of IL5, IL-13 and OVA-specific IgE compared with the OVA and PACAP treated mice (Fig. 6E–G). Additionally, H&E results demonstrated that OVA and PACAP-induced upregulation of infiltration of immune cells in nasal mucosa could be reversed by PAC1R knockdown or ERK inhibitor PD98059 (Fig. 6H). We got the similar results about the number of nose scratches, sneezes, and rhinorrhea (Fig. 6I). These data imply that PACAP induces allergic rhinitis via ERK pathway *in vivo*.

4. Discussion

Allergic rhinitis, like asthma, has a higher incidence rate in women, and symptoms such as nasal congestion and runny nose are more significant in women [29]. In addition, the immune system of female mice has unique gender differences, especially estrogen can regulate various inflammatory responses and cell activity, such as promoting Th2 type immune responses, increasing the secretion of IL-5 and IL-13, thereby exacerbating AR related inflammatory responses [30,31]. Furthermore, studies have shown that female mice are frequently used in the construction of AR models and the experimental results have high reproducibility [32,33]. Therefore, in this study, we chose female BALB/C mice to establish an OVA-induced AR model to better simulate the pathological characteristics of human diseases.

Several studies have shown that ILC2s control the nature of immune responses to airway allergens [34]. The expression of the ICL2 markers IL-5, IL-13, IL-25increased in the mouse model with allergic rhinitis, which also positively correlated with disease severity [35]. PACAP not only plays a neuroprotective role in the nervous system, but also participates in various immune regulatory responses [36]. Our study found that number of ILC2s and the concentration of PACAP were elevated in the OVA-induced AR model. However, the mechanism of action between PACAP and ILC2s needs further investigation in Allergic rhinitis.

ILC2 expansion and development need cytokines such as IL-2, TSLP, IL-25, and IL-33. IL-2 production by mast cells leads to expansion of $CD25^{+}$ ILC2 [37]. TSLP are the predominant ILC2 activators that mediate the production of type 2 cytokines [38]. IL-33 plays a critical

