

ORIGINAL ARTICLE

Asthma and Lower Airway Disease

Thrombin cleaves IL-33 and modulates IL-33-activated allergic lung inflammation

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Abstract

Background: Organisms have orchestrated coagulation and immune systems. Although a link between inflammation and haemostasis has been reported in asthma, the interaction mechanism has not been completely elucidated. Here, we investigated the direct link between the mammalian immune and coagulation systems.

Methods: Mice were administered protease or antigens intranasally to induce airway inflammation with or without thrombin inhibitors treatment. The effects of thrombin and its inhibitors on interleukin (IL)-33 were investigated both in vivo and in vitro. Peripheral blood mononuclear cells (PBMCs) and plasma from asthma patients are collected to verify the correlation between thrombin and group 2 innate lymphocytes (ILC2s).

Results: Low-molecular-weight heparin (LMWH, an indirect inhibitor of thrombin) restrained both papain- and fungus-induced type 2 immune responses in mice by inhibiting IL-33 cleavage. Upon examining the potential thrombin protease consensus sites, we found that IL-33 was directly cleaved by thrombin at specific amino acids (R48 and R106) to generate a mature form of IL-33 with potent biological activity. In addition, we found that bivalirudin TFA (a direct inhibitor of thrombin) inhibited a variety of type 2 inflammatory responses, such as those in house dust mite (HDM)- and ovalbumin (OVA)-mediated pulmonary inflammation models. We found that plasma thrombin-antithrombin complex (TATc) levels in asthma patients were positively associated with the number and function of IL-33-responder group 2 innate lymphocytes (ILC2s) among peripheral blood mononuclear cells (PBMCs) from asthma patients.

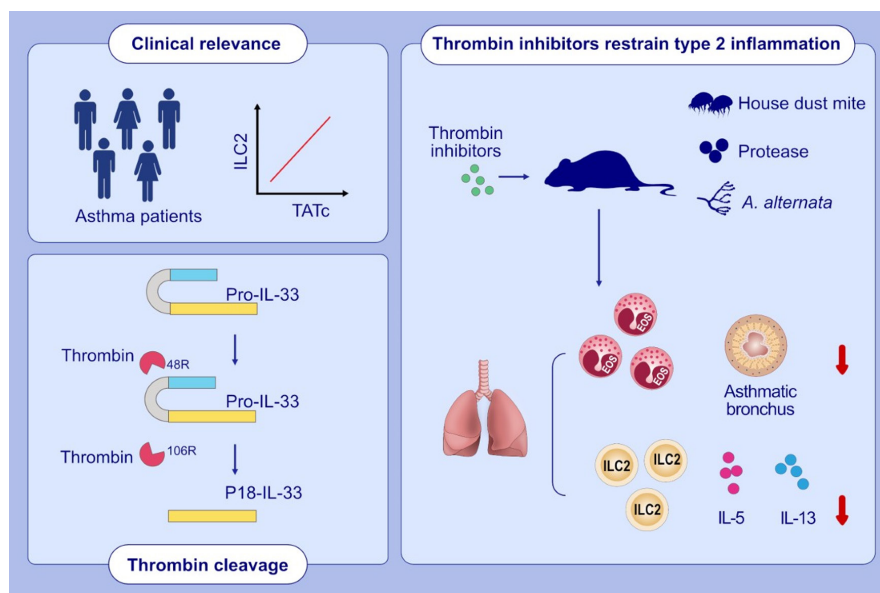
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Conclusion: The data suggested that thrombin inhibitors administration could be effective in treating lung inflammation by regulating ILC2s via IL-33 maturation, indicating that targeting thrombin is a potential way to treat allergic diseases.

KEYWORDS

asthma, group 2 innate lymphoid cell, IL-33, thrombin



GRAPHICAL ABSTRACT

Thrombin cleavage leads to IL-33 activation by targeting conserved thrombin consensus site. Thrombin inhibitors can restrain type 2 inflammation. Thrombin is associated with ILC2 activation in asthmatic patients.

Abbreviations: IL, interleukin; ILC2, group 2 innate lymphocytes; TATc, thrombin-antithrombin complex

1 | INTRODUCTION

From ancient times to the present, the greatest survival challenges faced by organisms have been infection and foreign antigen invasion, which are sometimes accompanied by bleeding and inflammation. The coexistence of coagulation and the inflammatory response supports the concept of regulatory cross-activation between the complex biological coagulation and inflammation systems. The coagulation system develops from the early innate immune system¹ and is induced by two core processes: blood clotting, which leads to fibrin formation, and platelet activation.^{2,3} Thrombin is the main substance involved in the coagulation process.⁴ In addition to playing a key role in coagulation, thrombin also exhibits proinflammatory activity, such as inducing endothelial cells, parietal cells, epithelial cells, adipose cells and immune cells to release a variety of proinflammatory cytokines.^{5–8} Although links between coagulation and immunity exist in mammals, the relationship is not yet clear.

Animal model research and clinical patient analyses identified the interleukin (IL)-1 family member IL-33 as an important target in several diseases, including asthma⁹ and other allergic diseases

such as rheumatoid arthritis, inflammatory bowel disease and cardiovascular diseases.¹⁰ IL-33 is a tissue-derived nuclear cytokine¹¹ that is produced mainly in endothelial cells, epithelial cells,¹² epidermal cells,¹³ fibroblast-like cells and myofibroblasts¹⁴ rather than in CD45⁺ haematopoietic cells. IL-33 can function as an alarm signal (alarmin) when released into the extracellular space after cellular damage or mechanical injury to alert the immune system of tissue damage following trauma or infection.^{12,15,16} Because the *IL-33* and *IL-33* receptor *IL1RL1/ST2* genes have been identified as the main susceptibility sites in several independent, large-scale genome-wide association studies of human asthma, IL-33 likely plays a crucial role in asthma.^{17,18} IL-33 is one of the key activators of the recently described group 2 innate lymphocytes (ILC2s), which highly express the IL-33 receptor *IL1RL1/ST2*,^{19–21} in addition to activate T helper 2 (Th2) cells and mast cells²² as originally described. ILC2s can produce extremely large amounts of the type 2 cytokines IL-5 and IL-13 and respond rapidly to IL-33, thus playing an important role in allergic airway inflammation.^{23–25}

Full-length human IL-33 (IL-33_{FL}) is a 270-amino acid protein that consists of two evolutionarily conserved domains, the N-terminal

nuclear domain and the C-terminal IL-1-like cytokine domain, separated by different central parts.²⁶ Some factors have been reported to regulate IL-33 function, such as cysteine oxidation,²⁷ apoptotic caspases,^{15,16} inflammatory proteases from neutrophils and mast cells^{28,29} and allergen proteases.³⁰ Due to the importance of IL-33 in airway inflammatory diseases such as asthma, the identification of endogenous or exogenous proteases that regulate IL-33 maturation in the lungs is important to better understand the physiological function of IL-33 and the pathogenic mechanism of airway inflammatory disease. Intriguingly, recent work has shown that thrombin can directly cleave pro-IL-1 α to generate an activated form, and these cleavage products have been identified in humans with sepsis-associated adult respiratory distress syndrome (ARDS).⁸ There is a strong correlation between thrombin levels and clinical asthma,^{31–33} but the specific mechanism has not been reported. Because IL-33 belongs to the IL-1 family and is highly homologous to IL-1 α at the sequence level,³⁴ we hypothesized that thrombin cleavage may also be involved in IL-33 activation and participate in related immune responses.

Here, we found that low-molecular-weight heparin (LMWH, an indirect thrombin inhibitor) suppressed the type 2 immune response *in vivo* by affecting IL-33 maturation. By using *in vitro* approaches, we discovered that IL-33 was processed into mature bioactive forms by thrombin at a highly conserved site, implying that coagulation might play an important role in the regulation of IL-33 activity during inflammation. Bivalirudin TFA, a direct inhibitor of thrombin, effectively inhibited ILC2-mediated or Th2 cell-mediated lung inflammation, indicating the potential clinical application of coagulation modifiers in the treatment of type 2 immune disease. We also found that plasma thrombin-antithrombin complex (TATc) levels were positively correlated with the number and function of IL-33-responder ILC2s among peripheral blood mononuclear cells (PBMCs) from allergic patients. These findings suggest that the elucidation of integrated blood coagulation and immune system pathways will provide new directions for the development of effective disease treatment strategies.

2 | MATERIALS AND METHODS

2.1 | Mice

C57BL/6 mice were purchased from the Shanghai Research Center for Model Organization (Shanghai, China). Rag1^{-/-} mice were maintained at the Chinese Academy of Sciences (Shanghai, China). IL-33^{-/-} mice were purchased from Shanghai Model Organisms Center Inc. (Shanghai, China). The mice were maintained under specific pathogen-free conditions at the Animal Care Facility of the Chinese Academy of Sciences (Shanghai, China) and used at 8–10 weeks old. The animal care and use procedures complied with the guidelines of CAS Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences.

2.2 | Preparation of cell suspensions from lung tissue

Mice were euthanized, and BALF (including cells) were collected from the lungs by two gentle washes with 800 μ l of ice-cold PBS each using 18 G plastic cannulas and 1 ml syringes. To isolate lung cells, the mice were perfused with 10 ml of cold DPBS via the right ventricle to remove blood from the lungs. The removed lungs were minced with scissors. Then, the shredded lung tissue was placed in RPMI-1640 medium containing 10% FCS (Thermo Fisher) and 1% penicillin-streptomycin (Gibco) and digested with 0.5 mg/ml collagenase type I (Invitrogen) for 30 min at 37°C with continuous agitation in an incubator. The digested lung fragments were passed through a 70 μ m filter to form a cell suspension, which was centrifuged at 350 g for 10 min. The supernatant was completely removed by aspiration. The pellets were suspended in 40% Percoll (GE Healthcare), and then, 80% Percoll was added; the gradient was centrifuged at 600 g for 20 min to obtain the fraction of lung mononuclear cells, which were isolated and washed with RPMI-1640 medium before further analysis.

