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Airway Uric Acid Is a Sensor of Inhaled Protease Allergens and Initiates Type 2 Immune Responses in Respiratory Mucosa

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Although type 2 immune responses to environmental Ags are thought to play pivotal roles in asthma and allergic airway diseases, the immunological mechanisms that initiate the responses are largely unknown. Many allergens have biologic activities, including enzymatic activities and abilities to engage innate pattern-recognition receptors such as TLR4. In this article, we report that IL-33 and thymic stromal lymphopoietin were produced quickly in the lungs of naive mice exposed to cysteine proteases, such as bromelain and papain, as a model for allergens. IL-33 and thymic stromal lymphopoietin sensitized naive animals to an innocuous airway Ag OVA, which resulted in production of type 2 cytokines and IgE Ab, and eosinophilic airway inflammation when mice were challenged with the same Ag. Importantly, upon exposure to proteases, uric acid (UA) was rapidly released into the airway lumen, and removal of this endogenous UA by uricase prevented type 2 immune responses. UA promoted secretion of IL-33 by airway epithelial cells in vitro, and administration of UA into the airways of naive animals induced extracellular release of IL-33, followed by both innate and adaptive type 2 immune responses in vivo. Finally, a potent UA synthesis inhibitor, febuxostat, mitigated asthma phenotypes that were caused by repeated exposure to natural airborne allergens. These findings provide mechanistic insights into the development of type 2 immunity to airborne allergens and recognize airway UA as a key player that regulates the process in respiratory mucosa. ***The Journal of Immunology*****, 2014, 192: 000–000.**

Asthma and allergic airway diseases have increased over the past 50 y. Globally, 300 million and 400 million people suffer from asthma and allergic rhinitis, respectively (1). Allergic asthma is generally caused by type 2 immune responses to innocuous airborne Ags, leading to eosinophilic airway inflammation, mucous production, structural changes to the airway wall, and variable airway obstruction (2). A substantial body of evidence suggests the importance of dendritic cells (DCs) in inducing or modulating Th2 responses (3–7). Basophils can prime Th2 responses by presenting Ags and producing IL-4 and thymic stromal lymphopoietin (TSLP) (8–11). Several other components of the innate immune system, including epithelial cells, recently identified innate lymphoid cells (ILCs), eosinophils, mast cells, and alternatively activated macrophages, are also likely involved (12–14). Despite recent advances in the cellular aspects of type 2 immune responses, the molecular mechanisms involved in sensing airborne allergens and initiating type 2 immunity in respiratory mucosa remain largely unknown.

One of the most enigmatic features of type 2 immunity is its propensity to be activated in response to a wide variety of inhaled Ags or environmental insults, including pollen, molds, crustaceans, and insects, as well as airborne irritants and respiratory viral infections. Accordingly, there may be multiple pathways that are tailored to respond against specific environmental factors. For example, inhaled house dust mite (HDM) extract stimulates Th2-type immune responses by acting on airway epithelial cells, DCs, basophils, and mast cells (3, 15). The response to HDM is mediated by recognition of a major HDM allergen (Der p 2) and perhaps endotoxin contained in fecal pellets through TLR4 (4, 16, 17). Alternatively, there may be a few shared pathways that drive type 2 immunity, regardless of the eliciting substance or Ag. Indeed, a common mechanism of sensing allergens is based on detection of their unique biological properties. For example, many Th2-inducing stimuli have enzymatic activities, such as proteases from HDM and fungi (18, 19), phospholipases from bee venom (20), and RNase ω-1 from Schistosoma egg extracts (21). However, the molecular mechanisms that explain how these enzymatic activities in allergens are detected by the immune system in airway mucosa are not well understood.

In this study, we sought to identify the early immunological mechanisms that detect protease allergens in the airways. When injected into the skin tissues, an authentic cysteine protease, papain, induces strong Th2-type immune responses (9, 22). Bromelain, another cysteine protease, is a strong airway sensitizer, and bromelain inhalation causes occupational asthma in humans (23, 24). Therefore, we used these proteases as a model. Administration of proteases into mouse airways induced robust Th2-type immune responses, which were mediated by IL-33 and TSLP. Upon exposure to proteases, uric acid (UA) was rapidly released into the airway lumen, and

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Abbreviations used in this article: BAL, bronchoalveolar lavage; DAMP, damage-associated molecular pattern; DC, dendritic cell; HDM, house dust mite; HMGB-1, high mobility group box protein B1; ILC, innate lymphoid cell; ILC2, group 2 ILC; i.n., intranasally; MSU, monosodium urate; NHBE, normal human bronchial airway epithelial; PAR2, protease-activated receptor 2; PAS, periodic acid–Schiff; TSLP, thymic stromal lymphopoietin; UA, uric acid; WT, wild-type.

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this endogenous UA initiated type 2 immunity by inducing IL-33 and TSLP. Importantly, inhibition of UA synthesis or removal of released UA from the airway lumen effectively inhibited type 2 immune responses induced by proteases, as well as natural allergens. Thus, monitoring of the epithelial environment by endogenous UA may be a strategy for responding to various environmental stresses in respiratory mucosa.