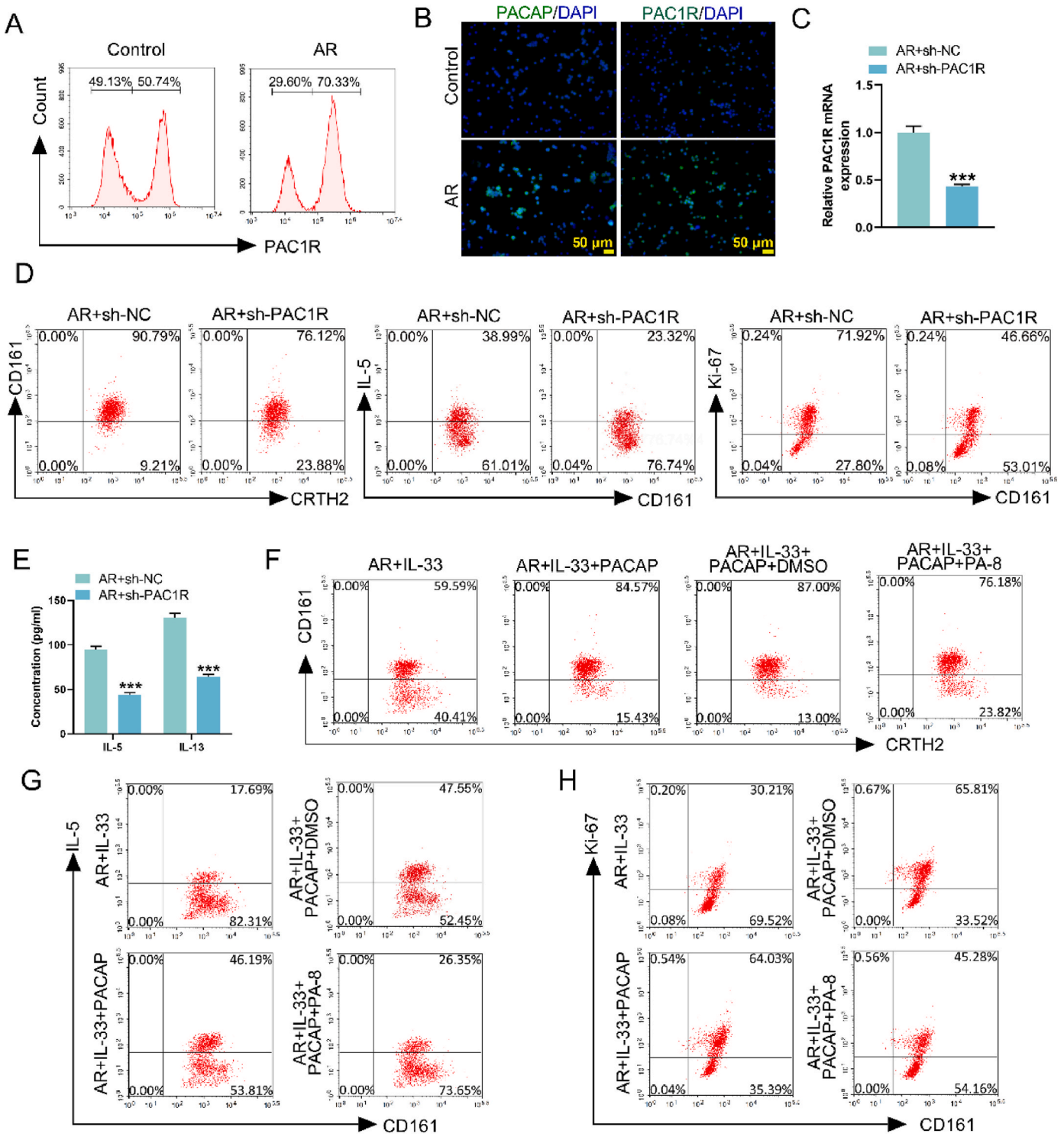


Fig. 3. PACAP induces the production of ILC2s via its specific receptor PAC1R.
(A) Representative flow cytometric analysis of PAC1R expression in ILC2s of Control and AR mice (n = 3). (B) The expression of PACAP and PAC1R was detected by immunofluorescence staining in ILC2s of Control and AR mice (n = 3). (C) The PAC1R mRNA level was analyzed by qRT-PCR in ILC2s after PAC1R knockdown (n = 3, F = 12.80). (D) ILC2s, the proliferation of ICL2s and the number of IL-5 positive ICL2s were assessed by flow cytometry in ILC2s after PAC1R knockdown. Representative flow cytometry plots are showed (n = 3). (E) ELISA was used to analyze the concentration of IL-5 and IL-13 after PAC1R knockdown (n = 3, $F_{IL\text{-}5}$ = 1.850, $F_{IL\text{-}13}$ = 2.483). (F–H) Flow cytometry of ILC2s, IL-5 positive ICL2s and the proliferation of ICL2s in ILC2s after 1 μg/μL IL-33, 15.7 ng/mL PACAP and 10 nmol/L PA-8 treatment for 24 h (n = 3). ***$p < 0.001$ vs AR + sh-NC.

role in the rapid induction of airway contraction by stimulating the prompt expansion of IL-13-producing ILC2, whereas IL-25-induced responses are slower and less potent [39]. ILC2 are GATA3(+) and secrete IL5 and IL13 [27,40]. Our data indicated that IL-33 and PACAP increased IL-5 positive and IL-13 positive ILC2s and promoted proliferation of ILC2. IL-33 was more effective in the promotion of ILC2s proliferation compared with PACAP. These results suggested that PACAP had direct regulation on ILC2s.