2.3 | Flow cytometric analysis and cell sorting

Single-cell suspensions were pretreated 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, Fc ϵ R1 and TCR γ/δ , to identify Lin⁻ cells. Antibodies specific to mouse CD45R (RA3-6B2), CD5 (53-7.3), NK1.1 (PK136), TCR γ/δ (UC7-13D5), Gr-1 (RB6-8C5) and a mouse erythroid cell marker (TER119) were purchased from BioLegend; antibodies specific to mouse CD3 (2C11), CD4 (RM4-5), CD127 (A7R34), CD11b (M1/70), CD11c (N418), Fc ϵ R1 (MAR-1), CD45.2 (104), IL-5 (TRFK5), IL-13 (eBio13A) and KLRG1 (2F1) were purchased from eBioscience; antibodies specific to GATA3 (L50-823) and Thy1.2 (53-2.1) were purchased from BD Biosciences, and antibodies specific to IL-33R (DJ8) were purchased from MD Bioproducts. Antibodies specific to human CD127 (A019D5), CD161 (HP-3G10), CRTH2 (BM16) and CD45 (HI30) were purchased from BioLegend, and the human lineage antibody cocktail was purchased from Thermo Fisher. A fixable viability stain (BD Biosciences) was used to discriminate viable from nonviable cells. For the intranuclear staining of transcription factors, nuclear protein cytokines or chemokines, cells were first stained with antibodies to surface antigens and then fixed and permeabilized according to the manufacturer's instructions (Foxp3/Transcription Factor Staining Buffer Set; eBioscience). For intracellular cytokine staining, cells were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), 1 μ g/ml ionomycin (Sigma-Aldrich) and 1 μ g/ml brefeldin A (BFA; eBioscience) for 4 h. An Annexin V-FITC/PI Apoptosis Detection Kit (Vazyme) was used

to differentiate cells in different stages of apoptosis. Flow cytometry data were collected on an LSRFortessa (BD Bioscience) or CytoFLEX3 flow cytometer (Beckman Coulter), and the data were analysed with FlowJo V10 (FlowJo). For flow cytometric sorting, mouse lung ILC2s (CD45⁺Lin⁻CD127⁺CD90.2⁺ST2⁺) were sorted with an Aria SORP or Aria III (BD Biosciences).

2.4 | ILC2 culture

Freshly sorted lung ILC2s (CD45⁺Lin⁻CD127⁺CD90.2⁺ST2⁺) from IL-33-treated mice were cultured in 96-well round-bottom plates at a density of 5×10^3 cells per well in RPMI-1640 medium (Thermo Fisher Scientific) containing 10% FCS, 100 U/ml penicillin and 100 mg/ml streptomycin (Thermo Fisher Scientific), mouse IL-2 (100 U/ml) and mouse IL-7 (20 ng/ml) in the presence or absence of various forms of IL-33 protein produced in rabbit reticulocyte lysate (RRL, Promega) with or without a neutralizing mIL-33 mAb (R&D). Then, the levels of cytokines in the culture supernatant were measured by ELISA. For intracellular cytokine staining, 50 ng/ml PMA (Sigma-Aldrich), 1 µg/ml ionomycin (Sigma-Aldrich) and 1 µg/ml BFA (eBioscience) were added to the cultures 2–4 h before staining.

2.5 | Lung inflammation mouse models

To establish papain-induced acute type 2 airway inflammation, mice were anaesthetized and administered papain (Sigma-Aldrich) intratracheally (5 µg of papain in 40 µl of PBS per mouse) on 5 consecutive days. BALF and lungs were harvested for analysis on day 6. For IL-33-induced airway inflammation, recombinant mouse IL-33 (50 ng per mouse; BioLegend) or PBS was intratracheally administered to mice on days 1, 2 and 3. Twenty-four hours after the final treatment, the mice were sacrificed, and samples were collected. For *A. alternata*-induced lung inflammation, mice were intratracheally administered 4–5 µg of *A. alternata* (Greer Laboratories) in 40 µl of PBS on 4 consecutive days. For HDM-induced chronic airway inflammation, we challenged the mice on 3 consecutive days with HDM (30 µg HDM per mouse; Greer Laboratories). Then, we allowed these mice to rest for 10 days before rechallenging them with HDM (6 µg per mouse) on 4 consecutive days. The mice were sacrificed 24 h after the last challenge, and BALF and lungs were collected for analysis. OVA (Sigma-Aldrich) was adsorbed onto Imject alum adjuvant (Thermo Fisher Scientific) by gentle shaking. The mice were primed with an intraperitoneal injection of 20 µg of OVA-alum in a 200 µl volume. On day 14, the mice were 'boosted' with the same protocol. On days 21–25, the mice were challenged with spraying of 10 mg/ml OVA in PBS. On day 26, the mice were killed. Mice received intratracheally LMWH (Selleck) or Bivalirudin TFA (MedChemExpress) throughout the study.

2.6 | Plasmid construction and protein production

IL-33 deletion mutants were amplified by PCR using human (NM_033439) and mouse (NM_133775) IL-33/NF-HEV cDNA as the template. The PCR fragments were cloned into the plasmid pcDNA3.1 (Invitrogen). PCR was used to generate the human IL-33_{FL} sequence; human IL-33_{FL-R48H}, IL-33_{FL-R106H}, IL-33₄₉₋₂₇₀, IL-33₁₀₇₋₂₇₀ and IL-33₁₁₂₋₂₇₀ mutants; the mouse IL-33_{FL} sequence; and mouse IL-33_{FL-R50H}, IL-33_{FL-R50Q}, IL-33_{FL-S51H}, IL-33_{FL-R87H}, IL-33₅₁₋₂₆₆ and IL-33₈₈₋₂₆₆ mutants. The sequences were cloned into the same expression vector. The wild-type (WT) and mutant IL-33 proteins were synthesized in vitro in RRL using a TNT-T7 kit (Promega). The protein concentration was determined by IL-33 ELISA (Thermo Scientific). The concentrations of IL-33 fragments produced in RRL were as follows: human IL-33_{FL}: 118.33 pg/ml; human IL-33_{FL-R48H}: 149.46 pg/ml; human IL-33_{FL-R106H}: 93.36 pg/ml; human IL-33₁₀₇₋₂₇₀: 49.95 pg/ml; human IL-33₁₁₂₋₂₇₀: 45.1 pg/ml; mouse IL-33_{FL}: 81 pg/ml; mouse IL-33₅₁₋₂₆₆: 53 pg/ml; mouse IL-33₈₈₋₂₆₆: 127 pg/ml; and mouse IL-33₁₁₄₋₂₆₆: 91 pg/ml. All IL-33 fragments were used at the same concentration (50 or 100 pg/well) in the experiment.

2.7 | Protein cleavage assays with thrombin

In vitro-translated proteins (RRL, 50–100 pg) were incubated in duplicate with thrombin (0.075 U or as indicated; MedChemExpress) in 20 mM Tris pH 8.4, 150 mM NaCl and 2.5 mM CaCl₂ for 1–1.5 h at room temperature (RT) with or without a neutralizing hIL-33 mAb (10 µg/ml, from our laboratory, S-730–8) in a 15 µl system.

2.8 | Preparation of lung homogenates

Whole lungs were homogenized by sonication in protein lysis buffer containing 1X protease cocktail inhibitor (Roche) in 1X PBS. Lung lysates were obtained by centrifugation at 10,000 xg for 10 min at 4°C and quantified with the BCA Protein Assay Kit (Thermo Fisher Scientific).

2.9 | IL-33 activity assays and ELISAs

In vitro-translated IL-33_{FL} or mature IL-33 forms (18 h treatment) were used to stimulate IL-33-responsive cultured ILC2s (5×10^3 cells per well in 96-well round-bottom plates). ILC2s cultured with LMWH-treated BALF from different lung inflammation models with or without a neutralizing mIL-33 pAb (R&D), and supernatants from cultured ILC2s were collected. The levels of the cytokines IL-5 and IL-13 were measured by ELISA following the manufacturer's instructions (Thermo Fisher). The concentrations of the alarmin cytokines IL-33, IL-25 and TSLP in BALF and lung homogenate supernatants

were analysed by ELISA (R&D for IL-33 and IL-25; eBioscience for TSLP) according to the manufacturers' instructions.

2.10 | Quantitative PCR (qRT-PCR)

Total RNA was extracted from lung homogenates or different cell types sorted from mice using TRIzol (Invitrogen) and reverse transcribed into cDNA with a synthesis kit (Takara). Gene expression was analysed by qRT-PCR with SYBR Green chemistry (Takara) using the following primer set: mL-33 forward, TTGGAGAATGGATGTTATGTG and reverse, CTCATAGTAGCGTAGTAGCA.

2.11 | Histological analysis

Mice were euthanized using CO₂. The lungs were fixed for at least 24 h with 4% formalin (paraformaldehyde), embedded in paraffin, cut into 4 µm sections and stained with H&E and alcian blue (AB)/PAS according to standard protocols. Sections were scanned for inflammation using a light microscope. Images of H&E-stained and AB/PAS-stained tissue slides were acquired with a Zeiss Axio Scan. Z1 slide scanner. Histologic images were analysed with ImageJ. Inflammation was determined by semiquantitative scoring (0–4: 0, none; 1, mild; 2, moderate; 3, marked; and 4, severe). An increment of 0.5 was used when the inflammation fell between two levels. Three fields were selected randomly for independent scoring by two treatment-blind pathologists.^{35,36}

2.12 | Human samples

Eighteen asthma patients aged 18–70 years were recruited from Ruijin Hospital, Shanghai Jiaotong University School of Medicine. The diagnosis of asthma was established based on respiratory symptoms and evidence of variable airflow obstruction according to the Global Initiative for Asthma (GINA) guidelines. A lung function test was performed, and the predicted forced expiratory volume in 1 s (FEV1) per cent (FEV1% pre), FEV1/forced vital capacity percentage (FEV1/FVC), disease duration and asthma control questionnaire

(ACQ) scores were recorded. Patients with an acute attack, autoimmune diseases or infectious diseases and those who had received systemic glucocorticoids and immunosuppressive agents within 1 month before the study were excluded. Written informed consent was obtained from all subjects before participation. This study was approved by the Research Ethics Board of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine.

2.13 | Human PBMC collection and culture

We collected fresh blood in EDTA anticoagulant tubes at Shanghai Ruijin Hospital, Shanghai Jiaotong University School of Medicine. Human PBMCs were obtained by using Ficoll-Paque PLUS (GE Healthcare). Isolated PBMCs were washed and resuspended in PBS containing 2% FBS. The cells were cultured in RPMI-1640 supplemented with hIL-2 (R&D), hIL-7 (R&D) and hIL-33 (R&D). IL-13 and IL-5 levels in the supernatant were then measured.

