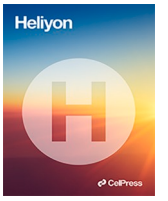
Heliyon 10 (2024) e37512

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Pseudomonas aeruginosa exotoxin A as a novel allergen induced Non-T_H2 inflammation in a murine model of steroid-insensitive asthma

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A R T I C L E I N F O

Keywords:
Allergen
Asthma
IL-17A
Pseudomonas aeruginosa exotoxin A
Steroid-insensitive

A B S T R A C T

Background: Despite the immediate *in vivo* occurrence of anaphylactic and allergic reactions following treatment with *Pseudomonas aeruginosa* exotoxin A (PEA)-based immunotoxins, the immunological role of PEA in asthma pathogenesis remains unclear.

Objective: This study investigated the allergenic potential of PEA and the specific type of asthma induced.

Methods: Recombinant PEA (rPEA) lacking domain Ia (to eliminate non-specific cytotoxicity) was expressed, purified, and employed to detect serum PEA-specific IgE levels in asthmatic patients. Competitive ELISA assays were used to assess rPEA's IgE binding capacity and allergenicity. Additionally, rPEA-challenged C57BL/6 mice were subjected to inflammatory endotyping and therapeutic assays to characterize the allergic nature of PEA.

Results: PEA-specific IgE was identified in 17 (14.2 %) of 120 asthma patients. The rPEA-sensitized and challenged mice had increased PEA-specific immunoglobulins (such as IgE, IgG1 and IgG2a) and developed asthma-like phenotypes with airway hyperresponsiveness, severe airway inflammation, and airway remodeling. Lungs from these mice displayed significant increases in neutrophils and IL-17A$^+$ cells. Innate lymphoid cells (ILCs) produced type 2 cytokines

Abbreviations: AHR, airway hyperresponsiveness; ASM, airway smooth muscle; BALF, bronchoalveolar lavage fluid; DEX, dexamethasone; ELISA, enzyme linked immunosorbent assay; HE, hematoxylin-eosin; ICS, inhaled corticosteroids; IFA, incomplete Freund's adjuvant; ILCs, innate lymphoid cells; LABA, long acting beta-agonists; NETs, neutrophil extracellular traps; PAS, periodic acid-Schiff; PEA, *P. aeruginosa* exotoxin A; RL, lung resistance; rPEA, recombinant PEA; sIgE, specific IgE; TSLP, thymic stromal lymphopoietin; ILC2s, type 2 innate lymphoid cells.

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https://doi.org/10.1016/j.heliyon.2024.e37512
Received 16 April 2024; Received in revised form 4 September 2024; Accepted 4 September 2024
Available online 5 September 2024
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(IL-4, IL-5, and IL-13), whereas Th cells did not. Nonetheless, airway inflammation, rather than hyperresponsiveness, was elicited in non-sensitized mice upon challenge with rPEA. Importantly, rPEA-induced asthmatic mice were unresponsive to dexamethasone treatment.
Conclusion: PEA is a novel allergen that sensitizes asthmatic patients. Furthermore, mice developed steroid-resistant asthma, characterized by an atypical cytokine profile associated with non-T_H2 inflammation, only after being sensitized and challenged with rPEA. These findings suggest a potentially significant role for PEA in asthma development, warranting consideration in clinical diagnosis and treatment strategies.

1. Introduction

Asthma as a chronic inflammatory respiratory disease can be causally grouped into allergic asthma and non-allergic asthma [1]. Alternatively, classification can occur based on the inflammatory cytokine profiles, resulting in type 2 asthma and non-type 2 asthma [2,3]. Allergens that cause asthma have been identified, such as dust mites, pollen, and animal dander [4]. Notably, fungi and bacteria also harbor various allergenic components. Examples include *Aspergillus fumigatus* allergens (Asp f 1, Asp f 2, Asp f 3, Asp f 4, and Asp f 6) and staphylococcal serine protease-like proteins (Spls) from *Staphylococcus aureus*. The mechanisms by which these allergens contribute to asthma pathogenesis are becoming increasingly studied [5–9]. *Pseudomonas aeruginosa,* a Gram-negative bacterium recognized as an opportunistic pathogen, has been isolated from the sputum of 15 % of patients with severe asthma [10]. However, the presence of an allergenic component within *P. aeruginosa* that specifically influences asthma remains unknown.

Pseudomonas aeruginosa exotoxin A (PEA), the most toxic virulence factor of the opportunistic bacterium *P. aeruginosa*, belongs to the mono-ADP-ribosyltransferase family and comprises three structural and functional domains: a receptor binding domain (Ia), a translocation domain (II), and an ADP-ribosyltransferase domain (Ib and III) [11]. When utilized as immunotoxin bullets, PEA induces apoptosis in target cells by catalyzing the ADP-ribosylation and inactivating elongation factor 2 [12]. However, the development of neutralizing antibodies against PEA-based immunotoxins in patients has hindered further treatment. The formation of these antibodies has been associated with serious adverse events, including allergic reactions and anaphylaxis [13]. For example, it has been reported that some patients have demonstrated allergic reactions related to the production of neutralizing antibodies [14]. While PEA's involvement in pneumonia development has been proposed [15], its specific role in asthma remains unclear.

Herein, we sought to detect the presence of PEA-specific immunoglobulins E (IgE) in the serum of patients diagnosed with asthma. Besides, we established a mouse model of asthma specifically triggered by recombinant PEA as an allergen. This model was subjected to comprehensive phenotyping, endotyping, and steroid treatment to elucidate the immunological contribution of PEA to the development of asthma.

2. Methods

2.1. Subjects and sample collections

Serum samples were obtained from 66 healthy controls who underwent a medical examination and 120 asthma patients, all recruited from the Center for Allergy of the Second Affiliated Hospital of Guangzhou Medical University. As established diagnostic criteria for *P. aeruginosa* allergy in asthma patients are lacking, we selected participants meeting the GINA 2020 asthma diagnosis criteria. The recruited asthma patients had no other comorbidities, such as bronchiectasis, bacterial pneumonia and other allergic diseases (allergic dermatitis, etc.). Specific IgEs (sIgE) of rPEA were detected by PEA- or *P. aeruginosa* extract-precoated ELISA. The PEA-sIgE negative cut-off value (mean + 2SD) was calculated based on the ELISA data of PEA-sIgE in control sera (n = 66) (Table S1). An OD value exceeding the cut-off value was considered positive.

2.2. Mice and treatment

Six-to eight-week-old female wild-type C57BL/6 mice were purchased from Zhuhai BesTest Biotechnology Limited Company. All mice were housed under a 12-h light/dark cycle in specific pathogen-free conditions and offered *ad libitum* access to sterile water and irradiated food.

For the asthma model, on days 0, 7, and 14, C57BL/6 mice were intraperitoneally injected with 200 μL PBS for sham group or 200 μL emulsion of rPEA and incomplete Freund's adjuvant (IFA, #F5506, Sigma, USA) for experimental group. On days 21, 22, and 23, the two groups of mice were challenged by nasal drip of 30 μL PBS or rPEA solution without IFA, respectively. In addition, we challenged non-sensitized mice with rPEA using nasal drip administration. The dose of rPEA was 1 mg/kg per mouse (Fig. S1A). Based on the asthma model, therapeutic assay was further conducted by administration of dexamethasone (DEX/Saline, i.p.) on the modeled mice at 1 h before rPEA challenge (Fig. S1B).