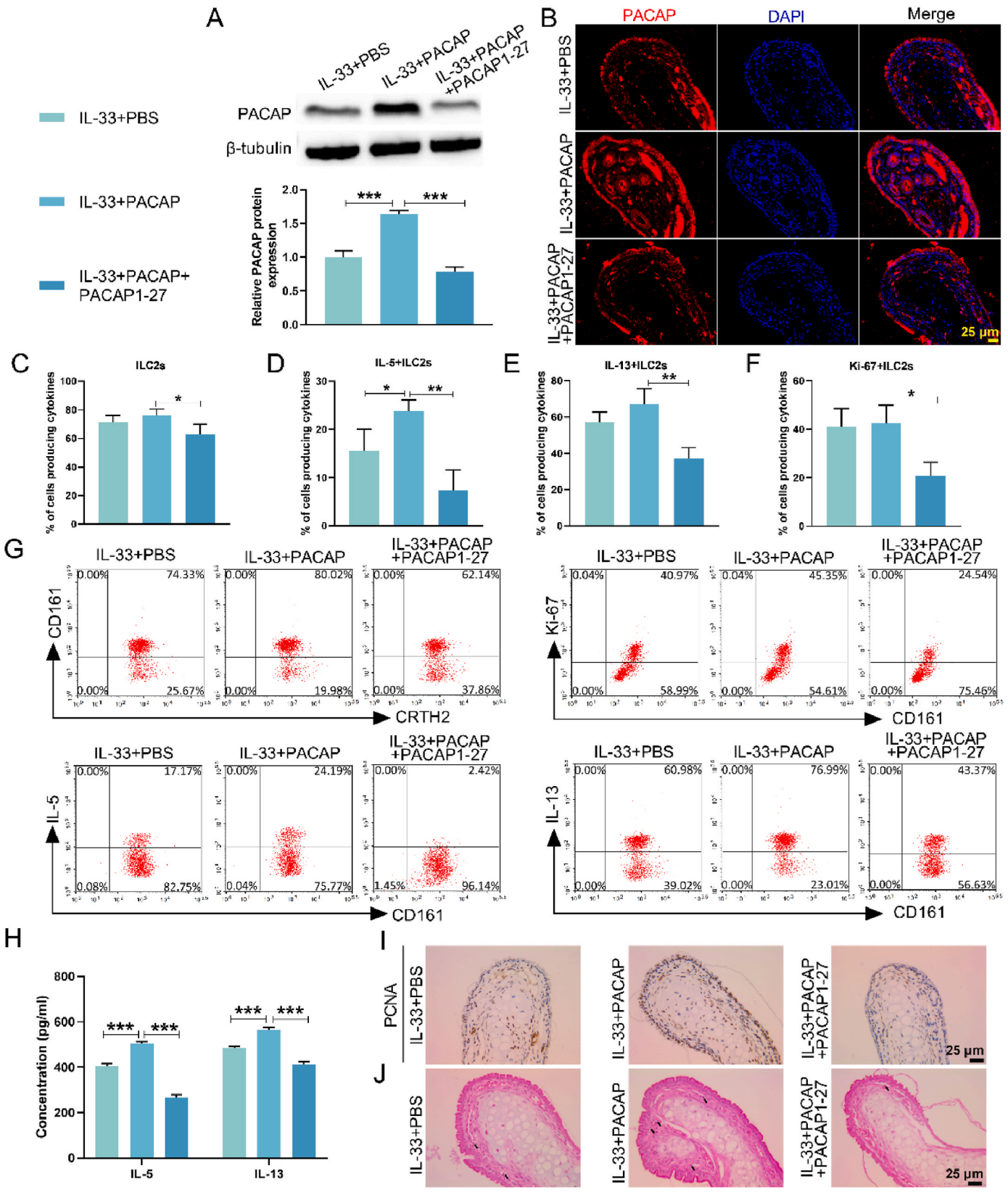


Fig. 4. PACAP antagonist PACAP 1–27 alleviate allergic rhinitis by decreasing number of ICL2s. The WT mice were administered with 6 μL 0.1 mg/mL PACAP liquid intranasally. 10 μL 0.5 μg/mL IL-33 was added to each nostril. PACAP 1–27 were given via inhaled aerosol (0.1 mM, 20 breaths). (A) The expression of PACAP protein was analyzed by Western blot (n = 3, F = 0.1353). (B) The nasal mucosa tissue was immunostained with anti-PACAP antibody and visualized in a fluorescence microscope. (C–G) Representative flow cytometry plots show the number of ICL2s, the number of IL-5 positive ICL2s, the number of IL-13 positive ICL2s and the proliferation of ICL2s (n = 3, $F_{ILC2s} = 0.2067$, $F_{IL\text{-}5+\ ILC2s} = 0.1470$, $F_{IL\text{-}13+\ ILC2s} = 0.08534$, $F_{Ki\text{-}67+\ ILC2s} = 0.07761$). (H) The concentration of IL-5 and IL-13 were detected via ELISA (n = 3, $F_{IL\text{-}5} = 0.1309$, $F_{IL\text{-}13} = 0.1274$). (I) Immunohistochemical staining was used to show the expression of PCNA. (J) H&E staining of the morphological changes in the submucous tissue. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs IL-33+PBS or IL-33+PACAP.