Materials and Methods

Mice and cells

BALB/c, C57BL/6, BALB/cByJ, *Tlr4*$^{Lps\text{-}d}$/J (*Tlr4*-d, BALB/cByJ background), *Rag1*$^{-/-}$ mice (BALB/c background), *Il1r1*$^{-/-}$ mice (C57BL/6 background), and *PAR2*$^{-/-}$ mice (C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME). *ST2*$^{-/-}$ (*Il1rl1*$^{-/-}$) mice (BALB/c background), *Il17rb*$^{-/-}$ mice (BALB/c background), and *Il13*$^{+/eGFP}$ mice (BALB/c background) were provided by Dr. Andrew McKenzie (Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.). *Tslpr*$^{-/-}$ mice (BALB/c background) were provided by Dr. Steven Ziegler (Benaroya Institute, Seattle, WA). *Il5*$^{+/venus}$ mice (BALB/c background) were provided by Dr. Kiyoshi Takatsu (University of Toyama, Toyama, Japan). *Nlrp3*$^{-/-}$ mice (C57BL/6 background) were provided by Dr. Jurg Tschopp (University of Lausanne, Lausanne, Switzerland). All knockout or transgenic mice were bred in the Mayo Clinic animal care facility, and female mice aged 6–10 wk were used for studies. All animal experiments and handling procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee and performed according to their guidelines.

Normal human bronchial airway epithelial (NHBE) cells were purchased from Lonza (Allendale, NJ) and maintained in serum-free bronchial epithelial cell growth medium (Lonza). NHBE cells were used within three passages.

Reagents

PE-conjugated anti-CD3 (17A2), anti-CD14 (rmC5-3), anti-CD16/32 (2.4G2), anti-B220 (RA3-6B2), PerCP-conjugated anti-CD44 (IM7), and allophycocyanin-conjugated anti-CD25 (PC61) Abs were purchased from BD Biosciences (San Jose, CA). A cysteine protease inhibitor (E64) and uricase from *Arthrobacter globiformis* were purchased from Sigma-Aldrich (St. Louis, MO). Endotoxin-free OVA (<0.5 endotoxin unit/mg protein) was purified from specific pathogen-free chicken eggs under sterile conditions. Recombinant mouse IL-33 (Ser^{109}-Ile^{266}, <0.01 endotoxin unit/μg protein) was purchased from R&D Systems (Minneapolis, MN). Monosodium urate (MSU) crystals were purchased from Sigma-Aldrich, suspended in PBS at 20 mg/ml, and sonicated for 20 min in an ultrasonic cleaner (BRANSON 2200; Branson Ultrasonics, Danbury, CT) before use. The endotoxin levels in the MSU crystal suspension were <0.005 endotoxin unit/ml. Bromelain (from pineapple stem) and papain (from *Carica papaya*) were purchased from Sigma-Aldrich and EMD Millipore (Billerica, MA), respectively. *Alternaria alternata* culture filtrate extract, *Aspergillus fumigatus* extract, and HDM extract were obtained from Greer Laboratories (Lenoir, NC); these extracts contained <2 endotoxin units/mg protein.

Acute airway inflammation model

Bromelain (10 μg/dose), papain (50 μg/dose), or MSU crystals (1 mg suspension/dose) in 50 μl PBS or PBS alone were administered intranasally (i.n.) once to naive wild-type (WT) mice or *ST2*$^{-/-}$ mice that were lightly anesthetized using isoflurane inhalation to examine acute airway immune responses. In some experiments, bromelain was administered together with uricase (1 U/dose). At the indicated time points, mice were sacrificed via an overdose of pentobarbital. The trachea was cannulated, and lungs were lavaged with 1 ml HBSS. The number of cells in bronchoalveolar lavage (BAL) fluids was counted using a hemacytometer, and cell differentials were determined in cytospin preparations stained with Wright–Giemsa; >200 cells were analyzed using conventional morphologic criteria. BAL fluid supernatants were collected and stored at −20°C for cytokine assays. Lungs were homogenized in 800 μl PBS and centrifuged for 5 min at 13,000 × *g* at 4°C, and protein concentrations in the supernatants were measured using the Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL). Supernatants were frozen at −20°C for cytokine analyses.

Airway sensitization and challenge model

Naive WT, *ST2*$^{-/-}$, *Il17rb*$^{-/-}$, *Tslpr*$^{-/-}$, *Il1r1*$^{-/-}$, *Rag1*$^{-/-}$, *Tlr4*-d, or *PAR2*$^{-/-}$ mice were anesthetized with isoflurane and administered i.n. with endotoxin-free OVA (100 μg/dose) with or without bromelain (10 μg/dose) in 50 μl PBS, bromelain alone, OVA alone, or PBS alone on days 0 and 7 to examine the effects of proteases or MSU crystals on adaptive type 2 immune response development. In some experiments, bromelain or papain were pretreated with the cysteine protease inhibitor E64 (10 μM) for 30 min at 4°C. In other experiments, uricase (1 U/dose) was added to the 50 μl bromelain and OVA mixture. On day 14, plasma was collected to analyze OVA-specific Abs. On days 21, 22, and 23, mice were challenged i.n. with 100 μg OVA. On day 24, mice were sacrificed by an overdose of pentobarbital, and BAL and lung specimens were collected and analyzed as described earlier. Fixed lung tissue sections were stained with H&E and periodic acid–Schiff (PAS) stain.

Repeated allergen challenge model

To examine the roles of UA in chronic airway inflammation, we gavaged naive mice once daily for 16 d with febuxostat (5 mg/kg/dose) or distilled water 2 d before i.n. administration of allergen extracts. The mice were exposed i.n. to a mixture of *Alternaria* extract, *Aspergillus* extract, and HDM extract (10 μg each/dose) in 50 μl PBS or PBS alone, 3 d/week for 2 wk, a total of seven times. Twenty-four hours after the last allergen exposure, mice were sacrificed, and BAL fluids and lungs were collected for analyses.