2.14 | Statistical analysis

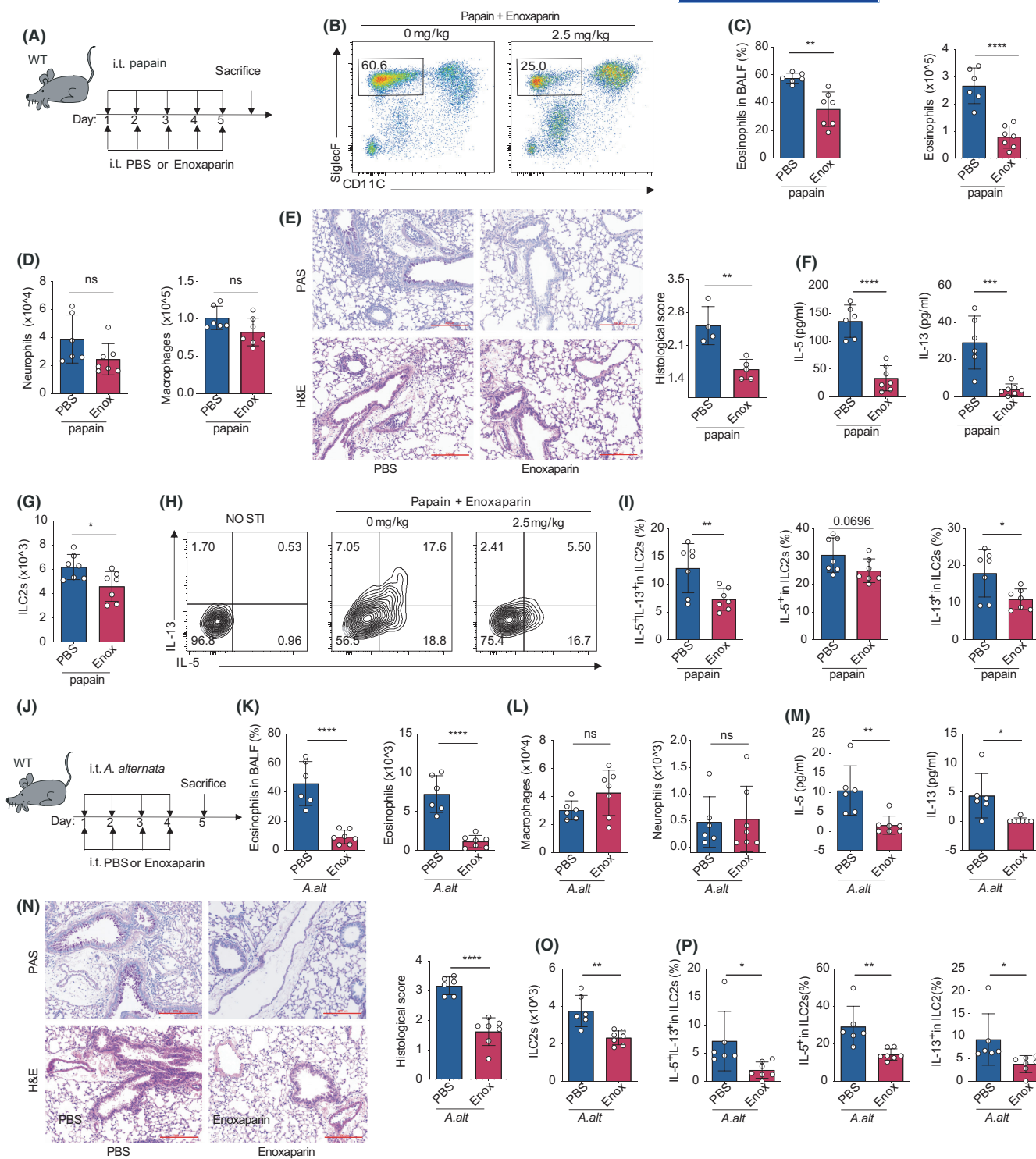
All data are presented as the mean ± standard error of the mean (SEM). A two-tailed Student's *t* test was applied for comparisons of unpaired data between two groups. For multigroup comparisons, we used one-way ANOVA with Tukey's post hoc test. Data were analysed with Prism Software (GraphPad Prism 8). Error bars represent the SEM. A *p* value < .05 was considered to denote statistical significance.

3 | RESULTS

3.1 | LMWH restrained type 2 acute pulmonary inflammation in mice

Heparin and LMWH are common antithrombotic drugs^{37,38} that indirectly inhibit thrombin activity by enhancing the function of antithrombin factors. We hypothesized that LMWH has the potential to inhibit IL-33-related inflammatory reactions. We selected an IL-33

FIGURE 1 LMWH suppresses type 2 acute pulmonary inflammation in mice. (A) Mice were intratracheally challenged with papain (5 µg per mouse) or PBS on five consecutive days, treated with PBS or LMWH (2.5 mg/kg) daily and sacrificed on day 6. (B) Flow cytometry plots of eosinophils (FVD⁺CD45⁺CD11c^{hi}SiglecF⁺) among live CD45⁺ cells in BALF. (C) Quantification of total eosinophils in BALF (*n* = 6–7 per group). (D) Number of macrophages (FVD⁺CD45⁺CD11c^{hi}SiglecF⁺) and neutrophils (FVD⁺CD45⁺CD11c^{hi}SiglecF⁺CD11b⁺Gr-1⁺) in BALF (*n* = 6–7 per group). (E) Representative images of H&E and PAS staining of lung sections (scale bar, 200 µm; *n* = 5 per group); inflammation was determined by semiquantitative scoring. (F) IL-5 and IL-13 levels in BALF were measured by ELISA (*n* = 6–7 per group). (G) Statistical analysis of the number of ILC2s in the lungs (*n* = 6–7 per group). (H) Flow cytometric analysis of IL-5 and IL-13 in lung ILC2s stimulated with PMA plus ionomycin and BFA for 4 h. (I) Statistical analysis of the percentages of IL-5⁺, IL-13⁺ and IL-5⁺IL-13⁺ cells among lung ILC2s (*n* = 6–7 per group). (J) Mice were intratracheally (i.t.) challenged with *A. alternata* (4 µg per mouse) or PBS on four consecutive days, treated with PBS or LMWH (2.5 mg/kg) daily and sacrificed on day 5. (K) Quantification of total eosinophils in BALF (*n* = 6–7 per group). (L) The number of macrophages and neutrophils in BALF (*n* = 6–7 per group). (M) IL-5 and IL-13 levels in BALF were measured by ELISA (*n* = 6–7 per group). (N) Representative images of H&E and PAS staining of lung sections (scale bar, 200 µm; *n* = 6–7 per group). (O) Statistical analysis of the number of ILC2s in the lungs (*n* = 6–7 per group). (P) Flow cytometric analysis of IL-5 and IL-13 in lung ILC2s stimulated with PMA plus ionomycin and BFA for 4 h. The bars and error bars represent the mean ± SEM. **p* < .05; ***p* < .01; ****p* < .001; ns, not significant by unpaired Student's *t* test. The data are representative of two independent experiments



responder ILC2-specific mouse model of acute pulmonary inflammation mediated by papain (Figure 1A, Figure S1A). The data showed that LMWH significantly reduced the proportion and number of eosinophils (Figure 1B-C) in bronchoalveolar lavage fluid (BALF). We also detected the number of neutrophils and macrophages in BALF and found no significant changes between PBS- and LMWH-treated mice stimulated with papain (Figure 1D). Immunohistochemical (haematoxylin and eosin, H&E) and periodic acid-Schiff (PAS) staining showed that inflammatory cell infiltration and mucus secretion

were reduced in papain-damaged lungs from LMWH-treated mice, indicating that type 2 inflammation was attenuated (Figure 1E). The levels of type 2 cytokines in BALF also decreased after LMWH treatment (Figure 1F). Moreover, LMWH-treated mice had fewer ILC2s than did PBS-treated control mice (Figure 1G), and the percentages of IL-5- and IL-13-producing ILC2s were lower in LMWH-treated mice than in control mice (Figure 1H-I). To confirm the role of LMWH in ILC2-mediated lung inflammation, we further assessed the response to LMWH in other ILC2-dependent type 2 immune mouse