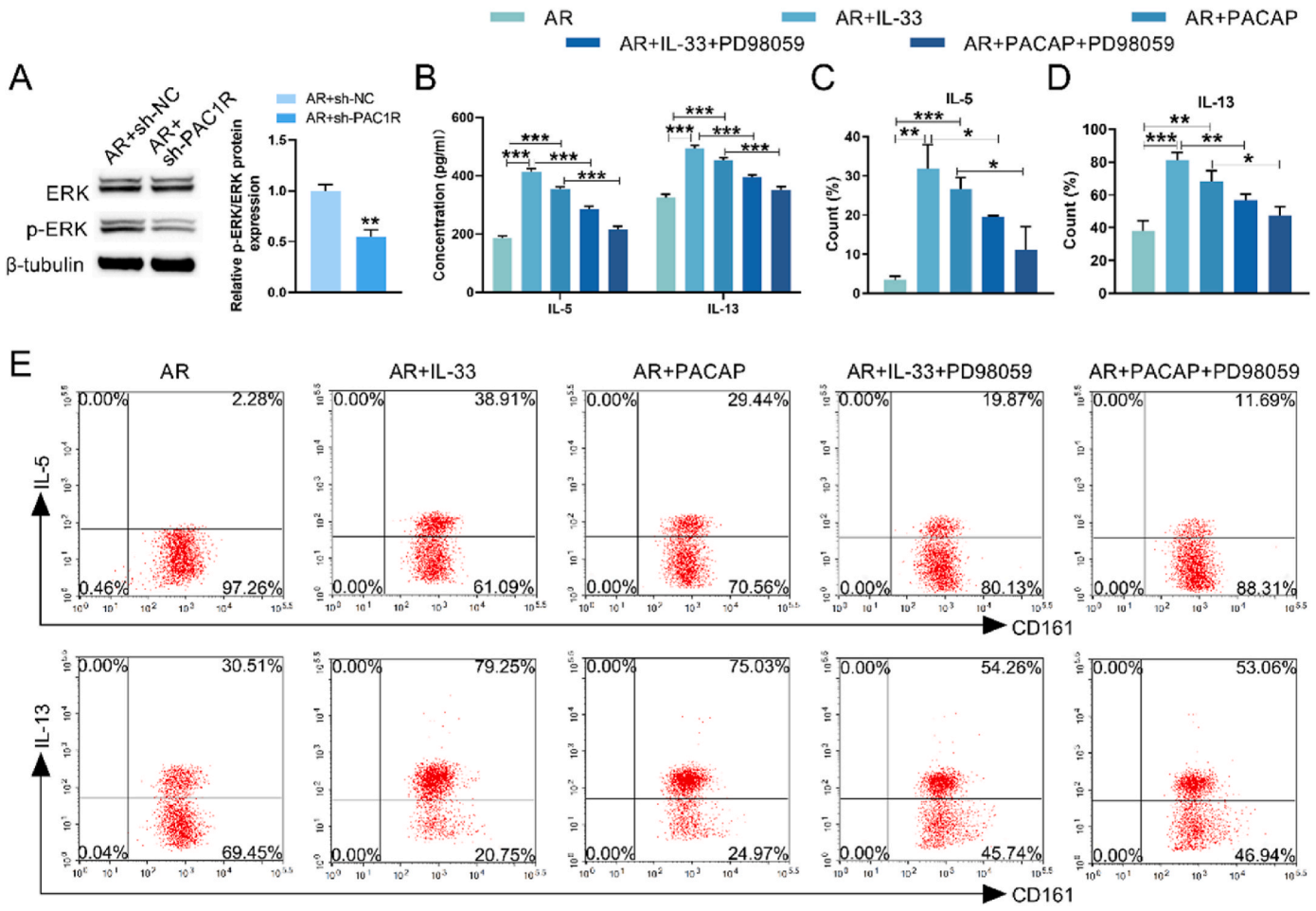


Fig. 5. PACAP induces the production of ILC2s via ERK pathway. (A) Western blot was used to detect the protein expression of ERK and p-ERK in ILC2s after PAC1R knockdown (n = 3, F = 1.118). (B) The concentrations of IL-5 and IL-13 in ILC2s of AR mice were detected by ELISA after 1 μg/μL IL-33, 15.7 ng/mL PACAP and 10 μmol/L PD98059 treatment for 24 h (n = 3, $F_{IL\text{-}5} = 0.2194$, $F_{IL\text{-}13} = 0.08556$). (C–E) Representative flow cytometry plots show the number of IL-5 positive ICL2s and the number of IL-13 positive ICL2s in ILC2s of AR mice after 1 μg/μL IL-33, 15.7 ng/mL PACAP and 10 μmol/L PD98059 treatment for 24 h (n = 3, $F_{IL\text{-}5} = 0.8243$,$F_{IL\text{-}13} = 0.1231$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs AR or AR + IL-33 or AR + PACAP.

Pituitary adenylate cyclase-activating peptide (PACAP) receptor (PAC1R) is a class B Gprotein-coupled receptor (GPCR) that is widely expressed in the human body [41]. Although there are reports that GPCR in regulating allergic rhinitis and allergic inflammation, the role of PAC1R in allergic rhinitis is still lacking [42,43]. Research shows that targeting PAC1 receptors can treat neurological and metabolic diseases [44]. Dong et al. confirmed that PACAP/PAC1R activation could induce the development of Parkinson's disease pain, and targeting PACAP/-PAC1R is an alternative strategy for treating Parkinson's disease pain [45]. In this study, we showed for the first time that the PAC1R affected the pathophysiology and progression of AR in a mouse model. PAC1R knockdown or antagonist PA-8 markedly suppressed ILC2s proliferation and cytokine production. Further experiments demonstrated that PACAP inhibition reduced ILC2 proliferation, thereby alleviating nasal mucosal inflammatory responses, confirming that PACAP exacerbates allergic inflammation through PAC1R-dependent activation of ILC2s.