Flow cytometric analyses of cytokine-producing cells by reporter mice

MSU crystals (1 mg suspension/dose) in 50 μl PBS or PBS alone were administered i.n. once a day for 3 d to *Il5*$^{+/venus}$ and *Il13*$^{+/eGFP}$ mice or WT mice. Twenty-four hours after the last administration, lungs were collected and minced using a gentleMACS Dissociator (Miltenyi Biotec, Auburn, CA), and digested with Liberase Research Grade (Roche, Mannheim, Germany) in RPMI 1640 medium in the presence of DNase I solution (STEMCELL Technologies, Vancouver, BC) for 1 h at 37°C. After digestion, single lung cells were hemolyzed with ammonium-chloride-potassium buffer and washed with PBS containing 0.1% sodium azide and 1% BSA. To examine the expression of cytokines by group 2 ILCs (ILC2s), we stained lung single-cell suspensions with a PE-conjugated lineage mixture (CD3 [145-2C11], CD14 [rmC5-3], CD16/32 [2.4G2], B220 [RA3-6B2]), allophycocyanin-CD25 (PC61), and PerCP-Cy5.5-CD44 (IM7; BD Biosciences). Lung ILC2s were identified as Lin^{-} CD25^{+}CD44^{hi} cells as previously described (25). The expression levels of IL-5venus and IL-13eGFP by CD3^{+} T cells or ILC2s were detected by FACS (BD FACSCalibur; BD Biosciences).

Analyses of Ag-specific IgE, IgG1, and IgG2a

To quantitate the levels of OVA-specific IgE Ab in plasma specimens, we coated ELISA plates (Immulon 4; Thermo Labsystems) with 5 μg/ml rat anti-mouse IgE mAb (Serotec) in 0.1 M carbonate buffer (pH 9.5) for 2 h at 37°C. The plates were blocked overnight with 300 μl PBS containing 1% BSA (Sigma-Aldrich) at 4°C. Plasma samples, which were diluted with PBS containing 1% BSA and 0.05% Tween 20 (1:40 for anti-OVA IgE), were added to the plates, and the plates were incubated for 2 h at room temperature. Thereafter, the plates were incubated for 1 h at room temperature with 1 μg/ml biotin-conjugated OVA, followed by 1:5000 streptavidin-poly-HRP (Pierce) for 30 min at room temperature. The plates were repeatedly washed with PBS containing 0.05% Tween 20 between each step. 3,3′,5,5′-Tetramethylbenzidine peroxidase substrate (Pierce) was added and after 15 min, the reaction was stopped with 1 M HCl. The OD at 450 nm was read in a microplate autoreader (SpectraMax 190; Molecular Devices).

To quantitate the levels of OVA-specific IgG1 and IgG2a, we coated ELISA plates with 10 μg/ml OVA, blocked them with BSA, and incubated them with plasma samples diluted in PBS (1:2000 for anti-OVA IgG1, 1:40 for anti-OVA IgG2a). After washing, plates were incubated with HRP-conjugated anti-mouse IgG1 or IgG2a (1:1000; BD Pharmingen), followed by 3,3′,5,5′-tetramethylbenzidine peroxidase substrate. After stopping the reaction with HCl, the absorbance was read in a microplate autoreader. Serial dilutions of plasma in these ELISAs showed linear correlations between Ab concentrations and OD values up to 1.5.

Measurement of cytokines, UA, and high mobility group box protein B1 levels

The levels of IL-4, IL-5, IL-13, IL-17A, IFN-γ, IL-33, and TSLP in the supernatants of BAL fluids and lung homogenates were measured using Quantikine ELISA kits (R&D Systems and GenWay Biotech, San Diego, CA). All ELISAs were performed per the manufacturer's instructions. To measure UA levels in BAL fluid supernatants, we used Amplex Red fluorogenic substrate UA/uricase assay kits (Invitrogen, Grand Island, NY). High mobility group box protein B1 (HMGB-1) levels in BAL fluid supernatants were analyzed using HMGB-1 ELISA kits (IBL International, Toronto,

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ON). UA levels in lung homogenates were measured using colorimetric UA assay kits (Biovision, Milpitas, CA).

Cytokine production and release by NHBE cells

NHBE cells were seeded in 24-well tissue culture plates (3×10^4 cells/well) and grown until 80% confluence (usually 4 d). Cells were stimulated with serial dilutions of MSU crystal suspensions or 100 μg/ml *Alternaria* extract for 3 h. Cell-free supernatants were collected, and IL-33 was analyzed by ELISA (R&D Systems).

Cell membrane integrity analyses of NHBE cells

The NHBE cell membrane integrity was examined using the Live/Dead Cellular Viability/Cytotoxicity kit (Invitrogen) that uses calcein AM and EthD-1 dyes to detect active esterase and compromised membrane integrity, respectively. After incubation for 3 h with media containing MSU crystals (100 μg/ml), NHBE cells were incubated for 30 min at room temperature with 2 μM calcein AM and 4 μM EthD-1. Using fluorescence microscopy, intact (calcein AM^+ and EthD-1^-) and damaged (EthD-1^+) cells in five randomly chosen fields were counted and expressed as the percentage of cells over the total number of cells (≥500 cells were counted).