2.3. Cloning, expression, and purification of rPEA

The cDNA sequence of PEA was obtained from the National Center for Biotechnology Information (NCBI) database (Gene ID:

877850). Biosune Biology and Technology Company (Shanghai, China) synthesized the coding sequence for rPEA. This sequence lacked the domain Ia to eliminate nonspecific cytotoxicity but contained a Strep II tag at the 3′ terminus for purification. The synthesized rPEA sequence was cloned into the plasmid vector pET-44 EK/LIC (#71144-3, Novagen, Germany) and subsequently transformed into ClearColi® BL21 (DE3) Electrocompetent Cells (#60810-1, Lucigen, USA). These ClearColi® BL21 (DE3) cells are LPS-naught expressing host which would not trigger the endotoxic response. Ideal clones were grown overnight in lysogeny broth (LB) medium containing 100 μg/mL ampicillin. The culture was then diluted 100-fold in prewarmed LB medium supplemented with 100 μg/mL ampicillin, 1 % glucose, 17 mM KH_2PO_4, and 72 mM K_2HPO_4, and incubated at 37 °C until the OD600nm reached 0.8. Protein expression was induced by adding isopropyl-β-d-thiogalactoside to a final concentration of 1 mM, followed by further incubation at 37 °C for 3 h. The cells were harvested by centrifugation and resuspended in 30 mL of ice-cold binding buffer (100 mM Tris-HCl, 1 M NaCl, 1 mM EDTA, pH 8.0). Mild sonication (AMPL 50 %, Pulse 5 s, Off 5 s, 30 min) was used for cell lysis. The lysed supernatant was collected by centrifugation (10,000 rpm, 4 °C, 30 min) and loaded onto a Strep Trap™ HR column (#28-9075-46, GE Healthcare, Illinois, USA). Following equilibration of the column with binding buffer, the bound rPEA was eluted with elution buffer (2.5 mM desthiobiotin dissolved in binding buffer). Elution fractions were collected, dialyzed against PBS, and stored at −20 °C for future use. Protein concentration was determined using the BCA Protein Assay Kit (#23225, Thermo Fisher, USA).

2.4. SDS-PAGE and Western blotting

Following overnight culture, *P. aeruginosa* lysate was obtained using sonication. Recombinant PEA protein or *P. aeruginosa* lysate were each mixed with SDS loading buffer and denatured at 100 °C for 10 min. Samples containing 2.5 μg of rPEA or 30 μg of *P. aeruginosa* lysate per well were loaded onto a 12 % (or 10 %) polyacrylamide gel and subjected to electrophoresis until the bromphenol blue tracking dye reached the bottom of the gel.

For SDS-PAGE analysis, the gels were stained with Coomassie brilliant blue solution (0.25 % (m/v) Coomassie brilliant blue R250, 45 % (v/v) methanol, 10 % (v/v) glacial acetic acid) for 2 h. Gels were then destained overnight in a solution containing 25 % (v/v) methanol and 8 % (v/v) glacial acetic acid. For Western blotting, proteins were transferred from the gel to a PVDF membrane (#1620177, Bio-rad, USA). The membrane was blocked with 5 % bovine serum albumin (BSA) for 2 h at room temperature (RT) and subsequently incubated overnight at 4 °C with either Strep•Tag ® II mouse monoclonal antibodies (#71590-3, Merck KGaA, Darmstadt, Germany, dilution 1:2000), patient sera (dilution 1:20), or PEA-sensitized mouse sera (dilution 1:50). The following day, the membrane was incubated with HRP-conjugated secondary antibodies: HRP-conjugated Affinipure Goat Anti-Mouse IgG antibodies (H + L) (#SA00001-1, Proteintech, USA, dilution 1:4000) or HRP-conjugated mouse anti-human IgE antibodies (#ab99806, Abcam, UK, dilution 1:10000) for 2 h at RT. Finally, the membrane was developed using ECL Western Blotting Substrate (#32106, Thermo Fisher, USA).

2.5. Assessment of airway hyperresponsiveness

Twenty-four hours after the final challenge with PEA or PBS, airway responsiveness to methacholine was assessed using the FinePointe Resistance and Compliance system (DSI-Buxco, St. Paul, MN, USA). Lung resistance (RL) was measured for 5 min following each nebulization step of methacholine (0, 6.25, 12.5, 25, and 50 mg/mL).

2.6. Collection and treatment of mouse sera and bronchoalveolar lavage fluid (BALF)

Following blood collection, sera were obtained by allowing the blood to coagulate and then centrifuging at 4 °C, 3000 rpm for 10 min. Subsequently, endotracheal intubation was performed on the mice. Bronchoalveolar lavage was then performed by flushing the lungs twice with a total of 0.8 mL PBS. The supernatants collected after centrifugation at 4 °C, 500 g for 5 min, were designated as BALF. The precipitates were resuspended in precooled PBS, and the total number of cells was counted. Both sera and BALF were stored at −80 °C for future use.

2.7. ELISA

Total IgE levels in mouse sera were determined by ELISA. Sera were diluted 10-fold and assayed using the IgE Mouse Uncoated ELISA Kit (#88-50460-88, Thermo Fisher, USA) according to the manufacturer's instructions.

The allergenic identity of rPEA was initially verified using a previously described ELISA protocol [16] with human or mouse serum IgE as antibodies. Briefly, 96-well plates were coated overnight at 4 °C with either 5 μg of rPEA, HDM or Bet v1 protein or 20 μg of *P. aeruginosa* lysate diluted in coating buffer (0.1 M Na_2CO_3, 0.1 M $NaHCO_3$, pH 9.6). Following incubation, the plates were blocked with 3 % BSA in PBS. Subsequently, 100 μL of diluted human or mouse sera were added to each well and incubated for 2 h at room temperature. This was followed by further incubation with the appropriate HRP-conjugated secondary antibody: HRP-conjugated mouse anti-human IgE antibodies for PEA-specific human IgE, HRP-conjugated goat anti-mouse IgE antibodies (#PA184764, Thermo Fisher, USA, 1:1000) for PEA-specific mouse IgE, HRP-conjugated goat anti-mouse IgG1 antibody (#ab97240, Abcam, USA, 1:1000) for PEA-specific mouse IgG1, or HRP-conjugated goat anti-mouse IgG2a antibody (#ab97245, Abcam, Cambridge, UK, 1:1000) for PEA-specific mouse IgG2a. After incubation, the reaction was developed colorimetrically using TMB and stopped with 2 M H_2SO_4. The absorbance was immediately measured at 450 nm. Sera dilutions were 1:10 for IgE detection and $1:10^5$ for IgG1 or IgG2a detection.

For competitive ELISA, patient sera diluted 1:10 were preincubated with various concentrations of rPEA or *P. aeruginosa* lysate at 37 °C for 2 h. The preincubated sera then functioned as primary antibodies when added to the plate wells. The remaining steps followed the identical protocol described above for detecting PEA-specific human IgE.

Cytokine levels in the BALF supernatants were measured for IFN-γ, IL-4, IL-5, IL-13, IL-17A, IL-1β, IL-23, IL-33, TSLP, IL-25 and IL-6 using commercially available mouse uncoated ELISA kits according to the manufacturer's instructions. The ELISA kits used were as follows: IFN gamma Mouse Uncoated ELISA Kit (#88-7314-88, Thermo Fisher, USA), IL-4 mouse Uncoated ELISA Kit (#1210402, Dakewe, China; #88-7044-88, Thermo Fisher, USA), IL-5 mouse Uncoated ELISA Kit (#88-7054-88, Thermo Fisher, USA), IL-13 mouse Uncoated ELISA Kit (#88-7137-88, Thermo Fisher, USA), IL-17A (homodimer) Mouse Uncoated ELISA Kit (#88-7371-88, Thermo Fisher, USA), IL-1 beta Mouse Uncoated ELISA Kit (#88-7013-88, Thermo Fisher, USA), IL-23 Mouse Uncoated ELISA Kit (#88-7230-88, Thermo Fisher, USA), IL-33 Mouse Uncoated ELISA Kit (#88-7333-88, Thermo Fisher, USA), TSLP Mouse Uncoated ELISA Kit (#88-7490-88, Thermo Fisher, USA), IL-25 Mouse Uncoated ELISA Kit (#88-7002-88, Thermo Fisher, USA), IL-6 Mouse Uncoated ELISA Kit (#88-7064-88, Thermo Fisher, USA).