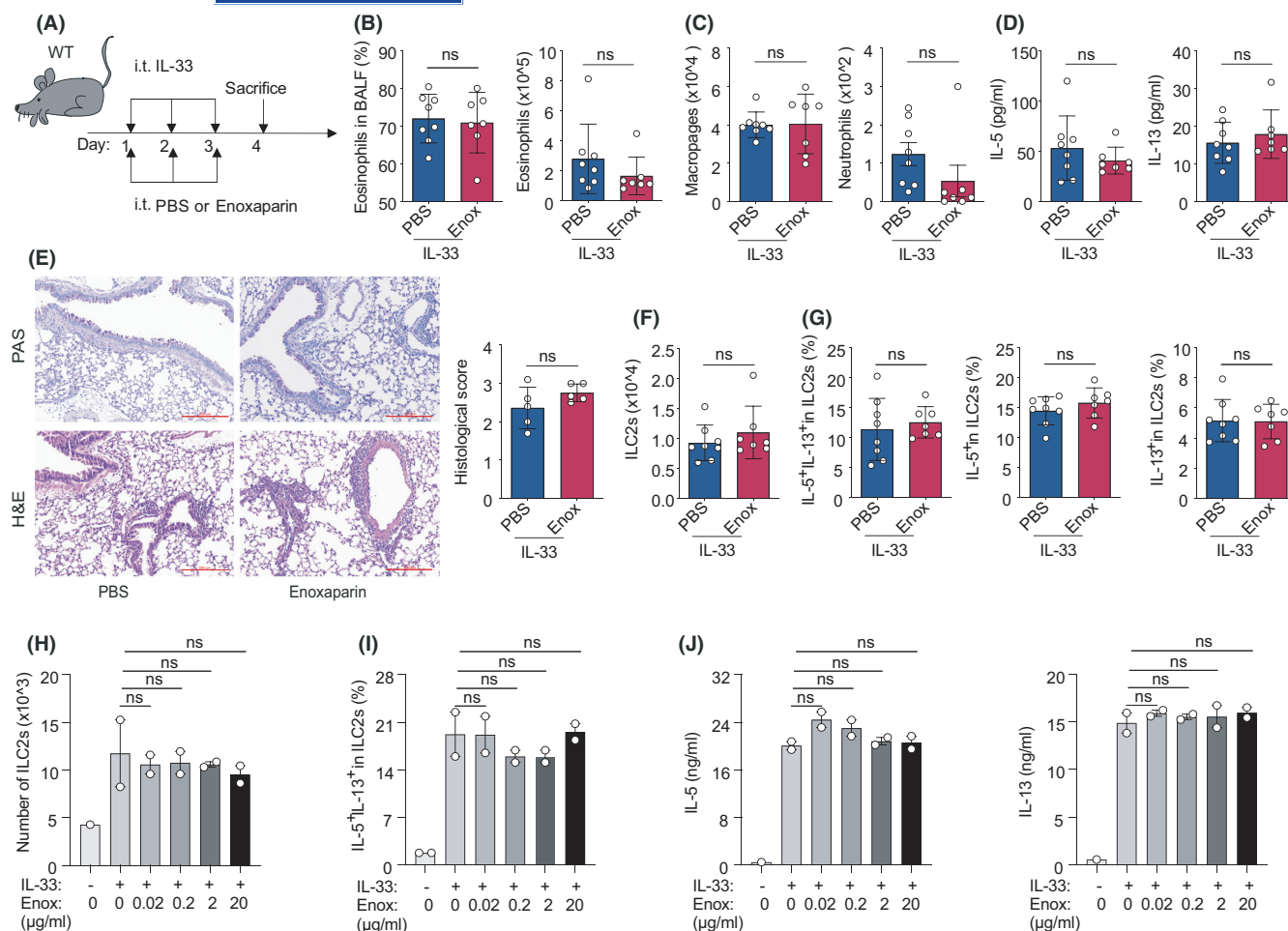


FIGURE 2 LMWH administration does not interfere with ILC2 responses in the IL-33-induced type 2 immune response. (A) Mice were intratracheally (i.t.) challenged with IL-33 (25 ng per mouse) on three consecutive days, treated with PBS or LMWH (2.5 mg/kg) daily and sacrificed on day 4. (B) The number and percentage of eosinophils in BALF ($n = 7-8$ per group). (C) The number of macrophages and neutrophils in BALF ($n = 7-8$ per group). (D) IL-5 and IL-13 levels in BALF were measured by ELISA ($n = 7-8$ per group). (E) Representative images of H&E and PAS staining of lung sections (scale bar, 200 μm); inflammation was determined by semiquantitative scoring ($n = 5$ per group). (F) The number of lung ILC2s ($n = 7-8$ per group). (G) Percentages of IL-5⁺, IL-13⁺ and IL-5⁺IL-13⁺ cells among lung ILC2s after stimulation with PMA, ionomycin and BFA for 4 h ($n = 7-8$ per group). (H) Statistical analysis of the number of ILC2s after 3 days of treatment with PBS or different doses of LMWH in vitro (5000 cells per well). (I) Statistical analysis of the percentage of IL-5⁺IL-13⁺ cells among ILC2s. (J) IL-5 and IL-13 secretion into the culture medium was measured by ELISA. Mouse CD45⁺Lin⁻CD127⁺CD90.2⁺ST2⁺ lung ILC2s sorted from IL-33-treated WT mice were cultured in vitro with IL-2 (100 U/ml), IL-7 (20 ng/ml) and IL-33 (0.5 ng/ml) in the presence or absence of LMWH for the above experiments. The bars and error bars represent the mean $\pm$ SEM; * $p < .05$; ** $p < .01$; *** $p < .001$; ns, not significant by unpaired Student's t test or one-way ANOVA with Tukey's multiple comparisons test. The data are representative of two independent experiments

models in vivo. Similarly, among mice exposed intratracheally to an extract of the fungal allergen *Alternaria alternata* (Figure 1J), those treated with LMWH had milder inflammation (Figure 1K-N), fewer ILC2s and lower ILC2 cytokine production in the lungs than those treated with PBS (control) (Figure 1O-P).

Some studies have reported that LMWH can restrain the allergic response by inhibiting CD4 T cell function in mice.^{39,40} To evaluate the contribution of ILC2s to the effects of LMWH treatment, Rag1^{-/-} mice lacking T and B cells were utilized (Figure S1B). The data showed that LMWH treatment reduced the eosinophil count and percentage in BALF (Figure S1C) and decreased IL-5 production in BALF (Figure S1D). Consistent with these findings, LMWH treatment

ameliorated inflammatory cell infiltration and mucus hypersecretion (Figure S1E) and reduced the numbers of ILC2s (Figure S1F) and IL-5- and IL-13-producing ILC2s (Figure S1G), indicating that IL-33 responder ILC2s are involved in the LMWH-mediated inhibition of type 2 pulmonary inflammation. Interestingly, LMWH did not attenuate type 2 inflammation or ILC2 function in mature IL-33-induced acute lung allergic inflammation (Figure 2A-G), in which the IL-33 stimulus is a mature form containing only the functional domain of pro-IL-33. These findings indicated that LMWH abolished IL-33 function before the maturation of this cytokine. To eliminate the possibility of a direct effect of LMWH on ILC2s, we cultured ILC2s with increasing concentrations of LMWH and found that LMWH did

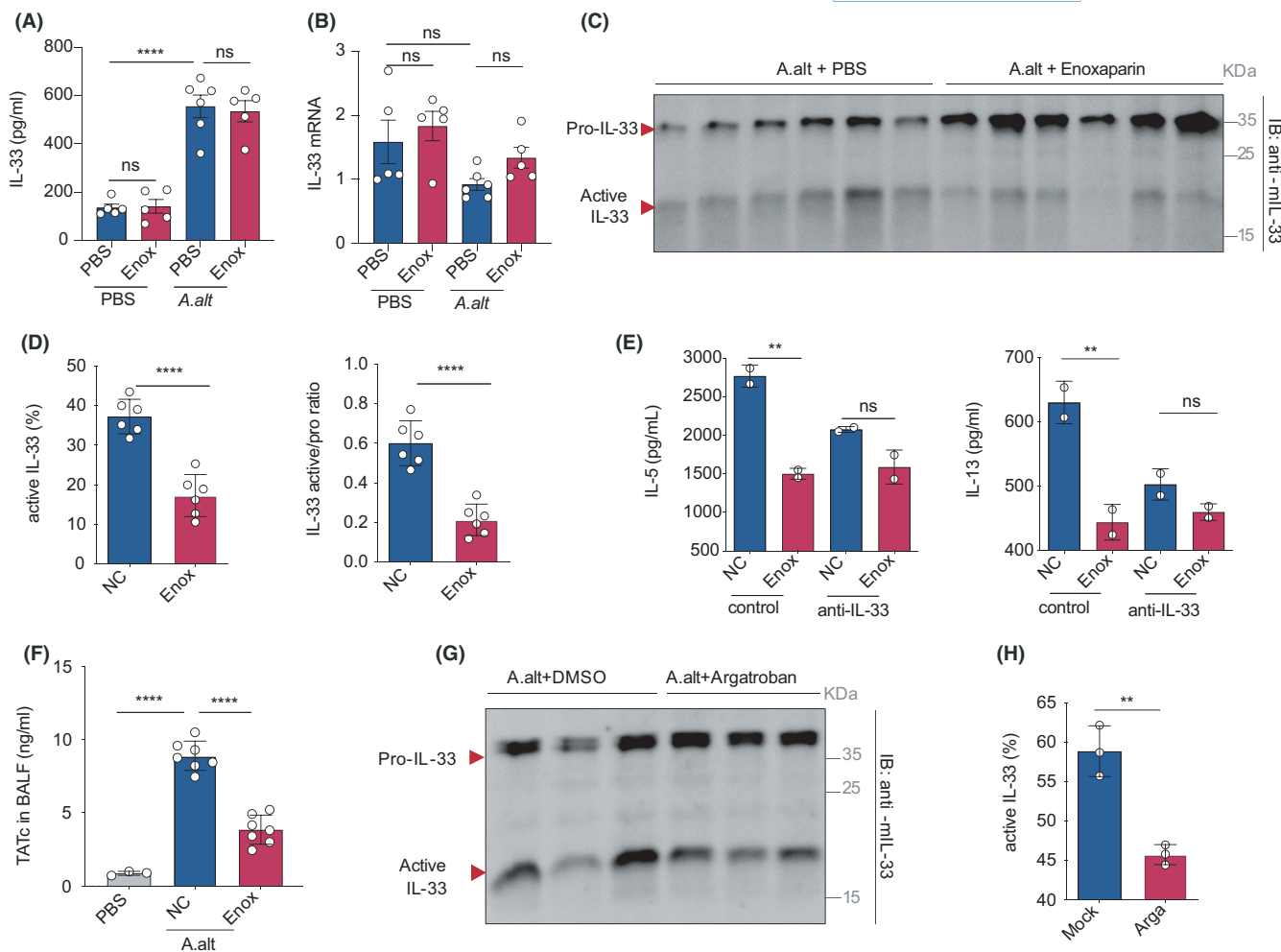


FIGURE 3 LMWH affects the maturation of endogenous IL-33, and cleaved endogenous IL-33 is detectable in vivo in a model of acute lung injury associated with thrombin. (A) ELISA results showing the total IL-33 concentration in BALF from PBS- or *A. alternata* (*A.alt*)-induced models (5 μ g/mouse) with or without LMWH treatment (Enox; 2.5 mg/kg) for 1 h ($n = 5-6$ per group). (B) qRT-PCR analysis of *IL-33* in mouse lungs with or without LMWH treatment (2.5 mg/kg) 1 h after PBS or *A. alternata* (4 μ g/mouse) administration ($n = 5-6$ per group). (C-D) Endogenous full-length and cleaved forms of IL-33 were released into BALF following *A. alternata*-induced acute lung injury with or without LMWH treatment. (C) Proteins in freeze-dried BALF were separated by SDS-PAGE and detected by Western blot analysis with endogenous anti-mouse IL-33 antibodies. (D) Statistical analysis of the different forms of IL-33 in (C) using ImageJ: active IL-33/total IL-33 ratio (left) and active IL-33/pro-IL-33 ratio (right). (E) ILC2s cultured with LMWH-treated BALF from *A. alternata*-induced models with or without a neutralizing mIL-33 pAb (10 μ g/ml). IL-5 and IL-13 protein levels in supernatants were detected by ELISA ($n = 2$ per sample). (F) ELISA results showed that TATc levels in BALF were increased in both *A. alternata*-induced models and were reduced by LMWH treatment. (G-H) Endogenous full-length and cleaved forms of IL-33 were released into BALF following *A. alternata*-induced acute lung injury with or without argatroban treatment. (G) Proteins in freeze-dried BALF were separated by SDS-PAGE and detected by Western blot analysis with endogenous anti-mouse IL-33 antibodies. (H) Statistical analysis of the different forms of IL-33 in (G) using ImageJ: active IL-33/pro-IL-33 ratio. Mouse CD45⁺Lin⁻CD127⁺CD90.2⁺ST2⁺ lung ILC2s sorted from IL-33-treated WT mice were cultured in vitro with IL-2 (100 U/ml) and IL-7 (20 ng/ml) for the above experiments. The bars and error bars represent the mean $\pm$ SEM; * $p < .05$; ** $p < .01$; *** $p < .001$; ns, not significant by unpaired Student's *t* test or one-way ANOVA with Tukey's multiple comparisons test. The results are representative of three independent experiments

not affect the quantity (Figure 2H) or cytokine production (Figure 2I-J) of ILC2s in vitro. Flow cytometry analysis also showed that LMWH did not affect the cell death, proliferation, or function of ILC2s or the expression of the key ILC2-related transcription factor GATA3 (Figure S2A). Taken together, these results suggested that LMWH might inhibit IL-33-related type 2 immune responses by affecting IL-33 maturation and ILC2 activation in vivo.