Localization of IL-33 in NHBE cells by confocal microscopy

NHBE cells were cultured on Lab-Tek 2 chamber slides (Fisher). After stimulation with MSU crystals (100 μg/ml) or medium for 3 h, the cells were washed with PBS and incubated with Golgi plug (BD Pharmingen) for 30 min at 4°C. The slides were fixed and permeabilized by Cytofix/Cytoperm reagents (BD Pharmingen) for 20 min at 4°C and then washed with BD Perm/Wash buffer. Fixed cells were blocked with 5% normal goat serum (Sigma) for 1 h and stained overnight with rabbit anti-human IL-33 (MBL International, Woburn, MA) or control normal rabbit IgG at 4°C. The cells were washed and then incubated with FITC-conjugated goat anti-rabbit IgG for 2 h at room temperature. After a final wash, the chambers were removed, and the slides were mounted with Vectashield mounting medium containing the DNA-binding dye, DAPI (Vector Laboratories). Fluorescent images were visualized using a confocal microscope (LSM580) and Zen software (both Carl Zeiss). The threshold for each negative control image was calibrated to a baseline value without positive pixels.

Statistical analyses

All data are reported as the mean ± SEM from the numbers of mice or samples as indicated. Two-sided differences between two samples were analyzed using Mann–Whitney *U* tests or Student *t* tests. Multiple comparisons

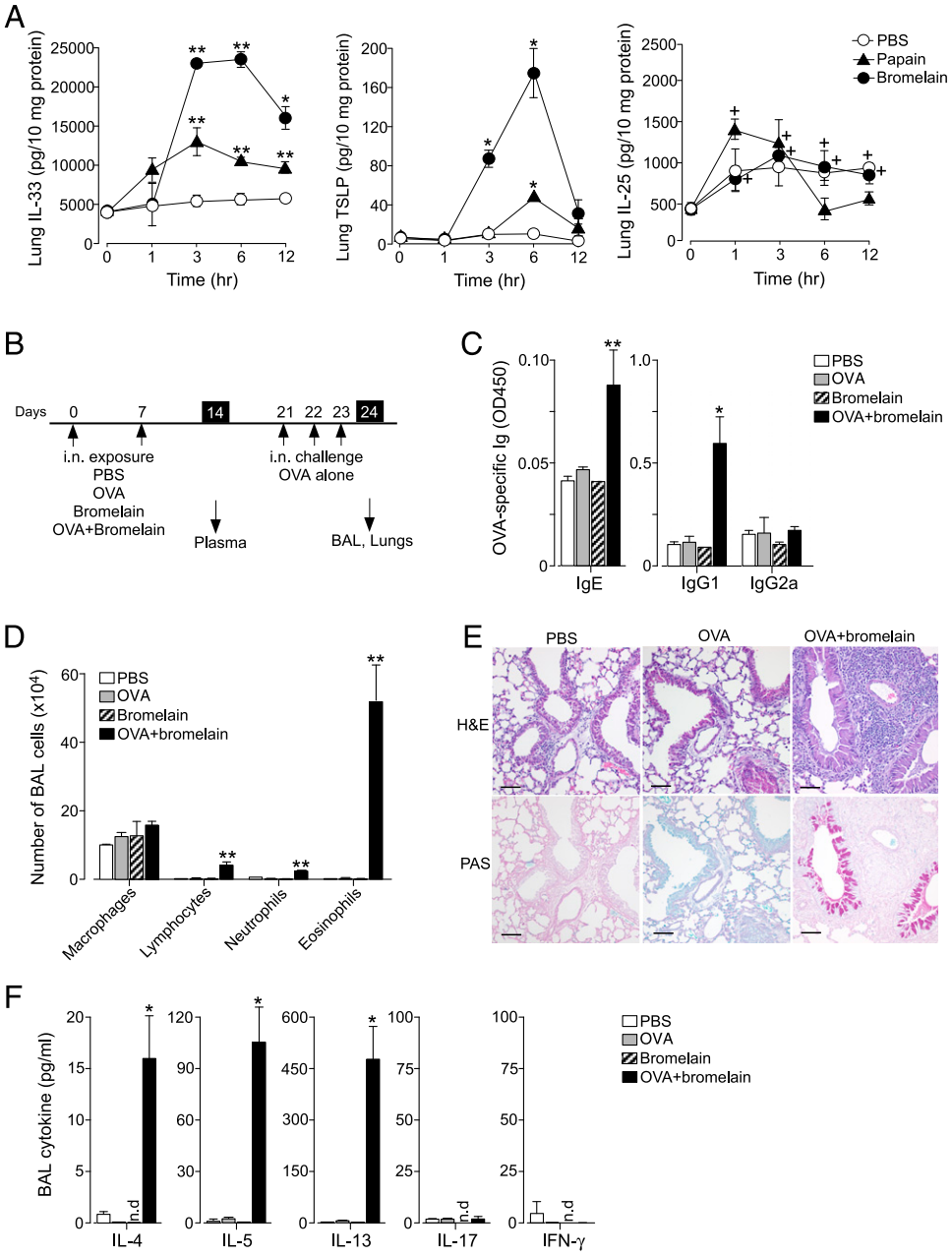

FIGURE 1. Airway exposure of naive mice to cysteine proteases promotes IL-33 and TSLP production and induces Th2-type immune responses to innocuous Ags. (**A**) Naive BALB/c mice were i.n. exposed to PBS, papain (50 μg/dose), or bromelain (10 μg/dose). Kinetic changes in cytokine levels in lung homogenates were analyzed using ELISA. Data shown are the mean ± SEM, $*p < 0.05$, $**p < 0.01$, compared with PBS, $^+p < 0.05$, compared with nontreated mice (i.e., 0 h), n = 5–8 mice/group. Experiments were repeated three times; data shown are one representative experiment. (**B**) Experimental protocol to study Ag-specific immune responses. On days 0 and 7, naive BALB/c mice were exposed i.n. to PBS, endotoxin-free OVA (100 μg/dose), bromelain (10 μg/dose), or OVA plus bromelain. Plasma was collected on day 14. All mice were challenged i.n. with OVA alone on days 21, 22, and 23. On day 24, BAL fluids and lungs were collected. (**C**) On day 14, plasma levels of anti-OVA Abs were determined using ELISA. (**D**) On day 24, total BAL cell number and differentials were determined. (**E**) Lung sections were stained with H&E and PAS. Scale bars, 100 μm. (**F**) Concentrations of cytokines in BAL fluids were analyzed using ELISA. Data shown are the mean ± SEM, $*p < 0.05$, $**p < 0.01$, compared with PBS group, $n = 6$ mice/group. Experiments were repeated twice; data shown are one representative experiment. n.d., not determined.