2.8. Lung single-cell suspension preparation

Approximately 80 mg of minced lung tissue were incubated with a solution of collagenase (1 mg/mL) and DNase I (0.2 mg/mL) in RPMI 1640 medium (all from Sigma-Aldrich, USA) for 40 min at 37 °C on a rotary shaker. The digested lung tissues were then filtered through a 70 μm cell strainer (#15–1070, Biologix, China). Red blood cells were subsequently removed using RBC lysis buffer (#420301, BioLegend, USA). Finally, the collected cells were washed with precooled 1 × PBS (Ca and Mg free).

2.9. Flow cytometry

Cells from BALF and digested lungs were incubated with Ms CD16/CD32 Pure 2.4G2 (#553141, BD, USA) to block with Fcγ III/II receptors before surface staining. The flow cytometry antibodies used were as follows: FITC Rat Anti-Mouse CD45 (#553079, BD, USA), BB700 Rat Anti-Mouse CD11b (#566416, BD, USA), APC Rat Anti-Mouse Ly-6G (#560599, BD, USA), PE Rat Anti-Mouse Siglec-F (#552126, BD, USA), BV421 Rat Anti-Mouse F4/80 (#565411, BD, USA), PE-Cy7 Rat Anti-Mouse TCRγδ (#118124, BioLegend, USA), APC Rat Anti-Mouse TCRβ (#109212, BioLegend, USA), BV421 Rat Anti-Mouse CD127 (#135027, BioLegend, USA), PE Rat Anti-Mouse IFN-γ (#505808, BioLegend, USA), BV421 Rat Anti-Mouse IL-4 (#504127, BioLegend, USA), BV421 Rat Anti-Mouse IL-5 (#504311, BioLegend, USA), PE Rat Anti-Mouse IL-13 (#159403, BioLegend, USA), PE Rat Anti-Mouse IL-17A (#506904, BioLegend, USA).

The flow cytometry gating strategies for neutrophils and eosinophils are shown in Fig. S2A. The total number of neutrophils or eosinophils in BALFs was calculated based on the proportion of these cell types within the total number of single cells enumerated by flow cytometry. Cells from digested lungs were incubated with a Cell Activation Cocktail (#423303, BioLegend, USA) for 6 h to promote further cytokine production by the cells (Fig. S3). The cells were then treated with the Zombie Aqua™ Fixable Viability Kit (#423102, BioLegend, USA) to distinguish living cells before blocking. For intracellular cytokine staining, cells were fixed and permeabilized with the FIX & PERM™ Cell Permeabilization Kit (#GAS004, Thermo Fisher, USA), and then incubated with flow cytometry antibodies against the cytokines as described above. Absolute cell counts were calculated on the basis of Precision Count Beads™ (#424902, BioLegend, USA). Data were obtained on a flow cytometer (BD Biosciences Fortessa) and analyzed with FlowJo software.

2.10. Lung tissue histopathology

Following euthanasia, the mice's left lungs were immersed in 4 % paraformaldehyde for 24 h and subsequently embedded in paraffin. Lung sections (4 μm thick) were obtained using a Leica microtome 2030 (Leica Microsystems Nussloch GmbH, Nussloch, Germany). The sections were then stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) for histopathological assessment performed in a blinded manner. Airway inflammation was graded semi-quantitatively based on the following criteria: 0, normal; 1, few cells; 2, a ring of inflammatory cells that was 1 cell-layer deep; 3, a ring of inflammatory cells that was 2–4 cell-layers deep; and 4, a ring of inflammatory cells that was >4 cell-layers deep.

2.11. Statistical analysis

Data were analyzed using GraphPad Prism version 8.3.0. An unpaired *t*-test was used for normally distributed data, and a Mann-Whitney test was used for non-normally distributed data. Multiple comparisons were analyzed using one-way ANOVA. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Positivity of PEA-specific IgE in asthma patients

The rPEA, lacking domain Ia to eliminate nonspecific cytotoxicity, was expressed, purified, and identified following established procedures (Fig. 1A & S4). To investigate the positivity of rPEA-specific IgE (sIgE) in patients, sera from 120 asthmatics were included

and rPEA-sIgE was detected by ELISA. The results revealed that 17 out of the 120 sera (14.2 %) tested positive for rPEA-specific IgE (Fig. 1B–Table S2). All but two patients with PEA-specific IgE-positive asthma had specific IgE for HDM and/or Bet v1 (Table S2). No correlation was found between total IgE and rPEA-specific IgE, a finding that corroborates the validity of the specific IgE test (Fig. S5).

To further confirm the ability of rPEA to bind IgE, six randomly selected positive sera were used in Western blot analysis. Immunoblots revealed distinct or slight rPEA-sIgE positive bands on the membrane (Fig. 1C). According to the competitive ELISA assay, the sIgE binding to immobilized rPEA was gradually blocked along with the ramp concentration of rPEA protein pre-incubated with the sIgE-contained sera, and the inhibition efficiency of rPEA (10 μg/mL) ranged from 40 % to 75 % (Fig. 1D). The reduced signal in Fig. 1D indicates that rPEA could work as an allergen to inhibit IgE binding in the analyzed sera.