3.2 | Effect of LMWH on endogenous IL-33 maturation in mice

Since IL-33 is critical for the type 2 immune response in the lungs,²² two mouse models of acute pulmonary injury induced by *A. alternata* and papain were used to detect IL-33 maturation in the lung microenvironment; both of these models are considered

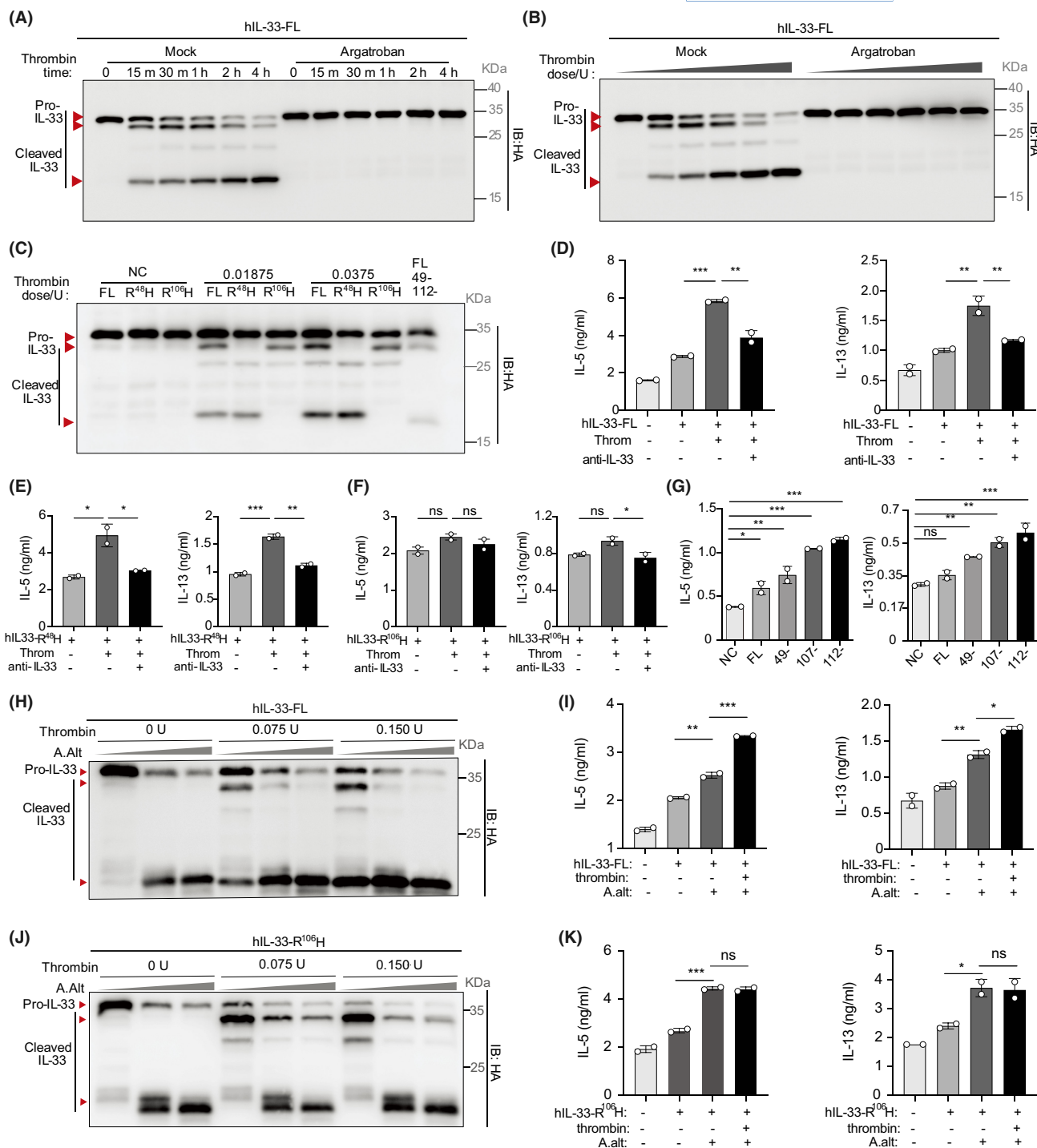
IL-33-mediated type 2 pulmonary inflammation models. First, we found that neither the concentration of IL-33 in BALF (Figure 3A) nor the transcription of IL-33 in the lungs (Figure 3B) changed after LMWH treatment in the *A. alternata* model. Similar results were obtained in the papain model at different times (Figure S3A–C). Owing to the extreme instability of IL-33 after release, we concentrated the BALF by freeze-drying after model induction. The different forms of IL-33 in freeze-dried BALF were detected with an endogenous IL-33 antibody through Western blot analysis. IL-33^{-/-} mice were used to verify the specificity of the IL-33 antibody, and we found that pro-IL-33 and cleaved IL-33 were the two main protein forms (Figure S3D). The quantified proportion of cleaved IL-33 among total IL-33 was used as a measure of active IL-33. With this detection system, we found that active IL-33 was significantly decreased in BALF from LMWH-treated mice challenged with *A. alternata* (Figure 3C–D). To detect the biological activity of endogenous IL-33, we cultured ILC2s with BALF supernatant from the *A. alternata* (Figure 3E) and papain (Figure S3E) mouse lung injury models and found that the activation of ILC2s by IL-33, as evidenced by type 2 cytokine secretion, was lower in BALF from LMWH-treated mice than in that from untreated mice. This phenotype was abrogated in the presence of an mIL-33 neutralizing antibody (Figure 3E and Figure S3E), suggesting that LMWH suppressed the maturation and biological activity of IL-33 in vivo. In addition, both *A. alternata* (Figure 3F) and papain (Figure S3F) elicited the local activation of the coagulation system in the lungs, as evidenced by elevated concentrations of thrombin-antithrombin (TAT) complexes, and this effect was inhibited by LMWH, indicating the involvement of thrombin in IL-33 maturation in vivo. Similar results were obtained using the direct thrombin inhibitor argatroban (Figure 3G). Argatroban treatment also resulted in a decreased proportion of cleaved IL-33 among total IL-33 protein (Figure 3H). Altogether, these data suggested that LMWH might play a role in the type 2 inflammatory response by inhibiting endogenous IL-33 maturation.

3.3 | IL-33_{FL} could be directly cleaved and activated by thrombin

To prove the direct interaction between thrombin and IL-33, we co-incubated these two proteins and found that thrombin-cleaved in vitro-translated full-length human IL-33₁₋₂₇₀ (IL-33_{FL}) to generate two major cleavage products of 25 kDa and 18 kDa. The amount of the 18 kDa product increased as the reaction time and enzyme activity increased. Moreover, this cleavage event was blocked by argatroban (Figure 4A and B). Upon examining the potential protease cleavage sites in IL-33_{FL} and reviewing the sequence characteristics of thrombin cleavage sites,⁴¹ we predicted and verified that two cleavage sites in IL-33_{FL}, arginine (Arg, R) 48 and R106, were critical for thrombin cleavage. These two thrombin cleavage sites in IL-33 were found to be conserved in different species (Figure S4A). Mutagenesis of R48 and R106 to histidine (IL-33_{FL-R48H} and IL-33_{FL-R106H}) abrogated the thrombin-mediated formation of the 25 and 18 kDa cleavage products, respectively (Figure 4C).

To verify the biological functions of the two forms of IL-33 after thrombin cleavage, we performed in vitro culture experiments to detect IL-33 activity in ILC2s that highly expressed ST2 and could respond to IL-33 stimulation. Secretion of the type 2 cytokines IL-5 and IL-13 from ILC2s was analysed to reveal the biological functions of different IL-33 forms. Compared with the control group not exposed to thrombin, the group exposed to thrombin-treated IL-33_{FL} exhibited significantly greater ILC2-mediated IL-5 and IL-13 secretion, and this increased secretion was abrogated by an IL-33 neutralizing antibody (Figure 4D). We also performed these experiments after mutating the two cleavage sites and found that the R106H mutation, but not the R48H mutation, significantly inhibited thrombin-mediated IL-33 activation (Figure 4E–F). In addition, we constructed and expressed a 25 kDa intermediate form (IL-33₄₉₋₂₇₀) and an 18 kDa form (IL-33₁₀₇₋₂₇₀) of human IL-33 and found that IL-33₁₀₇₋₂₇₀ and mature IL-33₁₁₂₋₂₇₀ showed comparable abilities to induce high levels of IL-5 and IL-13 secretion by ILC2s