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between treatment and control conditions were performed using one-way ANOVA. The p values <0.05 were considered significant.

Results

Cysteine proteases are potent adjuvants for induction of Th2-type immune responses in the airway

Many airborne allergens have intrinsic protease activities (26–29). Cysteine proteases, such as papain and bromelain, are potent allergens associated with occupational allergy in humans (30), and they have been used successfully to study mouse models of allergic diseases (9, 22, 31, 32). To examine the acute effects of cysteine proteases on airway immune responses, we administered 10 μg/dose bromelain or 50 μg/dose papain i.n. once into the airways of naive WT BALB/c mice. Substantial amounts of IL-33 and IL-25, but not TSLP, were detectable in lung homogenates of naive nontreated animals (Fig. 1A; please note y-axis scales). Upon exposure to the proteases, the lung levels of IL-33 and TSLP quickly increased within 3 h, peaking at 3–6 h (Fig. 1A). The 10 μg/dose bromelain appeared to be more potent than the 50 μg/dose papain.

Airway exposure to innocuous proteins, such as endotoxin-free OVA, generally induces immunologic tolerance (33, 34). To examine whether cysteine proteases can induce adaptive Th2-type immune response to innocuous Ags, we administered endotoxin-free OVA protein with or without bromelain into the airways of naive BALB/c mice on days 0 and 7 (Fig. 1B). On day 14, plasma levels of OVA-specific IgE and IgG1 Abs increased significantly in mice exposed to OVA plus bromelain (Fig. 1C). OVA alone or bromelain alone did not induce these Ab responses. No increase in IgG2a Ab was observed in mice exposed to OVA plus bromelain.

When these mice were challenged i.n. with OVA Ag (without bromelain) on days 21 through 23, mice previously exposed to OVA plus bromelain demonstrated marked airway eosinophilia, mucous hyperplasia, and peribronchial infiltration with inflammatory cells (Fig. 1D, 1E). These immunologic and pathologic changes were not observed in mice that were previously exposed to OVA alone or bromelain alone. Furthermore, increased BAL levels of IL-4, IL-5, and IL-13, but not IL-17 or IFN-γ, were observed in mice previously exposed to OVA plus bromelain, but not in mice previously exposed to