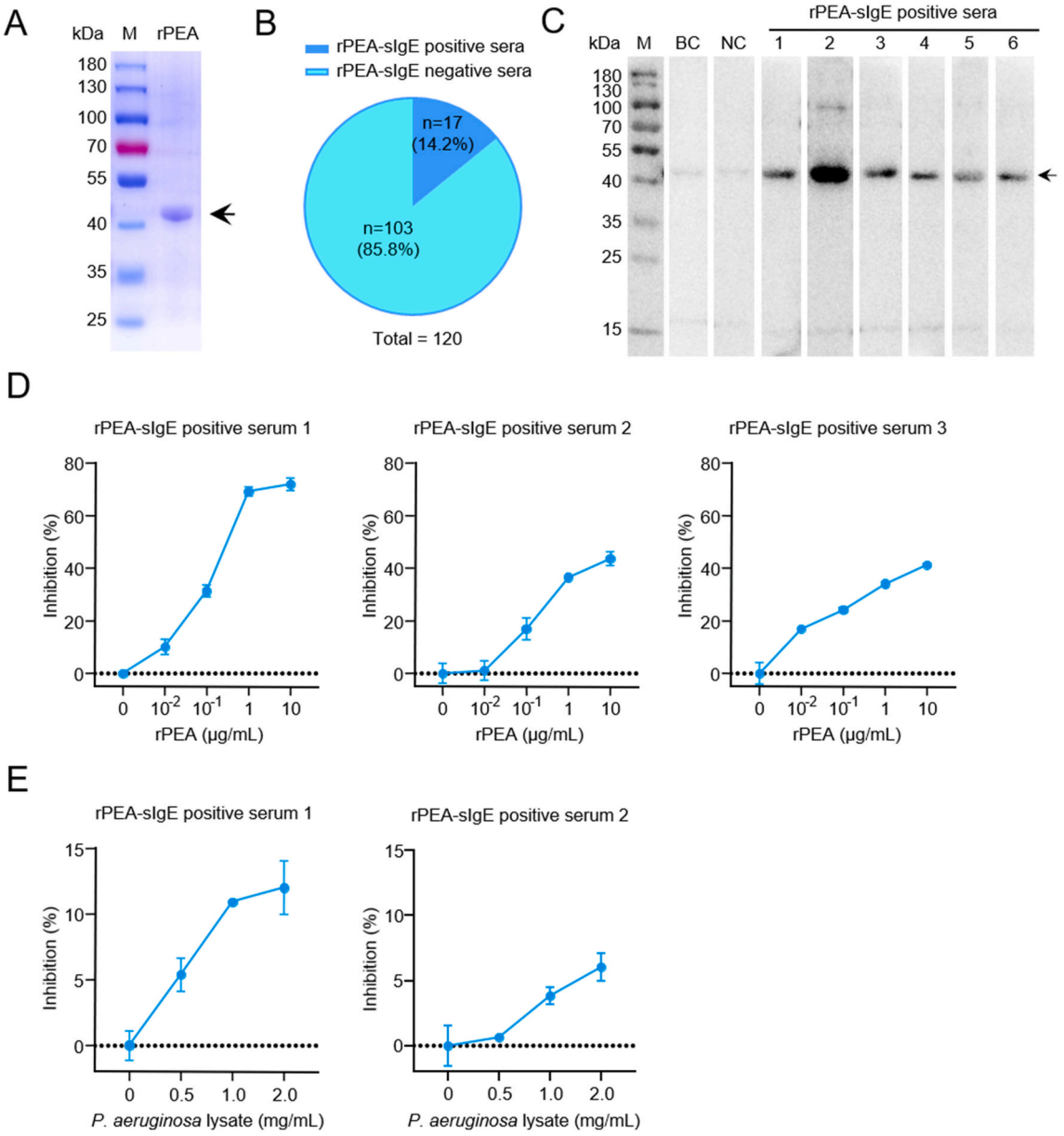

Fig. 1. Prokaryotic expression of rPEA and its serological identification in asthma patients. (**A**) The purity and apparent molecular weight of rPEA were determined by Coomassie brilliant blue. The target protein bands are located between 40 and 55 kDa. (**B**) Pie chart showing the proportion of positive and negative rPEA-sIgE in 120 asthma patients' sera. (**C**) Immunoblotting of IgE binding to rPEA using sera from six rPEA-sIgE positive patients (lanes 1–6). Samples of 2.5 μg per lane of rPEA was separated by electrophoresis on 12 % SDS-PAGE prior to transfer to PVDF membrane. Blots were incubated in patient sera (1:20) and then incubated with HRP-conjugated mouse anti-human IgE antibodies and visualized with ECL. M: molecular weight marker. BC: Blank control. NC: Negative control. **(D) and (E)** Specific IgE competitive ELISA assay. The rPEA-sIgE positive serum was incubated with increasing doses of rPEA (**D**) or *P. aeruginosa* lysate (**E**). The figure is shown as the inhibition of sIgE binding to plate-bound rPEA. The full, non-adjusted images of gels and blots are presented in Fig. S7.

To further examine the component allergenicity present in the lysate of *P. aeruginosa*, rPEA sensitized mouse sera were performed as primary antibody for immunoblotting analysis. It was evidenced that the lane of *P. aeruginosa* lysate appeared a band at 55–70 kDa (Fig. S6), which was consistent with the predicted size of natural PEA protein (nPEA, 66 kDa) [17]. Additionally, the IgE reactivity to recombinant PEA was inhibited by ~10 % by *P. aeruginosa* lysate (Fig. 1E). These results confirm that the recombinant PEA protein is substantially equivalent to the corresponding fragment of the natural PEA in *P. aeruginosa*, and that *P. aeruginosa* originated PEA could be a novel allergen for humans.

3.2. Recombinant PEA induces asthma-like phenotype in sensitized mice

To confirm the capacity of rPEA to induce allergic reactions, we investigated various pathological features in a mouse model using the rPEA protein. As anticipated, exposure to rPEA significantly increased airway responsiveness to methacholine in sensitized mice (Fig. 2A). Additionally, total cell counts in BALF were significantly higher in rPEA-treated mice than in controls (Fig. 2B). Flow cytometry analysis of cells obtained from mouse BALF revealed greater recruitment of neutrophils than eosinophils in the lungs (Fig. 2C & S2A).

H&E staining of lung tissue sections revealed typical pathological features of asthma in the rPEA group compared to the control group (Fig. 2D and E). These features included significant inflammatory cell infiltration in the lung parenchyma and surrounding airways, as well as airway epithelial cell proliferation and epithelial shedding (Fig. 2D and E).

Subsequently, airway remodeling, another pathological feature of asthma, was also observed in the rPEA group. Goblet cell hyperplasia, a hallmark of airway remodeling, was evident in the lungs of mice from the experimental group (Fig. 2F and G). Meanwhile,

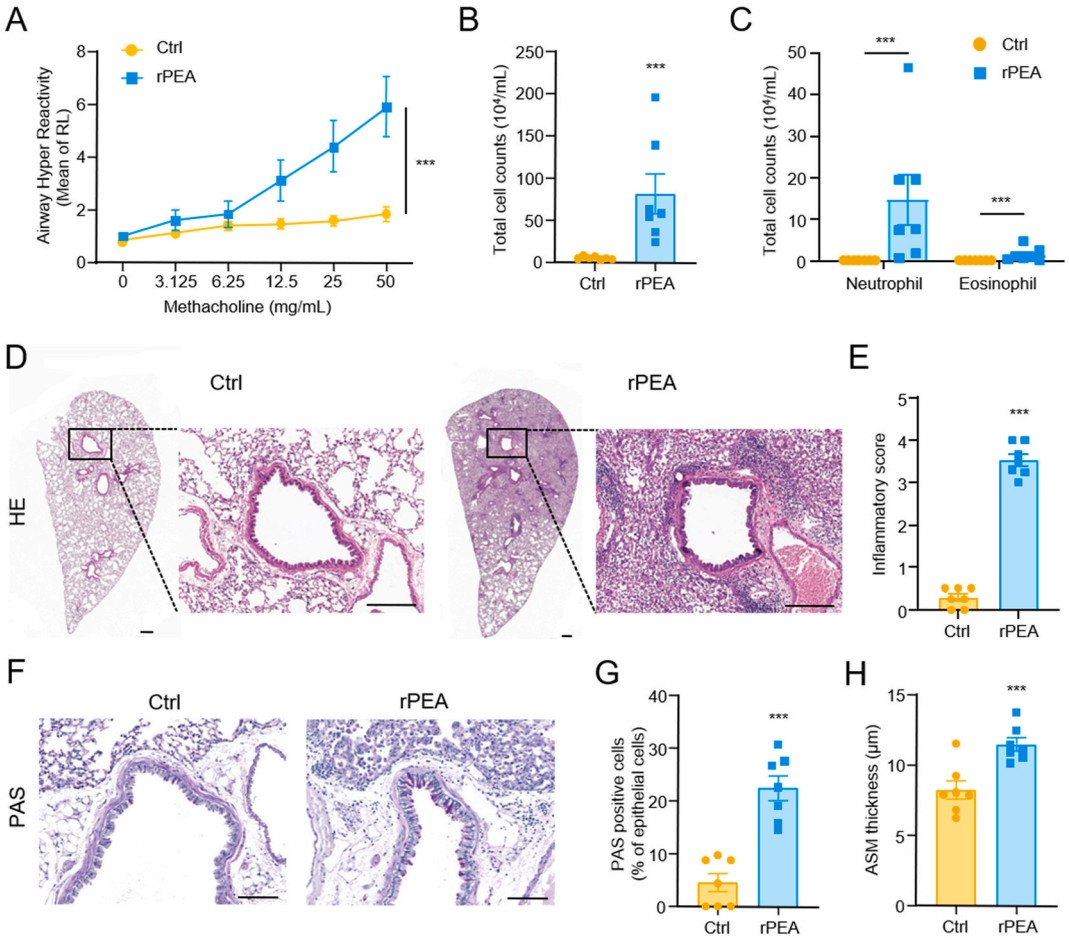

Fig. 2. Asthma-like pathological characteristics of rPEA-sensitized and challenged mice. (**A**) The airway hyperresponsiveness of mice was measured by lung resistance (RL) (n = 4–5). (**B**) Numbers of total inflammatory cell in bronchoalveolar lavage fluid (BALF) (n = 7). (**C**) Numbers of neutrophils and eosinophils in BALF (n = 7). (**D**) Representative HE-stained lung sections of mice. Scale bar: 200 μm. (**E**) Score of airway inflammation (n = 7). (**F**) Periodic acid-Schiff (PAS) stained lung sections of mice. Scale bar: 100 μm. (**G**) Quantification of PAS staining. The percentage of PAS positive cells in epithelial cells was calculated (n = 7). (**H**) Analysis of airway smooth muscle (ASM) thickness (n = 7). ***$p < 0.001$, Bar, mean ± SEM.

according to the measurement of HE-stained lung section, the airway smooth muscle (ASM) of rPEA-challenged mice was significantly thicker than that of the control group (Fig. 2H). These findings collectively demonstrate that rPEA can induce asthma-like pathology in sensitized mice.