FIGURE 4 Human IL-33 is directly activated by thrombin cleavage. (A, B) Western blots for the HA tag on RRL-expressed IL-33 showing the thrombin cleavage of human pro-IL-33 into the two main forms, ~18 and ~25 kDa, and the inhibition of these cleavage events by the thrombin inhibitor argatroban (50 µg/ml). Cleavage occurred in a time-dependent manner (15 min, 30 min, 1 h, 2 h and 4 h; 0.075 U thrombin; 15 µl buffer) (A) and a dose-dependent manner (0.01875, 0.0375, 0.075, 0.15 and 0.3 U; RT for 1 h; 15 µl buffer) (B). (C) Western blots for the HA tag on IL-33 showing inhibition of the cleavage of RRL-expressed human pro-IL-33 to the intermediate form (~25 kDa) and terminal form (~18 kDa) after mutation of R48 to His (IL-33_{FL-R48H}) and of R106 to His (IL-33_{FL-R106H}). (D) IL-33-dependent IL-5 and IL-13 production by ILC2s incubated with or without thrombin (50 pg RRL; 0.075 U thrombin; RT for 1 h) and with or without a neutralizing hIL-33 mAb. (E) Mutation of R48 to His (IL-33_{FL-R48H}) did not affect IL-33 activation by thrombin-mediated cleavage. (F) Mutation of R106 to His (IL-33_{FL-R106H}) prevented IL-33 activation by thrombin-mediated cleavage. (G) The capacity of different IL-33 forms (IL-33_{FL}, IL-33₄₉₋₂₇₀, IL-33₁₀₇₋₂₇₀ and IL-33₁₁₂₋₂₇₀) produced in RRL to activate IL-33-responsive ILC2s (5 × 10³ cells per well; 18 h stimulation). (H) Western blots for IL-33_{FL} showing cleavage of the RRL-produced protein by the combination of *A. alternata* (0, 50 and 100 µg/ml) and thrombin. (I) IL-33-dependent IL-5 and IL-13 production by ILC2s incubated with or without thrombin compared with *A. alternata* alone. (J) Western blots for IL-33_{FL-R106H} showing cleavage of the RRL-produced protein by the combination of *A. alternata* and thrombin. (K) IL-33-dependent IL-5 and IL-13 production by ILC2s incubated with or without thrombin compared with *A. alternata* alone. IL-5 and IL-13 protein levels in supernatants were detected by ELISA. Mouse CD45⁺Lin⁻CD127⁺CD90.2⁺ST2⁺ lung ILC2s sorted from IL-33-treated WT mice were cultured in vitro with IL-2 (100 U/ml) and IL-7 (20 ng/ml) for the above experiments. The bars and error bars represent the mean ± SEM; **p* < .05; ***p* < .01; ****p* < .001; ns, not significant by unpaired Student's *t* test or one-way ANOVA with Tukey's multiple comparisons test. The results are representative of three independent experiments



(Figure 4G), which indicated that the 18 kDa form (IL-33₁₀₇₋₂₇₀) was a highly activated form of IL-33 and that thrombin could directly activate IL-33 through R106-mediated cleavage. We also investigated whether similar thrombin cleavage of IL-33_{FL} occurred in mice. We found that the mouse full-length IL-33₁₋₂₆₆ protein, like its human counterpart, was cleaved by thrombin at R50 and R87, resulting in the production of a 25 kDa intermediate form (IL-33₅₁₋₂₆₆) and an 18 kDa form (IL-33₈₈₋₂₆₆), respectively (Figure S4B-D). All the forms were able to activate ILC2s (Figure S4E). To determine whether the

presence of both external protease and thrombin during inflammation could further enhance IL-33 maturation, we coincubated *A. alternata* and thrombin with in vitro-translated IL-33_{FL} and found that the combination of thrombin and *A. alternata* resulted in the production of IL-33 forms with greater activity (Figure 4H). Compared with the group treated with *A. alternata* alone, the combination treatment group exhibited stronger stimulation of type 2 cytokine secretion by ILC2s (Figure 4I). However, this enhancement did not occur upon incubation with IL-33_{FL-R106H} (Figure 4J-K). These data

suggested that the R106 site might play an important role in the coactivation of IL-33 by thrombin and *A. alternata*. Thus, thrombin specifically cleaved and activated IL-33_{FL} at a conserved site, revealing a fundamental link between coagulation and the IL-33-mediated immune response.

3.4 | Inhibition of type 2 acute pulmonary inflammation in mice by a direct thrombin inhibitor: bivalirudin TFA

Because of the wide-ranging biological functions of LMWH, it was difficult to further verify the direct regulation of IL-33 by thrombin in vivo using this agent. Thus, a direct inhibitor of thrombin, bivalirudin TFA, which is used clinically as an antithrombotic drug was selected to study the role of thrombin in type 2 acute inflammation. We found that bivalirudin TFA significantly suppressed acute type 2 inflammation. Mice were treated with bivalirudin TFA and *A. alternata* for 4 consecutive days (Figure 5A). The number and percentage of eosinophils, but not the number of macrophages or neutrophils, were significantly lower in bivalirudin TFA-treated mice than in PBS-treated mice (Figure 5B–D). Furthermore, lung inflammation was ameliorated, IL-5 and IL-13 production reduced (Figure 5E and F), and the number of ILC2s decreased (Figure 5G) after treatment with bivalirudin TFA. Similarly, among mice that received papain extract intratracheally (Figure 5H), those treated with bivalirudin TFA had less type 2 pulmonary inflammation (Figure 5I–L), fewer ILC2s and lower ILC2 cytokine production in the lungs than those treated with PBS (control) (Figure 5M and N). Collectively, these results indicated that bivalirudin TFA could restrict ILC2-mediated type 2 acute pulmonary inflammation in different mouse models.

3.5 | Effects and clinical prospects of bivalirudin TFA as a direct thrombin inhibitor for allergic disease

Dust mites are common allergens among allergic asthma patients.⁴² To better understand the possible clinical significance of antithrombotic drugs for allergy treatment, we used a house dust mite (HDM)-induced mouse model and investigated whether bivalirudin TFA treatment affected the characteristics of human-like allergic lung inflammation (Figure 6A). HDM administration significantly increased airway resistance in the isotype-treated cohort but not in the bivalirudin TFA-treated cohort, suggesting that bivalirudin TFA engagement could prevent HDM-induced airway hyper-responsiveness (AHR) (Figure 6B). Bivalirudin TFA also decreased eosinophil accumulation (Figure 6C) and type 2 cytokine production (Figure 6D) in BALF from HMD-exposed mice but did not decrease the numbers of macrophages and neutrophils (Figure 6E). Lung inflammation (Figure 6F) and ILC2 function (Figure 6G) were also ameliorated by bivalirudin TFA treatment. Moreover, we found that bivalirudin TFA treatment reduced the characteristics of T/B cell-dependent allergic responses, such as the high plasma

concentrations of total IgE, HDM-IgE and HDM-IgG1 (Figure 6H). Th2 cells can express ST2 and thus respond to IL-33⁹; therefore, we demonstrated that IL-33_{FL} cleaved in vitro by thrombin was able to activate ST2⁺ CD4⁺ T cells from the lungs to produce type 2 cytokines (Figure 6I). Moreover, bivalirudin TFA inhibited the inflammatory response in an ovalbumin (OVA)–alum-mediated allergic mouse model (Figure 55A). BALF from bivalirudin TFA-treated mice contained significantly less IL-4/5/13 than that from control mice (Figure 55B). Likewise, we observed much lower absolute numbers of ILC2s in the lungs of bivalirudin TFA-treated mice than in those of control mice (Figure 55C). Total IgE and OVA-specific IgE and IgG1 levels, but not IgG2a levels, in serum were decreased in bivalirudin TFA-treated mice (Figure 55D). Similarly, lung lymphocytes from bivalirudin TFA-treated mice produced less IL-4/5/13 ex vivo than those from control mice (Figure 55E). The data from the HDM and OVA models suggested that thrombin-mediated IL-33 maturation was critical for type 2 inflammation promoted by ILC2s and Th2 cells, indicating the clinical application value of bivalirudin TFA for the treatment of allergic diseases.

3.6 | Thrombin in asthmatic patients is associated with IL-33-mediated ILC2 activation

An increased thromboembolic risk has been reported in asthmatic patients,^{31–33} thereby providing a thrombin-related physiological condition to detect changes in the function of IL-33 responder ILC2s. Consistent with previous reports, plasma TATc levels were significantly increased in asthma patients compared to healthy controls (Figure 7A). The gating strategy for human ILC2s is shown in Figure S6A. Asthma patients were divided into two categories according to TATc levels: the high-thrombin and low-thrombin categories (Figure S6B). Interestingly, the percentage and number of ILC2s in PBMCs were higher in the TATc^{High} group than in the TATc^{Low} group (Figure 7B). To identify the sources of IL-5 and IL-13 within the human PBMC population, we isolated equal numbers of ILC2s, total CD4⁺ T cells, and Th2 cells and activated them with IL-33. We found that ILC2s were the major source of type 2 cytokines among IL-33-activated PBMCs (Figure S6C). To assess the IL-33-mediated type 2 immune response in allergic patients, we cultured PBMCs in vitro with IL-2, IL-7 and IL-33 for 5 days. Then, the number of activated ILC2s and cytokine (IL-5 and IL-13) production was measured. We found that PBMCs derived from patients with low TATc levels secreted less IL-5 and IL-13 than those from patients with higher TATc levels after IL-33 stimulation (Figure 7C). It was obvious that the percentage and number of ILC2s in PBMCs from asthma patients were positively correlated with plasma TATc levels (Figure 7D–E). IL-13 secretion from asthma patients' PBMCs was positively correlated with plasma thrombin levels, and IL-5 secretion showed a similar tendency (Figure 7F–G). All patient information is shown in the table in Figure S6D. We also collected PBMCs from healthy donors and found that, unlike the phenomenon observed in asthma patients, there was no significant correlation between plasma thrombin content and the