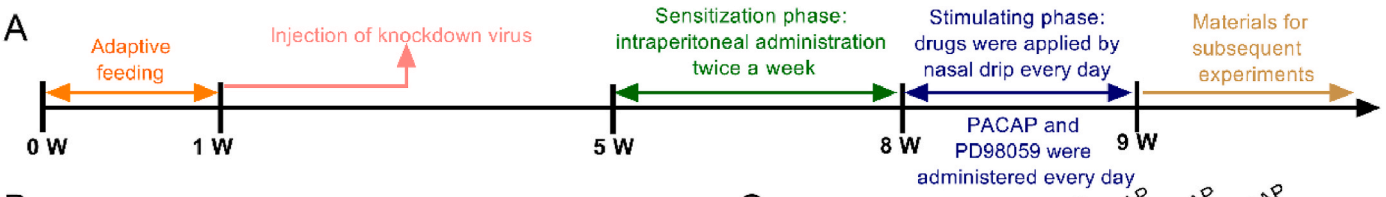
The ERK signaling pathway has been extensively studied in the field of tumor diseases and is considered a potential therapeutic target for cancer [46]. There is evidence to suggest that the ERK pathway also plays an important role in allergic immunity. Shikonin alleviates allergic airway remodeling by inhibiting the ERK–NF–κB signaling pathway, and Imperatorin improves mast cell-mediated allergic airway inflammation by inhibiting the CamKII/ERK signaling pathways [47,48]. Moreover, studies have shown that Neuromedin U induces activation of ILC2s through the ERK pathway in AR. It is currently unclear whether PACAP induces the activation of ILC2s. In our study, PACAP upregulated the expression of p-ERK expression and increased IL-5/IL-13 secretion in ILC2s. However, PAC1R knockdown or ERK inhibition significantly decreased p-ERK expression and ILC2s proliferation, while ameliorating AR pathological features. These results demonstrated that PACAP/-PAC1R activation promoted ILC2s proliferation through ERK pathway in AR.

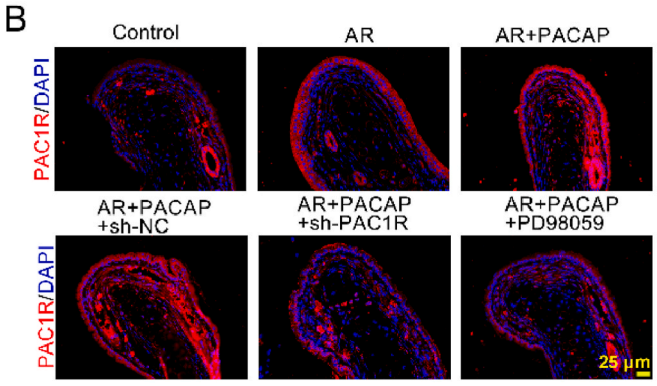
There are several intriguing issues remain unanswered. First, the animal studies observed relatively acute effects. Long-term effect studies are also necessary in the future. Furthermore, whether the IL-33 or PACAP also influences other immune cells needs to be investigated.

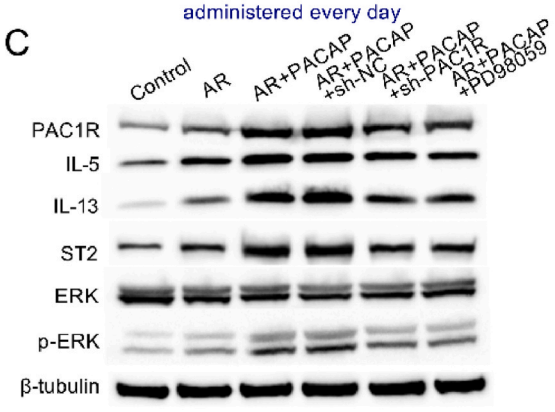
Collectively, the abundance of ILC2s in AR was significantly higher compared with that in controls. The IL-33 and PACAP further increased the frequency of IL-5 positive and IL-13 positive ILC2s in AR mice. Knocking down PAC1R resulted in a substantial reduction in the number of ICL2, IL5 positive ICL2s, IL-13 positive ICL2s cells and the proliferation of ICL2 in the nasal mucosa of AR mice. Our findings revealed that PACAP/PAC1R activation promoted ILC2s proliferation and allergic inflammation through ERK pathway, which provides novel insights into the regulation of ILC2s and potential therapeutic targets in allergic rhinitis.