3.3. Non-T_H2 airway inflammation develops in sensitized mice challenged with rPEA

To gain insight into the endotypes of sensitized mice challenged with rPEA, we investigated various indicators of the immune response. Levels of both total IgE and rPEA-specific IgE were significantly increased in the sera of these mice (Fig. 3A). In addition, levels of specific IgG1 and IgG2a were also elevated in rPEA-challenged mice compared to controls (Fig. 3B). Moreover, the expression of cytokines IL-1β, IL-33, TSLP, IL-25, IL-4, IL-5, IL-13, and IL-17A was significantly up-regulated in the BALF of rPEA-challenged mice (Fig. 3C).

To elucidate the cellular sources of the inflammatory response, we investigated the abundance of innate lymphoid cells (ILCs), αβ T cells, and γδ T cells in mouse lung tissues using flow cytometry (Fig. 4A, Fig. S2B). The abundance of ILCs expressing various cytokines (IL-17A, IL-4, IL-5, and IL-13) was significantly increased in rPEA-challenged mice compared to controls (Fig. 4B). Notably, the percentage of IL-17A-positive cells within the αβ T cell population showed a significant rise in rPEA-challenged mice (Fig. 4B). Interestingly, the increase in the abundance of cells positive for IL-4, IL-5, and IL-13 was observed only in ILCs from rPEA-challenged mice, not in αβ T cells (Fig. 4B). This suggests that IL-4, IL-5, and IL-13 are primarily secreted by ILCs in this model, while IL-17A mainly originates from both ILCs and αβ T cells. Consequently, these findings indicate that the observed asthma-like inflammation can be characterized as non-T helper cell type 2 (T_H2) airway inflammation.

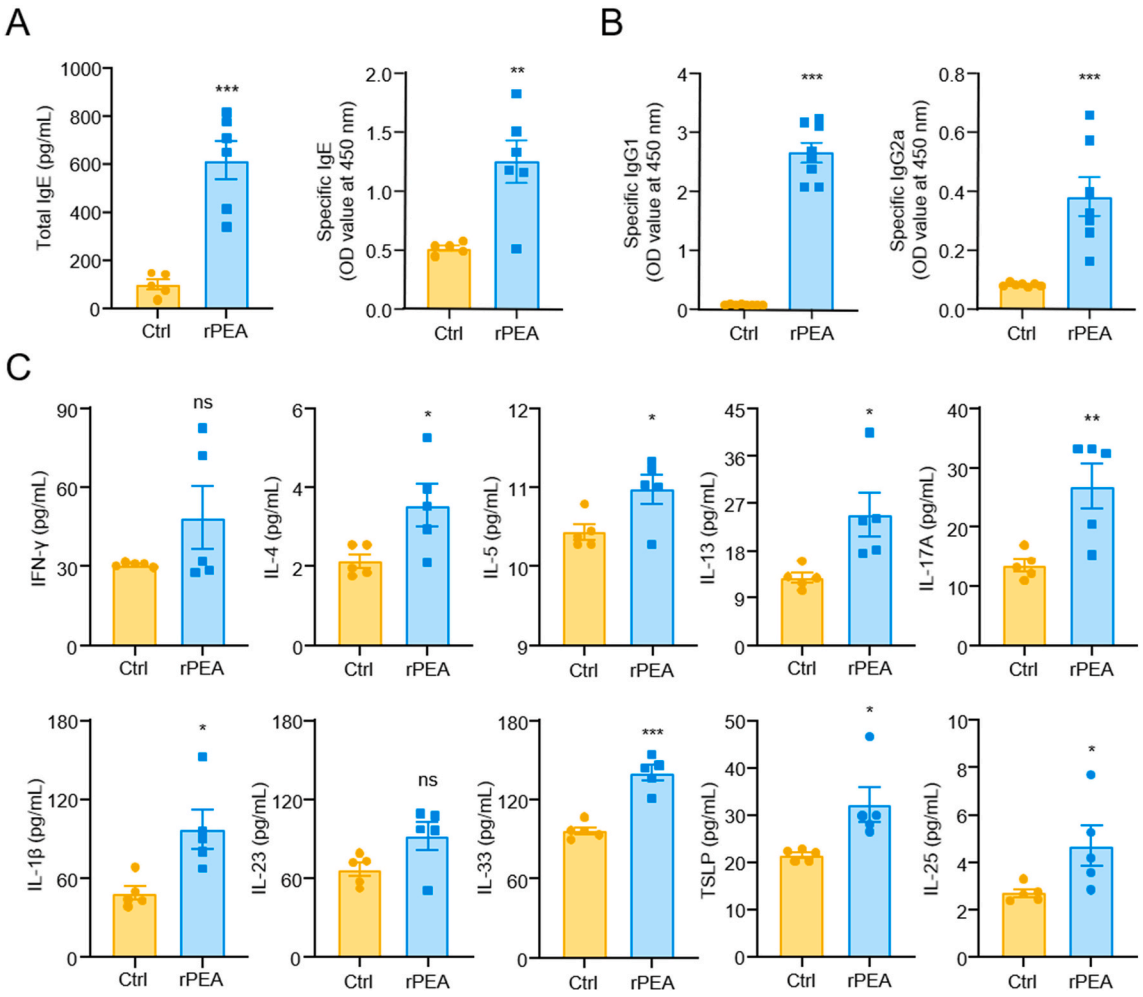

Fig. 3. Levels of serum immunoglobulin E and BALF cytokines from rPEA-sensitized and challenged mice. (**A**) The total IgE and rPEA-specific IgE in sera (n = 5–6). The sera were diluted with 1:10. (**B**) Detection of rPEA specific IgG1 and IgG2a in sera (n = 7). The serum was diluted with $1:10^5$. (**C**) Levels of IFN-γ, IL-4, IL-5, IL-13, IL-17A, IL-1β, IL-23, IL-33, TSLP, and IL-25 in BALF (n = 5). ns, not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Bar, mean ± SEM.