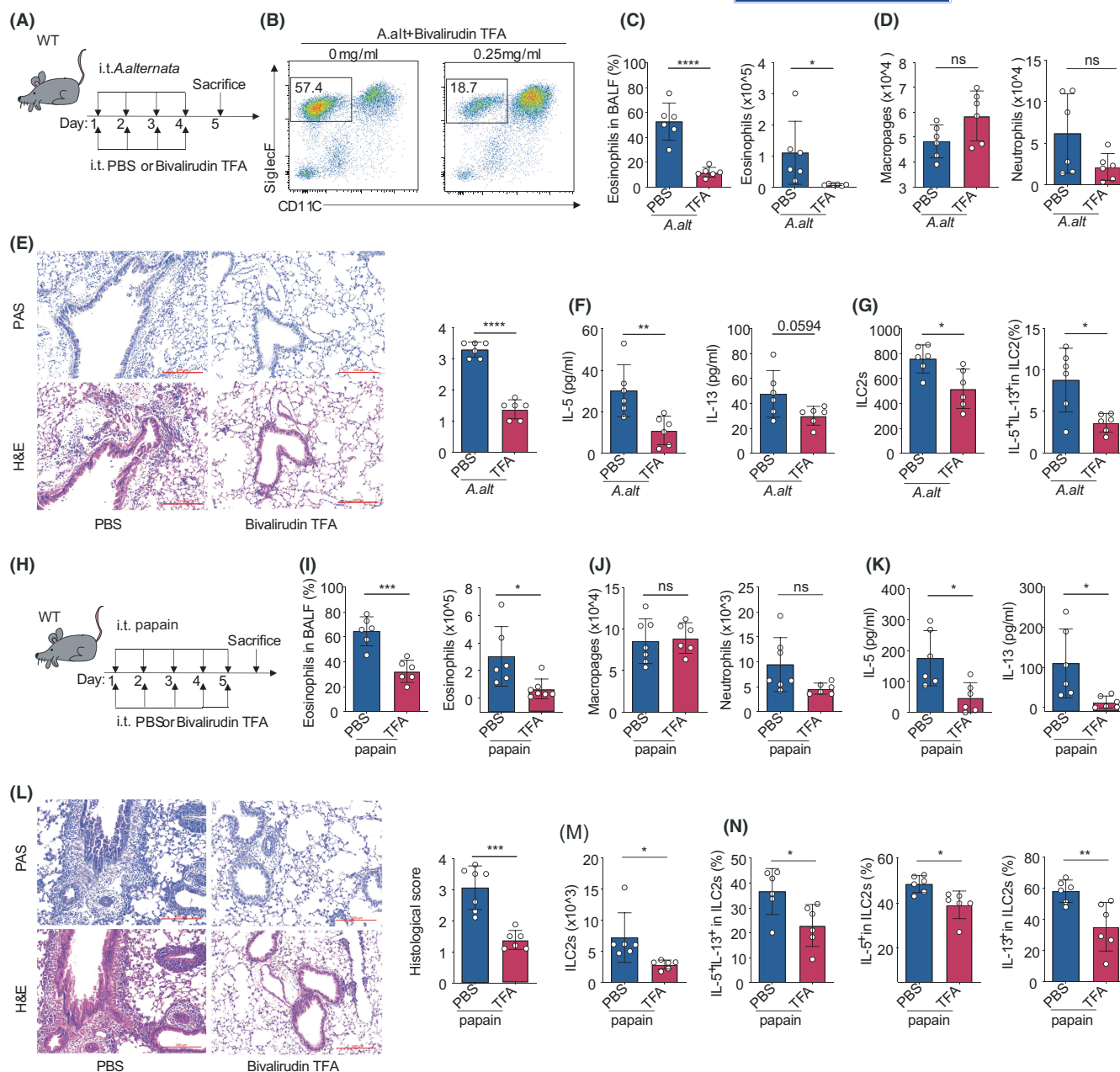


FIGURE 5 Bivalirudin TFA constrains type 2 acute pulmonary inflammation in mice. (A) Schematic diagram of intratracheal (i.t.) bivalirudin TFA treatment (0.25 mg/kg) in the context of an *A. alternata*-induced type 2 immune response. (B) Flow cytometry plots of eosinophil percentages in BALF. (C) Percentage and number of total eosinophils (CD45⁺CD11c^{+/lo}SiglecF⁺) in BALF ($n = 6-7$ per group). (D) The number of macrophages and neutrophils in BALF ($n = 6$ per group). (E) Representative images of H&E and PAS staining of lung sections (scale bar, 200 μ m); inflammation was determined by semiquantitative scoring ($n = 6$ per group). (F) IL-5 and IL-13 levels in BALF were measured by ELISA. (G) Statistical analysis of the number of ILC2s in the lungs; percentages of IL-5⁺IL-13⁺ cells among lung ILC2s after stimulation with PMA, ionomycin and BFA for 4 h ($n = 6$ per group). (H) Mice were intratracheally challenged with papain or PBS on five consecutive days, treated with PBS or bivalirudin TFA (0.25 mg/kg) daily and sacrificed on day 6. (I) Number and percentage of eosinophils in BALF ($n = 6-7$ per group). (J) The number of macrophages and neutrophils in BALF ($n = 6$ per group). (K) IL-5 and IL-13 levels in BALF were measured by ELISA. (L) Representative images of H&E and PAS staining of lung sections (scale bar, 200 μ m); inflammation was determined by semiquantitative scoring ($n = 6$ per group). (M) The number of ILC2s in the lungs. (N) Percentages of IL-5⁺, IL-13⁺ and IL-5⁺IL-13⁺ cells among lung ILC2s after stimulation with PMA, ionomycin and BFA for 4 h. The bars and error bars represent the mean $\pm$ SEM; * $p < .05$; ** $p < .01$; *** $p < .001$; ns, not significant by unpaired Student's *t* test. The data are representative of two independent experiments

percentage and quantity of ILC2s in PBMCs (Figure S6E). Similarly, cytokine production by PBMCs after IL-33 stimulation did not correlate with plasma thrombin levels in healthy donors (Figure S6F).

Collectively, our clinical results suggested that thrombin levels in allergic patients might affect the type 2 immune response by influencing ILC2 activation.

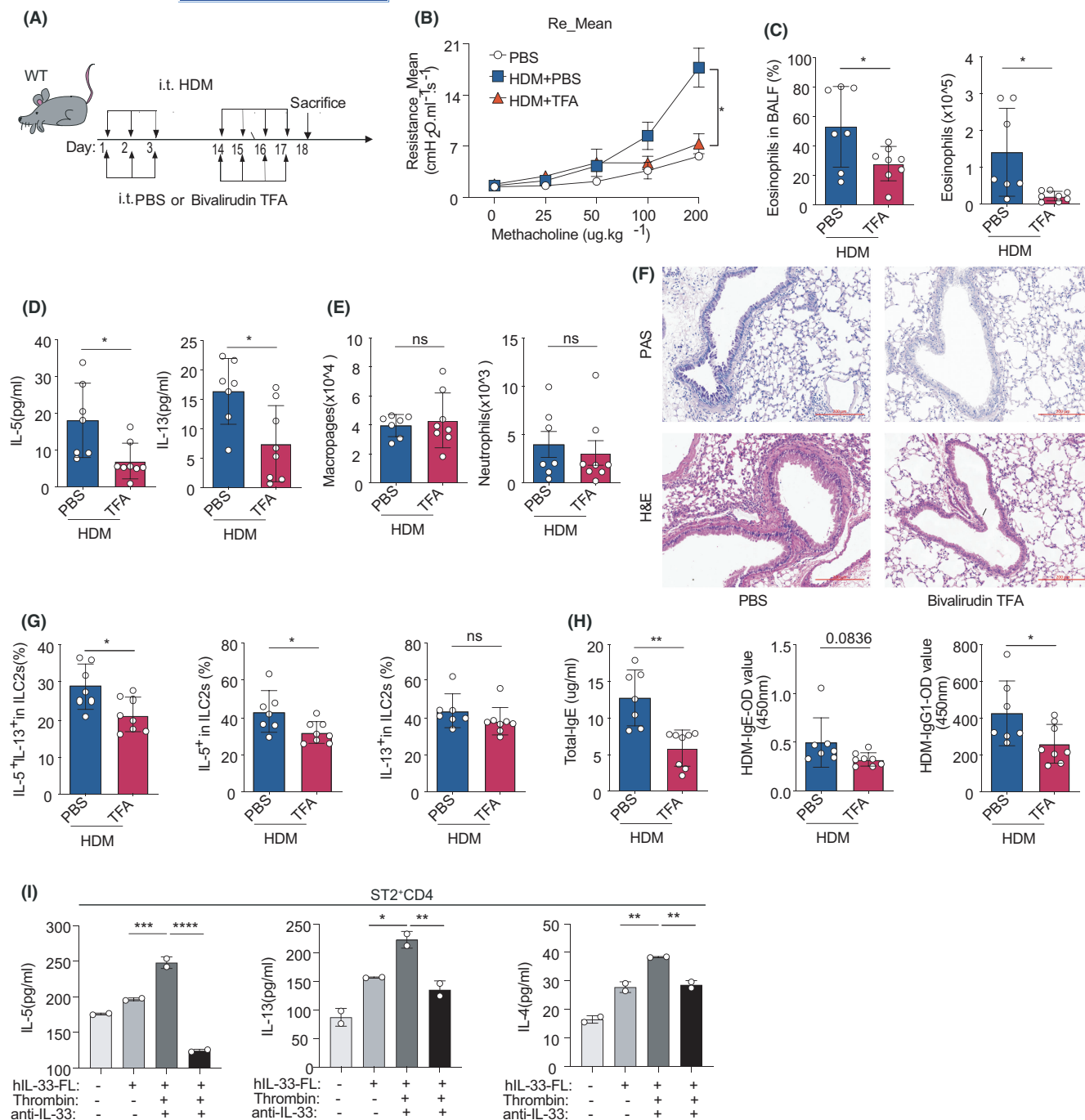


FIGURE 6 Role of bivalirudin TFA in HDM-mediated chronic lung inflammation. (A) Mice were challenged on three consecutive days with HDM (30 μg HDM per mouse) and treated with PBS or bivalirudin TFA (0.25 mg/kg) daily. Then, these mice were allowed to rest for 10 days, rechallenged with HDM (6 μg per mouse) on four consecutive days and sacrificed on day 18. (B) Line graphs show the airway resistance in response to increasing doses of methacholine. (C) Number and percentage of eosinophils in BALF ($n = 7-8$ per group). (D) IL-5 and IL-13 levels in BALF were measured by ELISA. (E) The number of macrophages and neutrophils in BALF ($n = 6$ per group). (F) Representative images of H&E and PAS staining of lung sections (scale bar, 200 μm). (G) The percentages of IL-5⁺, IL-13⁺ and IL-5⁺IL-13⁺ cells among lung ILC2s after stimulation with PMA, ionomycin and BFA for 4 h. (H) Total IgE, HDM-specific IgE and HDM-specific IgG1 levels in serum from PBS- and bivalirudin TFA-treated mice were measured by ELISA; the results are shown as the antibody titre extrapolated from a standard curve (if available) or as the absorbance at 450 nm (OD450 value) for a dilution of serum at which all samples were in the range of the assay. (I) IL-33-dependent IL-5, IL-13 and IL-4 production by ST2⁺ CD4 cells incubated with or without thrombin (50 pg RRL; 0.075 U thrombin; RT for 1 h) and with or without a neutralizing hIL-33 mAb. The bars and error bars represent the mean ± SEM; * $p < .05$; ** $p < .01$; *** $p < .001$; ns, not significant by unpaired Student's *t* test or one-way ANOVA with Tukey's multiple comparisons test. The data are representative of two independent experiments