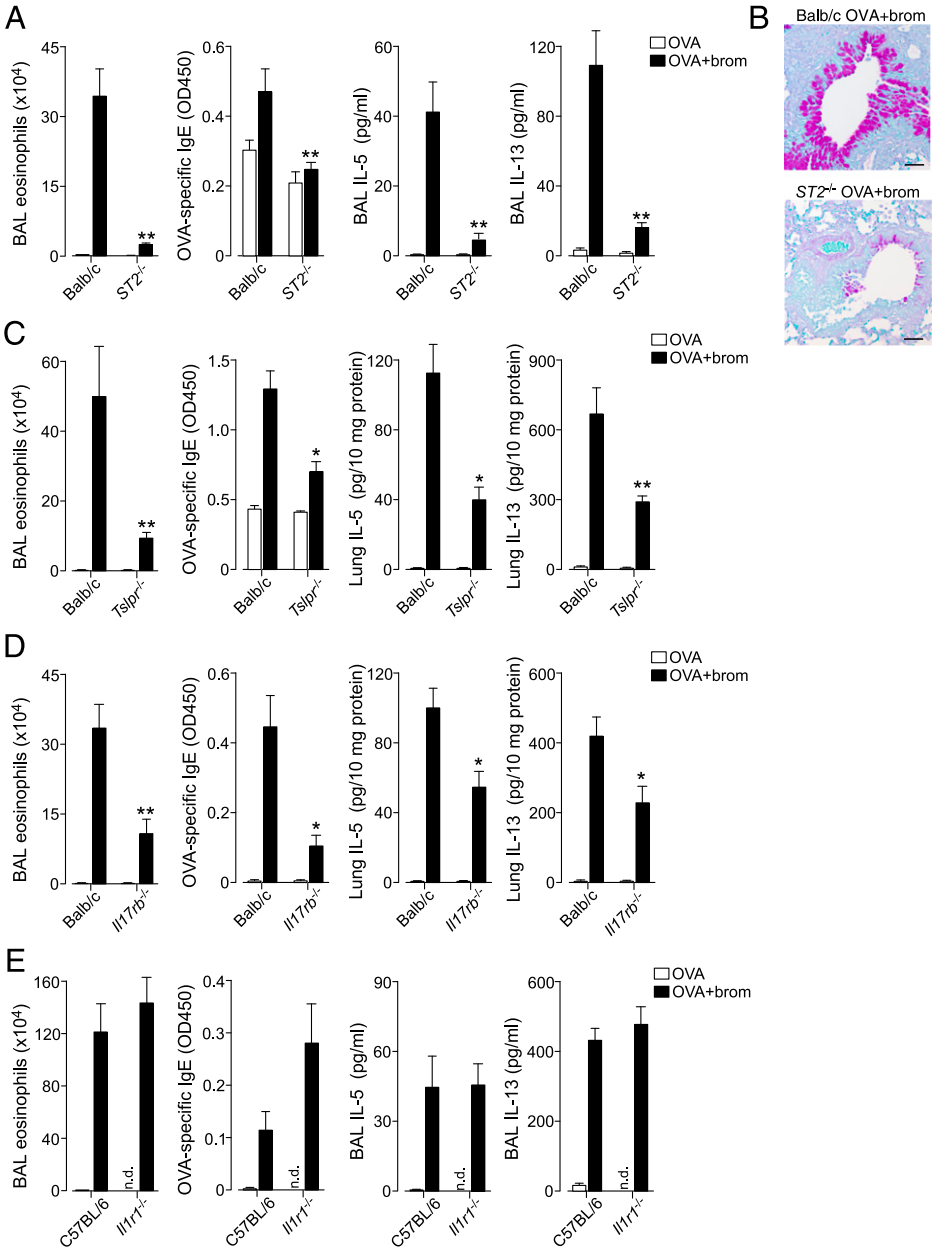

FIGURE 2. Bromelain-induced Ag-specific Th2-type immune responses to OVA are dependent on IL-33, TSLP, and IL-25. Naive WT BALB/c, C57BL/6 mice, or *ST2*$^{-/-}$ mice (**A**, **B**), *Tslpr*$^{-/-}$ mice (**C**), *Il17rb*$^{-/-}$ mice (**D**), or *Il1r1*$^{-/-}$ mice (**E**) were exposed to OVA alone or OVA plus bromelain, and challenged with OVA using the same protocol as described in Fig. 1B. Total numbers of eosinophils in BAL fluids, plasma levels of anti-OVA IgE Ab, and BAL or lung levels of cytokines were determined. Lung sections were stained with PAS. Scale bars, 100 μm. Data shown are the mean ± SEM, $*p < 0.05$, $**p < 0.01$, compared with WT mice, n = 5–6 mice/group. Experiments were repeated twice (A, B, and D) or once (C and E); data shown are one representative experiment. n.d., not determined.

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OVA alone or bromelain alone (Fig. 1F). These immunological responses to OVA were abolished in *Rag1*$^{-/-}$ mice (Supplemental Fig. 1A), suggesting that they are indeed mediated by adaptive immunity. Furthermore, these adjuvant activities of bromelain, as well as papain, were dependent on its cysteine protease activity, which was abolished by treating them with the protease inhibitor E64 (Supplemental Fig. 1B, 1C). These findings suggest that cysteine proteases, when administered into the airways, possess potent adjuvant activity, leading to the development of humoral and cellular Th2-type immune responses to innocuous airborne Ags.

Protease-induced IL-33 and TSLP play key roles in mediating type 2 immune responses

Pro-Th2 cytokines, such as IL-33, IL-25, and TSLP, likely play central roles in regulating type 2 immunity by acting on a variety of immune cell types (35–39). To examine whether these pro-Th2 cytokines play any role in the adjuvant activities of cysteine proteases as described earlier, we exposed mice deficient in cytokine receptors i.n. to endotoxin-free OVA plus bromelain and challenged with OVA alone. Mice deficient for IL-33R (*ST2*$^{-/-}$ mice) showed >80% reduction in BAL eosinophils and BAL IL-5 and IL-13 levels as compared with WT mice (Fig. 2A). Anti-OVA IgE Ab was significantly inhibited, and an apparent decrease in airway mucous hyperplasia was observed in *ST2*$^{-/-}$ mice (Fig. 2B). Mice deficient for TSLP receptor (*Tslpr*$^{-/-}$ mice) and mice deficient for IL-25R (*Il17rb*$^{-/-}$ mice) also showed significant decreases in BAL eosinophils, lung IL-5 and IL-13 levels, and serum IgE Ab (Fig. 2C, 2D). In contrast, no differences in these immunological parameters were observed in mice deficient in IL-1R (*Il1r1*$^{-/-}$), the receptor for IL-1α and IL-1β (Fig. 2E). Thus, IL-33, as well as IL-25 and TSLP, likely play pivotal roles in the potent Th2-type adjuvant activities of cysteine proteases.

Endogenous UA is involved in protease-induced type 2 immune responses

There are major questions regarding how these proteases are sensed in airway mucosa and how production of IL-33 and other pro-Th2 cytokines is initiated. Recent studies suggest that the ability of allergens to promote allergic responses is generally mediated by three major mechanisms: 1) engagement of pattern-recognition receptors, 2) molecular mimicry of TLR signaling complex molecules, and 3) proteolytic activity (27, 40). In particular, TLR4 plays critical roles in type 2 immune responses to inhaled HDM allergens (4, 16, 17), low-dose LPS in the airways (41), and papain injected into skin (22).

However, we found that mice deficient in TLR4 developed comparable levels of airway eosinophilia compared with WT mice when they were exposed to OVA plus bromelain and challenged with OVA (Supplemental Fig. 2A). We actually observed significant increases in BAL IL-5 and IL-13 levels in TLR4-deficient mice. Therefore, TLR4 is unlikely to be required for recognition of proteases in airways. Another candidate receptor, protease-activated receptor 2 (PAR2) (29), is also unlikely to be required because *PAR2*$^{-/-}$ mice showed comparable responses to WT mice (Supplemental Fig. 2B). Therefore, we speculated that an alternative mechanism(s) exists to sense protease activities in respiratory mucosa.