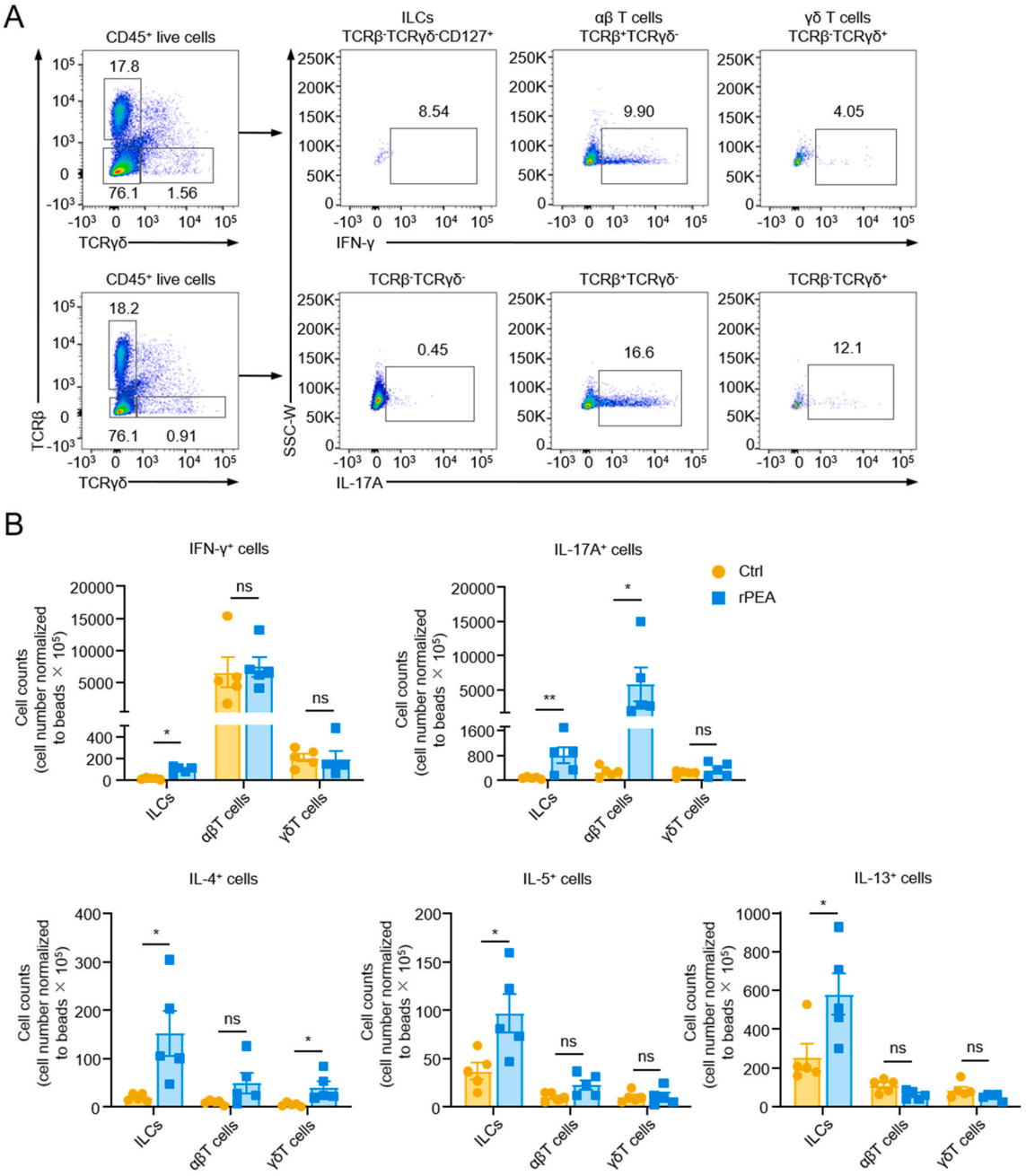

Fig. 4. A single-cell suspension of lung tissue from mice that had been sensitized and challenged with rPEA was investigated by flow cytometry. (**A**) Flow cytometry gating strategy for representative cytokines (IFN-γ and IL-17A) of innate lymphoid cells (ILCs), αβ T cells, and γδ T cells in the lung tissue. It is worth noting that NK cells need to be excluded with CD127 before evaluating IFN-γ of ILCs. The gating strategy of other cytokines (IL-4, IL-5 and IL-13) was the same as that of IL-17A (seen Fig. S2B). (**B**) Absolute number of IFN-γ, IL-4, IL-5, IL-13, IL-17A positive cells from ILCs, αβ T and γδ T cells (n = 5). ns, not significant, $*p < 0.05$, $**p < 0.01$. Bar, mean ± SEM.

3.4. Recombinant PEA induces inflammation but not airway hyperresponsiveness in non-sensitized mice

In addition, we performed experiments in which non-sensitized mice were challenged with rPEA. It is interesting to note that no difference in airway hyperresponsiveness was observed between the rPEA-challenged murine group and the control group (Fig. 5A). Nonetheless, a marked increment in the aggregate inflammatory cell population, including neutrophils but excluding eosinophils, was detected within the BALF of rPEA-challenged mice (Fig. 5B and C). H&E staining of lung tissue sections from rPEA-challenged mice revealed significant infiltration of inflammatory cells in the lung parenchyma and surrounding airways (Fig. 5D and E). Flow

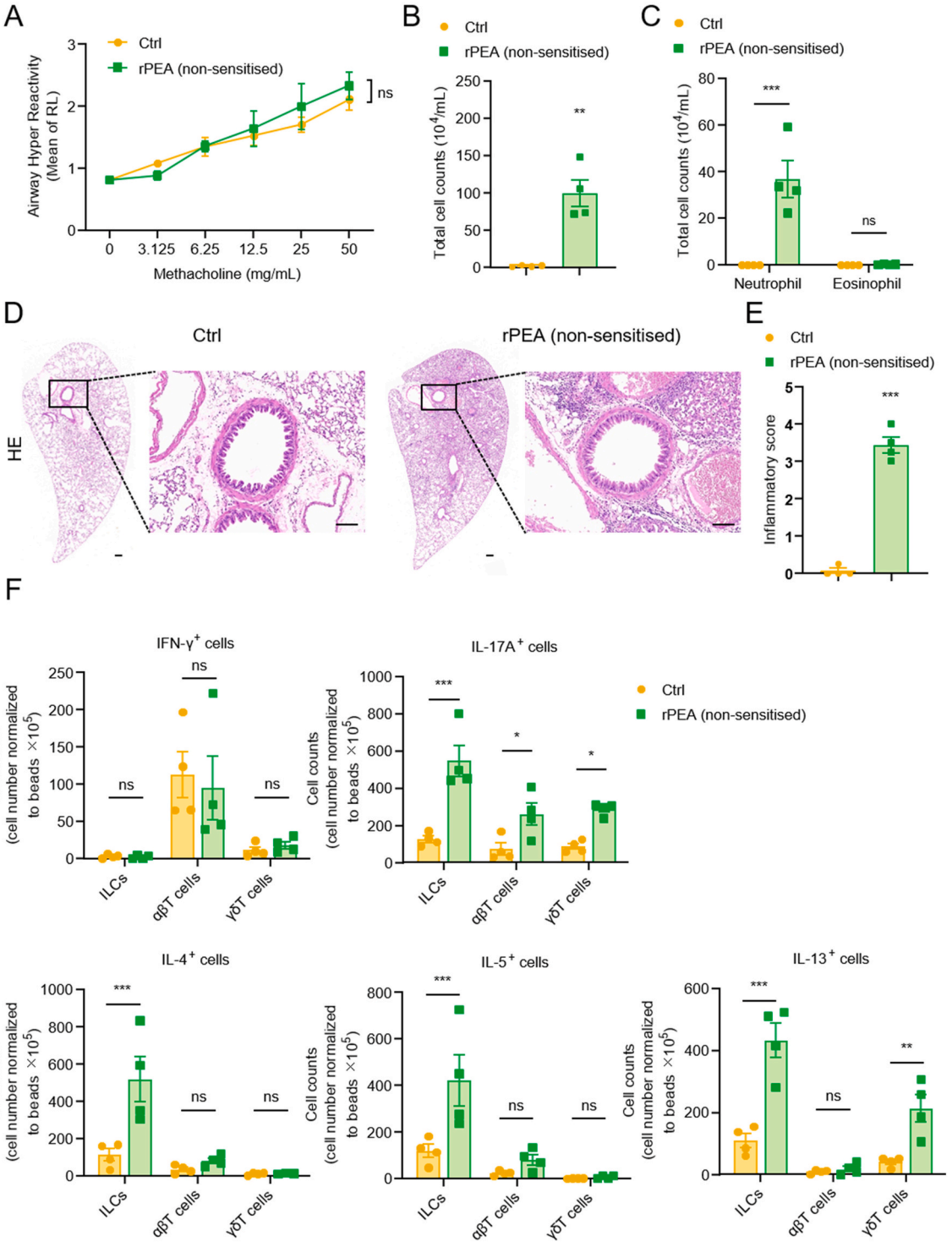

Fig. 5. Measures of airway hyperresponsiveness and lung inflammation in non-sensitized mice challenged with rPEA. (**A**) The airway hyperresponsiveness of mice was measured by lung resistance (RL) (n = 3–4). (**B**) Numbers of total inflammatory cell in bronchoalveolar lavage fluid (BALF) (n = 4). (**C**) Numbers of neutrophils and eosinophils in BALF (n = 4). (**D**) Representative HE-stained lung sections of mice. Scale bar: 200 μm. (**E**) Score of airway inflammation (n = 4). (**F**) Absolute number of IFN-γ, IL-4, IL-5, IL-13, IL-17A positive cells from ILCs, αβ T and γδ T cells (n = 5). ns, not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Bar, mean ± SEM.

cytometry revealed a large increase in the number of ILCs expressing IL-4, IL-5, IL-13, and IL-17A, while the increase in the number of αβT cells and γδ T cells expressing IL-17A was relatively less significant (Fig. 5F). These data suggest that sensitization is required to induce airway hyperresponsiveness, but not to induce inflammation, such as the activation of ILCs and γδ T cells.