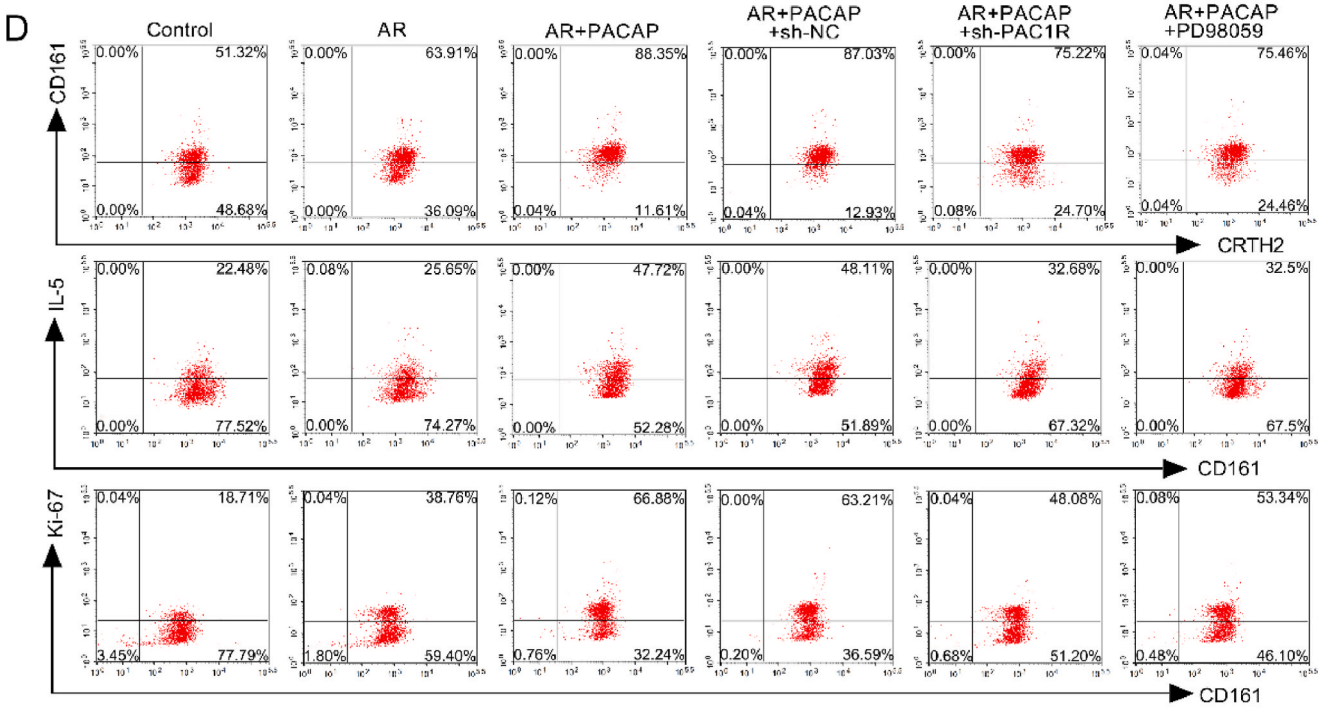
CRediT authorship contribution statement

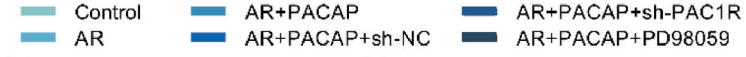
Huigang Wang: Investigation, Conceptualization, Writing – review