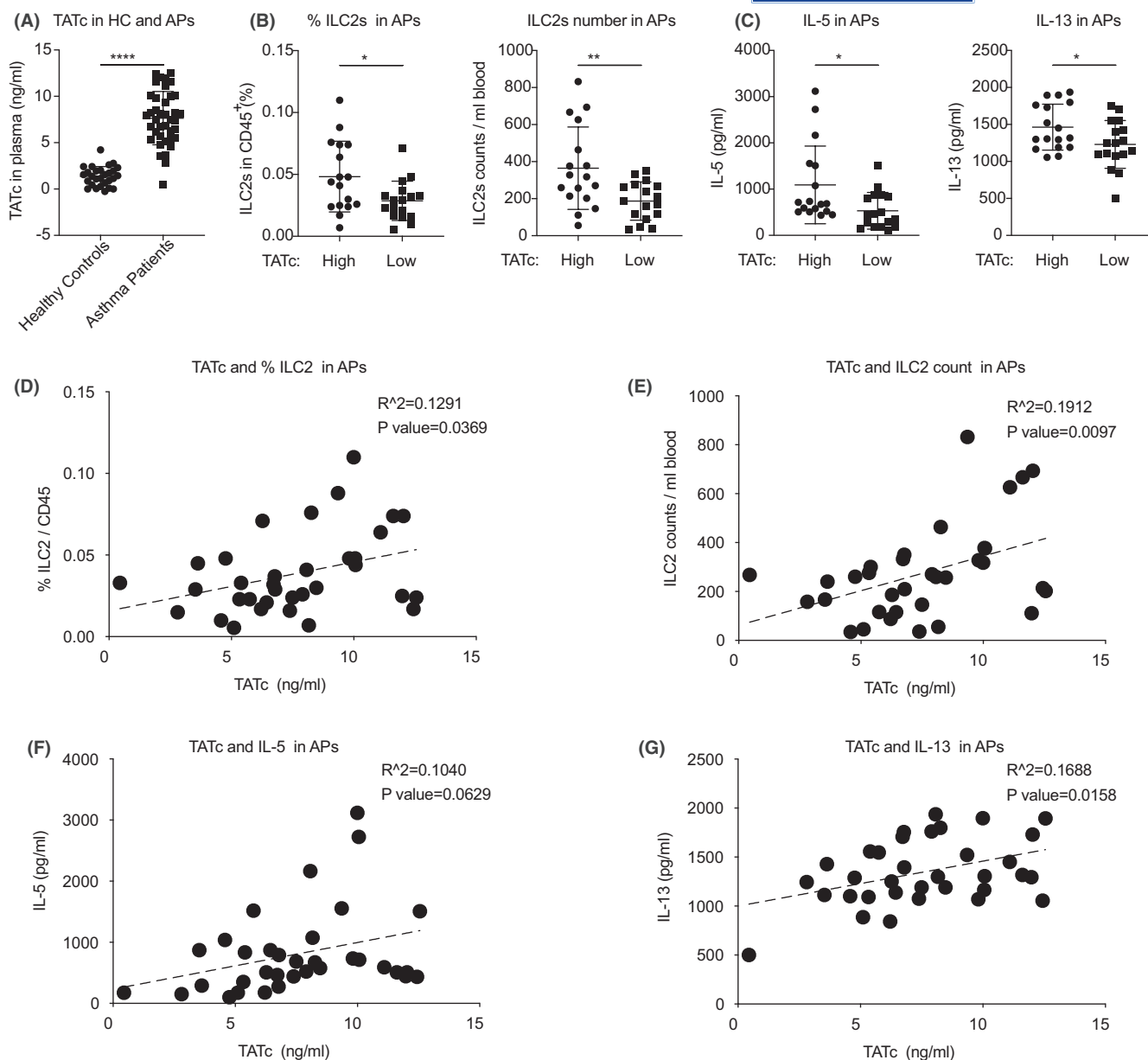


FIGURE 7 IL-33-mediated ILC2 activation in PBMCs from asthma patients (APs) is positively associated with thrombin levels. (A) ELISA data for the thrombin-antithrombin complex (TATc) in plasma from healthy donors ($n = 28$) and asthma patients ($n = 34$). (B) Percentages and numbers of hILC2s among PBMCs from TAT^{High} and TAT^{Low} asthma patients. (C) Secretion of the type 2 immune response cytokines IL-5 and IL-13 from PBMCs after IL-33 activation. (D–E) Correlation analysis between the percentage and number of hILC2s among PBMCs and thrombin levels in the plasma of APs. (F–G) Correlation analysis between IL-5 or IL-13 and thrombin levels in the plasma of APs. PBMCs were isolated from APs and then cultured in vitro with hIL-2 (100 U/ml), hIL-7 (20 ng/ml) and hIL-33 (50 ng/ml). The supernatants were collected on day 5, and the levels of the cytokines IL-5 and IL-13 were detected by ELISA. PBMCs with onefold higher IL-5 secretion after treatment with IL-2, IL-7 and IL-33 than after treatment with IL-2 and IL-7 were used for data analysis. The bars and error bars represent the mean $\pm$ SEM; * $p < .05$; ** $p < .01$; *** $p < .001$; ns, not significant by unpaired Student's t test

4 | DISCUSSION

In our present study, we found that LMWH was able to suppress both papain- and *A. alternata*-induced type 2 immune responses through ILC2s. A mechanistic study revealed that thrombin inhibitors regulated pulmonary type 2 inflammation mainly by targeting IL-33 maturation. We found that thrombin directly cleaved IL-33_{FL} at specific amino acid residues (R48 and R106) to generate

a mature, biologically active form of IL-33. Different forms of thrombin-cleaved IL-33 showed different biological activities. The shorter spliced form of IL-33 had stronger activity than the longer spliced form. In addition to ILC2-mediated acute pulmonary inflammation, bivalirudin TFA also alleviated HDM- and OVA-alum-induced chronic pulmonary inflammation. These findings indicated that the direct thrombin inhibitor bivalirudin TFA might be a good candidate drug for the treatment of type 2 diseases such

as allergic diseases. Consistent with previous clinical observations, we also found that plasma thrombin content was associated with changes in ILC2 number and function in PBMCs from asthma patients. Overall, we elucidated a new regulatory circuit in which thrombin regulates the immune system by affecting IL-33 maturation to impact type 2 inflammation.

There are still several issues that remain to be discussed. (1) Although some reports have stated that thrombin induces IL-33 expression in human aortic smooth muscle cells (HASMCs) in a time-dependent manner,⁴³ there are no significant changes in IL-33 at the transcriptome and protein levels in models of lung inflammation caused by epithelial injury, which might be due to IL-33 tissue heterogeneity or different temporal regulation. During allergic pulmonary inflammation, IL-33 is released from the nucleus and then quickly cleaved and activated by endogenous or environmental allergen proteases.³⁰ (2) We detected the IL-33 intermediate form (IL-33₄₉₋₂₇₀) produced by thrombin-specific cleavage in the in vitro protein expression system, but we did not detect endogenous intermediate forms of IL-33 in mouse BALF. We believe that a complex protease environment might result in the rapid degradation of IL-33 intermediate forms produced by thrombin cleavage. (3) The function of the thrombin-cleaved IL-33 intermediate form IL-33₄₉₋₂₇₀ remains to be further determined. Notably, researchers have found that a reduction in IL-33 chromatin binding can increase extracellular IL-33 release by affecting intracellular retention.⁴⁴ Interestingly, R48 is within the chromatin binding domain of IL-33, and mutation of R48 is known to specifically diminish the interaction of IL-33 with the nucleosome acidic patch.⁴⁵ Therefore, we suspect that IL-33₄₉₋₂₇₀ may be related to histone binding, which likely changes IL-33 activity to affect the downstream signal. More research on the role of R48 in IL-33 function needs to be conducted. (4) IL-33 is considered an important target in several important diseases.^{9,10} In our study, we used only the most classic asthma models. However, we believe that our findings might indicate the potential for the wide application of thrombin inhibitors in IL-33-related diseases.

Although steroid drugs are usually sufficient for the treatment of asthma, some patients exhibit a disease that is refractory to currently available treatments.⁴⁶ Because poorly controlled allergic pulmonary inflammation can lead to the irreversible loss of normal lung function,⁴⁷ new anti-inflammatory treatments are urgently needed. The wide range of biological activities of LMWH limits its clinical use. We hypothesized that a more specific thrombin inhibitor would be beneficial for the treatment of pulmonary inflammation. A previous study explored the effects of dabigatran, an oral direct thrombin inhibitor, and found that it had only a modest effect on HDM-induced lung inflammation, suggesting that dabigatran is unlikely to be very useful as adjunctive therapy in refractory asthma.⁴⁸ In the current study, we explored bivalirudin TFA-mediated thrombin inhibition as a potential anti-inflammatory treatment option in a mouse asthma model. Importantly, bivalirudin TFA has been found to be associated with a lower risk of bleeding than heparin.⁴⁹

Over the past two decades, pulmonary coagulation has been shown to play an important role in allergic lung inflammation processes such as asthma.⁵⁰⁻⁵⁴ Pulmonary coagulation factors may arise from plasma proteins that leak through inflamed blood vessels or from the local synthesis and secretion by lung epithelial cells.⁵⁵⁻⁵⁷ Physiological levels of thrombin generation in the airways may contribute to mucosal haemostasis, fibrin-mediated bacterial capture or enhanced defence mechanisms.⁵¹ Under pathological conditions such as allergic mucosal inflammation, the regulatory mechanism may be disrupted by excessive thrombin production and fibrin deposition, which may have harmful effects on the airways.^{56,58} In addition, exacerbation of the allergic response by thrombin during activation of the clotting system may be mediated by T cells, mast cells and dendritic cells (DCs).⁵⁹ However, the specific molecular mechanism is not yet clear. Here, we propose a potential mechanism in which thrombin directly activates the cytokine IL-33 and participates in mediating IL-33 maturation and regulating IL-33-driven ILC2 activation. Since IL-33 functions in many diseases involving immune cells, these findings may also be relevant in many other IL-33-related diseases. In summary, our work shows that bioactive forms of IL-33 are generated by caspase- and protease-independent mechanisms and that thrombin plays an important role in type 2 pulmonary inflammation mainly by affecting IL-33 maturation. Thus, our findings provide a potential drug target for the treatment of type 2 pulmonary inflammatory diseases.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Y. Huang, X. Li and L. Zhu designed and performed the experiments and analysed the data. C. Huang collected the clinical samples. W. Chen, C. Yan, S. Zhu, X. Feng, X. Su, W. Gu, C. Yi and J. Wang provided protocols and suggestions. L. Ma and Z. Ling prepared cell lines and provided reagents. Y. Huang and Y. Zhang wrote the manuscript. Y. Zhang, B. Sun, G. Shi and R. Dai supervised the project and revised the manuscript.

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SUPPORTING INFORMATION

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