3.5. Recombinant PEA-induced asthmatic mice are insensitive to hormones

To assess the efficacy of DEX in the rPEA-induced asthma model, mice in the rPEA + DEX group were pre-treated with DEX (1 mg/kg) via intraperitoneal injection daily for 1 h before rPEA challenge (Fig. S1B). DEX treatment did not suppress the airway hyperresponsiveness (AHR) exacerbated by rPEA (Fig. 6A). Total cell counts in BALF remained significantly elevated to similar levels in both the rPEA + DEX and rPEA groups (Fig. 6B). This pattern was also observed for eosinophil and neutrophil counts in the BALF (Fig. 6C). Furthermore, DEX exhibited no inhibitory effect on inflammatory infiltration or goblet cell hyperplasia in the lungs of rPEA-challenged mice (Fig. 6D–F). ASM thickness remained unchanged following DEX administration in the rPEA group (Fig. 6G). Similarly, DEX treatment did not alter the expression levels of cytokines (IFN-γ, IL-4, IL-6, IL-17A, and IL-1β) in the BALF of rPEA-induced asthmatic mice (Fig. 6H). Collectively, these findings suggest that rPEA-induced asthma is resistant to steroid therapy.

4. Discussion

The immunogenicity of PEA has been recognized as a significant challenge in the development of recombinant targeted therapies [13]. Motivated by this observation, we investigated the potential role of PEA in triggering immune responses. Notably, limited research has explored the association between PEA and allergic diseases. Therefore, we expressed and purified rPEA protein (domain Ia deleted to eliminate the nonspecific cytotoxicity) and then identified rPEA-sIgE in the serum of patients with asthma for the first time (Fig. 1 & S4 & S6). These findings collectively provide hitherto undocumented evidence that rPEA is a novel allergen.

While there has been extensive research on isolating *P. aeruginosa* from asthmatics and targeting *P. aeruginosa* infection with antibiotics [10,18–20], less attention has been paid to the potential role of specific *P. aeruginosa* components in asthma development and progression. The colonization of *P. aeruginosa* in asthmatics is generally not unique, and it often colonizes airways with other bacteria [18]. Patients who underwent skin testing against *P. aeruginosa* lysates also tended to have a reaction to other bacterial lysates (data not shown). Furthermore, data on allergy to *P. aeruginosa* in asthmatic patients is scarce. Given that this study involved asthma patients who had not previously been identified as having an allergy to *P. aeruginosa*, it is possible that the specific IgE detected was the result of an allergen cross-reaction. Additionally, there is a problem here that the presence of specific IgE in some sera does not yet prove that PEA is a relevant allergen for human asthma pathogenesis. To address these limitations, we employed a mouse model to validate PEA's role as an allergen. More clinical studies are needed to demonstrate the sensitization probability of PEA in asthma patients and to rule out cross-reaction between PEA and other allergens in the future. This study opens avenues for exploring additional bacterial allergens. In addition, PEA may be used as a marker for clinical diagnosis of *P. aeruginosa* allergy. It is used to perform skin tests on patients or to detect specific IgE in patients.

Over the past decades, asthma has been classified into allergic and non-allergic subtypes based on IgE diagnoses. However, the presence of specific IgE does not rule out that PEA contributes to asthma through other mechanisms unrelated to IgE. It is now firmly accepted that asthma is a heterogeneous disease, varying in endotypes of cellular and molecular profile and treatment response. "Strong T_H2-related inflammation" is further replaced by "type 2 inflammation" which involves both the innate (type 2 innate lymphoid cells) and adaptive (T-helper type 2 cells) immune systems. Epithelial cell alarmins such as thymic stromal lymphopoietin (TSLP), IL-33, and IL-25 become the star-shining molecules involved in the pathogenesis of asthma [21,22]. On the other hand, "type 2 low" asthma is estimated to account for one-third of severe asthma and presents a challenge to clinicians [1,23]. Nevertheless, allergy never loses its IgE and T_H2 signature and "type 2 low" seems to be equal to "non-allergic" or "non-eosinophilic". This belief has long hampered the precise treatment of asthma and other airway diseases for decades.

In the present study, the pathogenesis of modeled asthma follows the pathways involving ILCs, γδ T cell, T_H17 beyond the alarmins pathways. Notably, the asthma subtypes exhibit dominant IL-33, IL-1β, IL-17A, and type 2 cytokines such as IL-5, IL-13, and IL-4 relatively low but significantly obvious, which comes from type 2 innate immune cells, rather than T_H2 cells. These endotypes are different from those obtained by Chu DK et al., in 2013 that T_H2 inflammation was only observed in mite and peanut allergies which mainly triggered also by IL-33, but not thymic stromal lymphopoietin or IL-25 [24]. IL-33 is a versatile cytokine that plays a critical role in a variety of biological responses and a variety of human diseases. Especially, IL-33 and its receptor ST2 are associated with asthma susceptibility [25]. Type 2 cytokines produced by type 2 innate lymphoid cells (ILC2s) are known to initiate and amplify airway inflammation in these diseases through the activation of eosinophils, B cells, mast cells, macrophages, fibroblasts, and epithelial cells [26]. Hence, these cocktail cytokines would exacerbate not only asthma, but also other airway conditions, including allergic rhinitis, chronic rhinosinusitis with nasal polyps, etc. [26].

While "type 2 inflammation" suggests effective treatment with inhaled corticosteroids (ICS) and LABA (long-acting β2 agonist) in asthma, there remains a significant proportion of people with "type 2-low" or "type 17" severe asthma, with bad treatment response even with biologic agents targeting the major type 2 cytokines. The present study showed that PEA induces IL-17A secretion from αβT cells and ILCs, as well as type 2 inflammation from ILC2s and γδ T cells. The activation of ILCs and γδ T cells is largely independent of the sensitization process and plays an important role in the development of inflammation in asthma. It is important to note that type 2 cytokines were not detected in αβT cells (αβT cells including Th cells) of the model mice, that is, T_H2 cells were not present. This means that T_H2 cells do not contribute to the fancy profile of PEA-induced inflammation. We additionally demonstrate that in the constructed asthma model, rPEA-sensitized and challenged mice develop airway hyperresponsiveness, severe airway inflammation, and airway