Exposure to proteases could cause stress, damage, or both to tissue cells and trigger the release of damage-associated molecular patterns (DAMPs). DAMPs are generally produced and stored within cells and are released extracellularly upon cellular injury (42, 43). UA is produced in all cells by the catabolism of purines from DNA and RNA, and has been considered a DAMP molecule (44). Furthermore, in the airways, UA is constitutively secreted on the surface of mucosal epithelial tissues without apparent pathologic consequences (45). When the fluids in the airway lumen

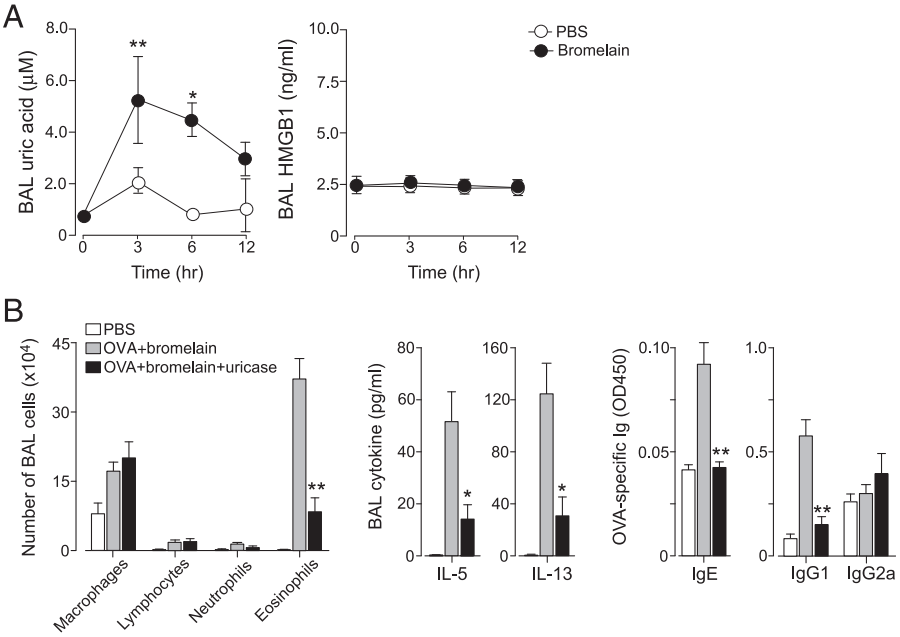

FIGURE 3. Endogenous UA in the airways plays a pivotal role in type 2 immune responses induced by bromelain. (**A**) Naive BALB/c mice were exposed once i.n. to bromelain (10 μg) or PBS. At the indicated times, BAL fluids were collected, and the levels of UA and HMGB-1 in the supernatants were measured using fluorogenic UA assay kits and HMGB-1 ELISA kits, respectively. Data shown are the mean ± SEM, $*p < 0.05$, $**p < 0.01$, compared with PBS, $n = 6$ mice/group. Experiments were repeated twice; data shown are pools of two experiments. (**B**) Using the same protocol as shown in Fig. 1B, naive WT BALB/c mice were exposed i.n. to PBS or OVA (100 μg/dose) plus bromelain (10 μg/dose) with or without uricase (1 U/dose) on days 0 and 7. On day 14, plasma was collected for analyses of anti-OVA Abs. All mice were challenged i.n. with OVA alone on days 21, 22, and 23, and BAL fluids were analyzed for cell numbers and cytokine levels on day 24. Data shown are the mean ± SEM, $*p < 0.05$, $**p < 0.01$, compared with OVA plus bromelain, $n = 5–6$ mice/group. Experiments were repeated twice; data shown are one representative experiment.

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(i.e., BAL fluids) were collected and analyzed quantitatively, UA levels increased rapidly within 3 h after a single airway exposure of mice to bromelain (Fig. 3A). In contrast, BAL levels of an authentic DAMP molecule, HMGB-1 (44), did not change significantly upon bromelain exposure.

We therefore examined whether endogenous UA in respiratory mucosa is involved in the Th2-type adjuvant activities of bromelain. Uricase depletes UA by oxidizing UA into allantoin and water (46). Using the protocol shown in Fig. 1B, we exposed naive mice i.n. to endotoxin-free OVA plus bromelain with or without uricase, and they were subsequently challenged with OVA alone. As expected, mice previously exposed to OVA plus bromelain showed airway eosinophilia, increased BAL levels of IL-5 and IL-13, and increased serum levels of OVA-specific IgE and IgG1 Abs (Fig. 3B). These immune responses were significantly inhibited when uricase was administered into the airways at the time of OVA plus bromelain exposure.

To rule out nonspecific inhibitory effects of uricase on the development of type 2 immune responses in the airways, we used IL-33 as an "adjuvant" in place of bromelain to sensitize animals to OVA through the airways (47). When these mice were challenged subsequently with OVA, lung levels of IL-5 and IL-13 were not affected in mice administered uricase (Supplemental Fig. 3). Taken together, the results indicate that endogenous UA in respiratory mucosa is likely required for type 2 immune responses when mice are exposed to proteases.