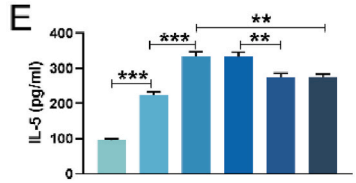


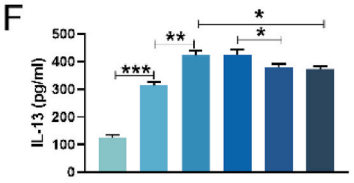


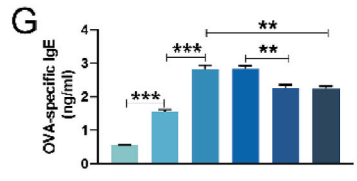


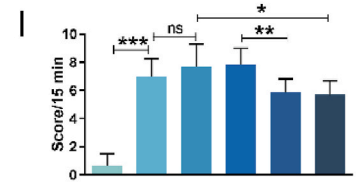












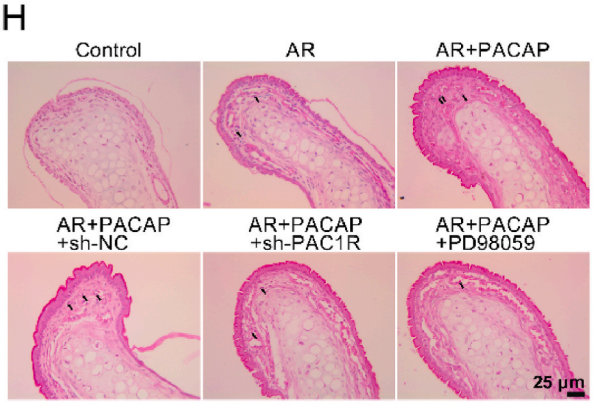


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Fig. 6. PACAP induces allergic rhinitis via ERK pathway *in vivo*. The mice were injected with sh-PAC1R lentivirus and the AR model was induced with OVA. The AR mice were administered with 6 μL 0.1 mg/mL PACAP liquid intranasally. The AR mice were given 1 μmol/mL PD98059 intranasally. (A) A schematic view of the treatment plan. (B) Representative immunofluorescence images of PAC1R. (C) The protein expressions of PAC1R, IL-5, IL-13, ST2, ERK and p-ERK were detected by Western blot (n = 3). (D) Representative flow cytometry plots show the number of ICL2s, the number of IL-5 positive ICL2s, the number of IL-13 positive ICL2s and the proliferation of ICL2s (n = 3). (E–G) ELISA was used to detect the concentration of IL-5, IL-13 and OVA-specific IgE (n = 3, $F_{IL\text{-}5} = 0.3653$, $F_{IL\text{-}13} = 0.2761$, $F_{OVA\text{-}specific\ IgE} = 0.6466$). (H) H&E staining of the morphological changes of the submucous tissue. (I) The sneezing, rubbing, symptom score of Control and AR mice (n = 6, F = 0.6602). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs Control or AR or AR + PACAP or AR + PACAP-sh-NC.

& editing, Formal analysis, Writing – original draft, Data curation. **Yifei Ma:** Writing – review & editing, Data curation, Writing – original draft, Formal analysis, Conceptualization. **Jianyao Li:** Validation, Writing – review & editing, Methodology, Writing – original draft, Formal analysis. **Qingming Bao:** Writing – review & editing, Methodology, Validation, Software. **Guodong Yu:** Project administration, Writing – review & editing, Resources, Funding acquisition, Supervision, Conceptualization.

Ethics approval

All animal experiments were conducted with the approval of the Experimental Animal Ethics Committee of The Affiliated Hospital of Guizhou Medical University.

Consent for publication

Not applicable.

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Competing interest

The authors declare no competing interests.

Abbreviation

AR	Allergic rhinitis
H&E staining	Hematoxylin and eosin staining
IgE	immunoglobulin E
ILC2s	group 2 innate lymphoid cells
IL-5	interleukin-5
IL-13	interleukin-13
OVA	ovalbumin
PACAP	Pituitary Adenylate Cyclase-Activating Polypeptide
qRT-PCR	quantitative real-time PCR analysis

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.abb.2025.110564.

Data availability

All data generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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