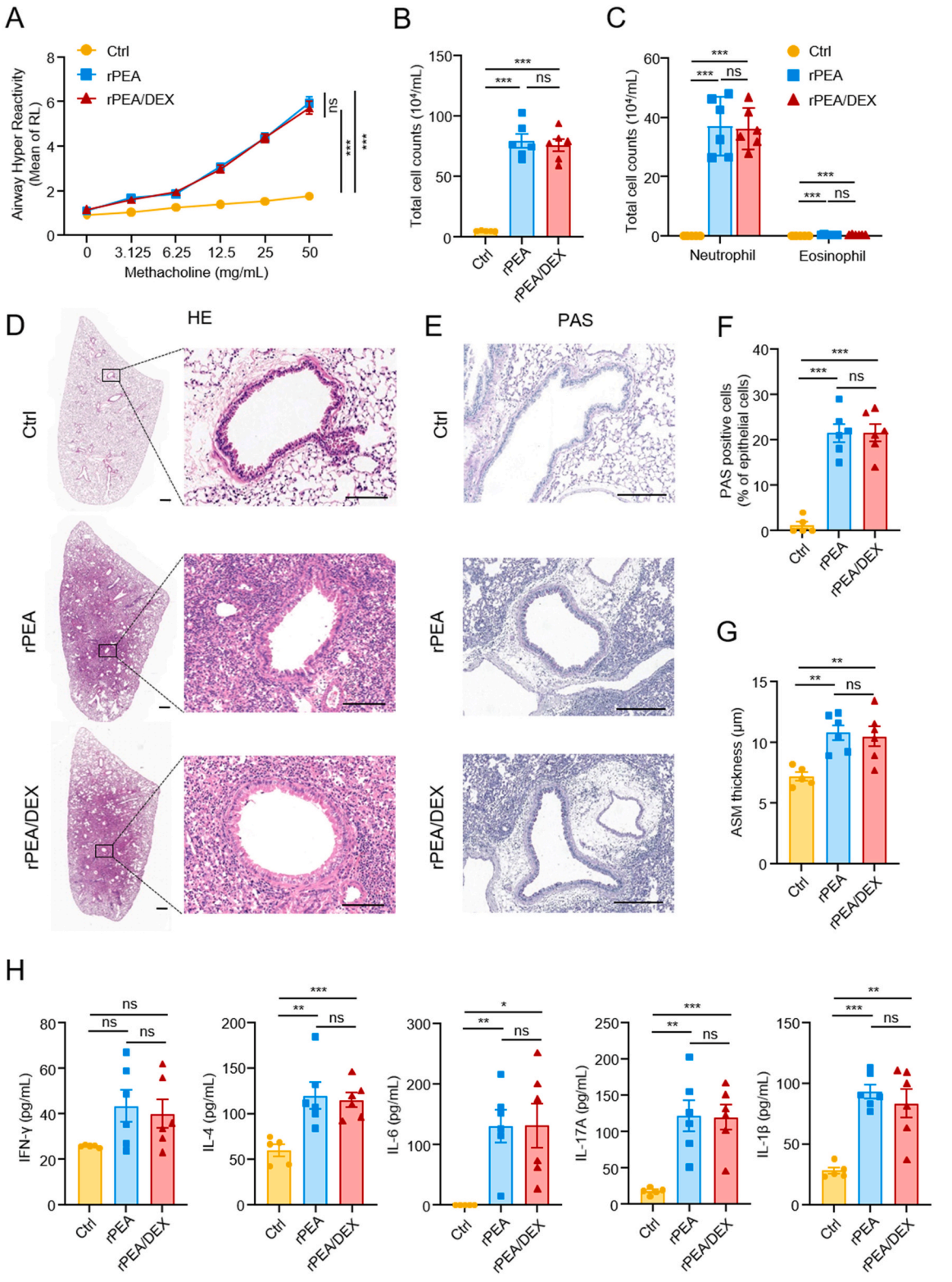

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Fig. 6. Asthma in mice induced by rPEA exposure are corticosteroid resistant. Dexamethasone (1 mg/kg) or PBS was daily administered i.p. 1 h before rPEA challenge on days 21–23. (**A**) The AHR of mice was measured by lung resistance (RL) (n = 5–6). (**B**) Numbers of total inflammatory cell in bronchoalveolar lavage fluid (BALF) (n = 5–6). (**C**) Differential BALF cell counts of neutrophils and eosinophils (n = 5–6). (**D**) Representative HE-stained lung sections of mice. Scale bar: 200 μm. (**E**) PAS-stained lung sections of mice. Scale bar: 200 μm. (**F**) Quantification of PAS staining. The percentage of PAS positive cells in epithelial cells was calculated (n = 5–6). (**G**) Analysis of airway smooth muscle (ASM) thickness (n = 5–6). (**H**) Levels of IFN-γ, IL-4, IL-6, IL-17A and IL-1β in BALF (n = 5–6). ns, not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Bar, mean ± SEM.

remodeling, exhibiting the pathophysiological signatures of severe asthma. However, rPEA induced lung inflammation (such as the activation of ILCs and γδ T cells) in non-sensitized mice, but it did not induce airway hyperresponsiveness. This suggests that challenges to mice without prior sensitization do not induce asthma. Similar steroid-insensitive asthma, induced by toluene diisocyanate, can only be attenuated by early IL-17A prevention rather than late IL-17A neutralization [27,28]. We speculate that the ICS + LABA regimen would earn a poor treatment response in these scenarios of asthma induced by PEA. It is therefore tempting to propose that PEA would act as a driving force in the vicious cycle of inflammation that promotes steroid-insensitive severe asthma. Thus, early disruption of the PEA-triggered inflammation pathway would be more effective than late targeting of the potent type 17/type 2 cytokines.

This study demonstrates that PEA can trigger severe asthma in mice with a cocktail of non-canonical inflammatory cytokines. A mouse model in which previous sensitization with an adjuvant is required in order to induce hyperresponsiveness and inflammation could be set up with many different protein antigens that have no relationship to asthma pathogenesis. For example, ovalbumin, which is used to develop asthma in mice, is a food allergen. Many adjuvants also enhance the immune response. For example, complete Freund's adjuvant has an added *Mycobacterium tuberculosis* component. Therefore, in this study, we tried to use a mild adjuvant (incomplete Freund's adjuvant), which does not contain *Mycobacterium tuberculosis* components but merely contains oil to encapsulate the allergen for slow and sustained release in the peritoneal cavity of mice. This was done to minimize the effect of the adjuvant on the mouse test.

This study does pose a significant challenge to the routine medical administration of PEA-based immunotoxins as well. For the clinically safe utilization of PEA-based immunotoxins, it is therefore necessary to reduce the allergenicity of PEA-based immunotoxins through protein engineering. It is intriguing to propose that this strategy can be followed for other immunotoxins to resolve and attenuate their allergenicities.

PEA could act as a specific allergen and elicit specific IgE responses in asthma patients. Recombinant PEA triggered severe asthma in sensitized mice with a significant increase in the production of type 2 cytokines, mainly from ILC2s, and in IL-17A from ILCs and T cells. What's more, rPEA-induced asthma in mice requires both sensitization and challenge. Additionally, the observed steroid insensitivity in this model highlights the need for further investigation into alternative therapeutic strategies for PEA-induced asthma. Collectively, our findings advocate for increased attention to PEA in the clinical diagnosis and development of novel treatment approaches for asthma.

Ethical approval

Subject recruitment and sample collection were approved by the Clinical Research and Application Ethics Committee of the Second Affiliated Hospital of Guangzhou Medical University (Approval No. 2023-hg-ks-01). All patients provided written informed consent.

All animal care and experimental procedures were in accordance with the guidelines of the Animal Experimental Committee of South China Agricultural University on the use and care of animals and were authorized by the Animal Experimental Committee of South China Agricultural University (Approval No. 2022D020).

Data availability

Data will be made available on request.

CRediT authorship contribution statement

Huancheng Xie: Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation, Conceptualization. **Linmei Li:** Writing – original draft, Visualization, Validation, Methodology, Funding acquisition, Data curation, Conceptualization. **Yuhe Guo:** Writing – review & editing, Visualization, Validation, Methodology, Data curation, Conceptualization. **Linghui Zhou:** Validation, Methodology, Investigation. **Linyi Ma:** Validation, Methodology, Investigation. **Andong He:** Validation, Resources. **He Lai:** Validation, Resources. **Ying He:** Validation, Resources. **Yongping Liu:** Validation, Resources. **Huifang Chen:** Validation, Resources. **Liping Luo:** Validation, Resources. **Yuyi Huang:** Resources, Funding acquisition. **Xiangyin Sha:** Resources, Funding acquisition. **Huanping Zhang:** Validation, Resources. **Jie Yan:** Validation, Resources. **Qingling Zhang:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Ailin Tao:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

Acknowledgments

This work was supported by National Natural Science Foundation of China (81871266, 82100025), Tertiary Education Scientific research project of Guangzhou Municipal Education Bureau (202235411) and Guangzhou Science & Technology Developing Program (202102010045).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37512.

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