Exogenous UA induces IL-33 and TSLP production and initiates innate and adaptive type 2 immune response

UA crystals administered into the peritoneal cavity trigger acute neutrophilic inflammation by stimulating IL-1β production and engaging the IL-1R on tissue cells (48, 49). Such systemic effects of UA crystals are typically represented in the human disease condition gout (50). To investigate whether UA is capable of inducing type 2 immune responses in respiratory mucosa, we administered MSU crystals i.n. into the airways of naive BALB/c mice. Lung levels of IL-33 and TSLP, but not IL-25, increased significantly 3 h after a single airway administration of MSU crystals (Fig. 4A) to naive mice. Increased BAL levels of IL-33 (Fig. 4B), but not IL-25 or TSLP (data not shown), were also observed in mice exposed to MSU crystals, suggesting that IL-33 protein is released extracellularly. IL-33 release into the airway lumen was partially inhibited in mice deficient in NALP3, a component of inflammasomes (Supplemental Fig. 4A). Similarly, lung levels of IL-5 and IL-13, but not IL-17 or IFN-γ, increased in WT mice after airway administration of MSU crystals (Fig. 4C). IL-5 and IL-13 production

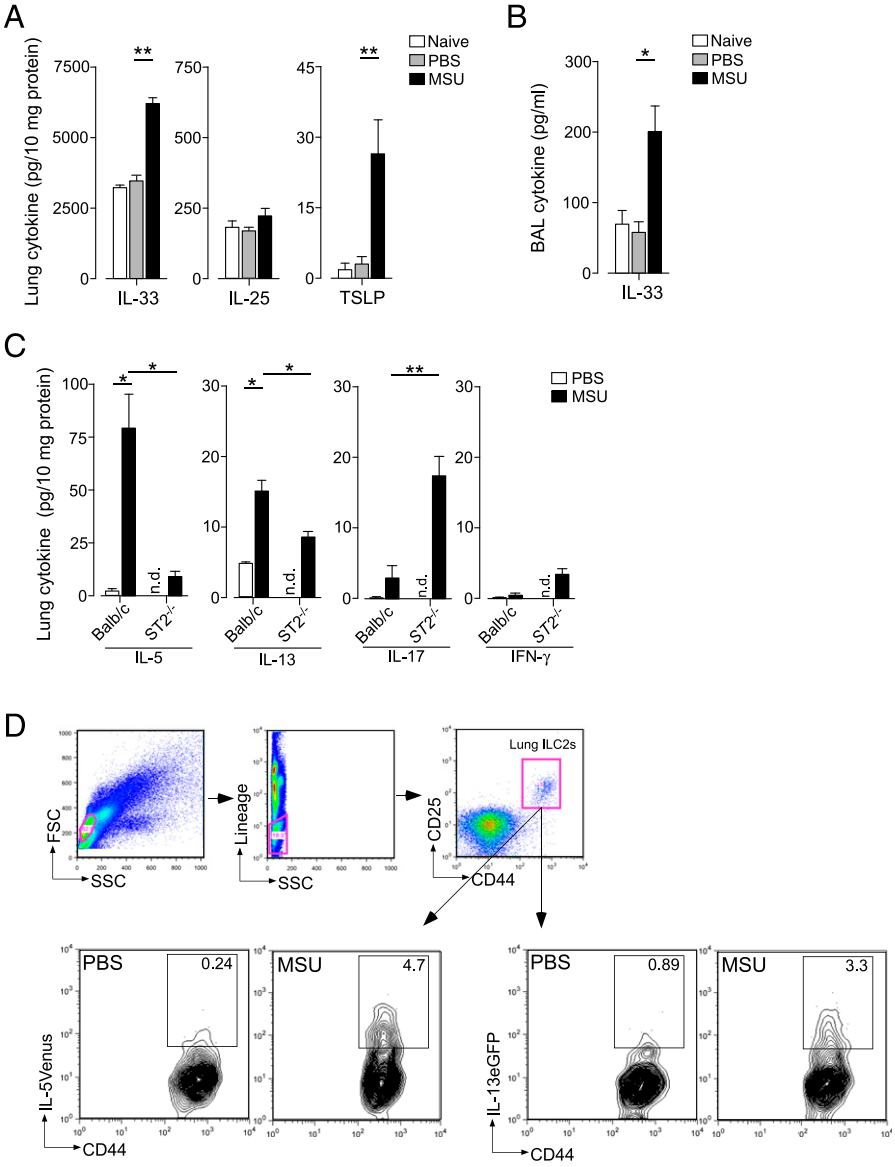

FIGURE 4. Airway administration of MSU crystals induces IL-33 and TSLP production in the lungs and triggers innate type 2 responses. (**A** and **B**) Naive WT BALB/c mice were untreated or administered once i.n. with MSU crystals (1 mg/dose) or PBS. After 3 h, cytokines levels in lung homogenates (A) or BAL supernatants (B) were analyzed using ELISA. Data shown are the mean ± SEM, $*p < 0.05$, $**p < 0.01$, $n = 5$ mice/group. Experiments were repeated three times; data shown are one representative experiment. (**C**) Naive WT BALB/c mice or $ST2^{-/-}$ mice were administered once i.n. with MSU crystals. After 3 h, cytokine levels in lung homogenates were analyzed using ELISA. Data shown are the mean ± SEM, $*p < 0.05$, $**p < 0.01$, $n = 5$ mice/group. Experiments were repeated twice; data shown are one representative experiment. (**D**) IL-$5^{+/venus}$ mice or IL-$13^{+/eGFP}$ mice were exposed to PBS or MSU crystals (1 mg/dose), once daily, for 3 d. Lung single-cell suspensions were gated on lung ILC2s as described in the *upper panels*, and the expression levels of IL-5venus and IL-13eGFP in the ILC2 population were analyzed by flow cytometry (*lower panels*). Experiments were repeated twice; data shown are one representative experiment.

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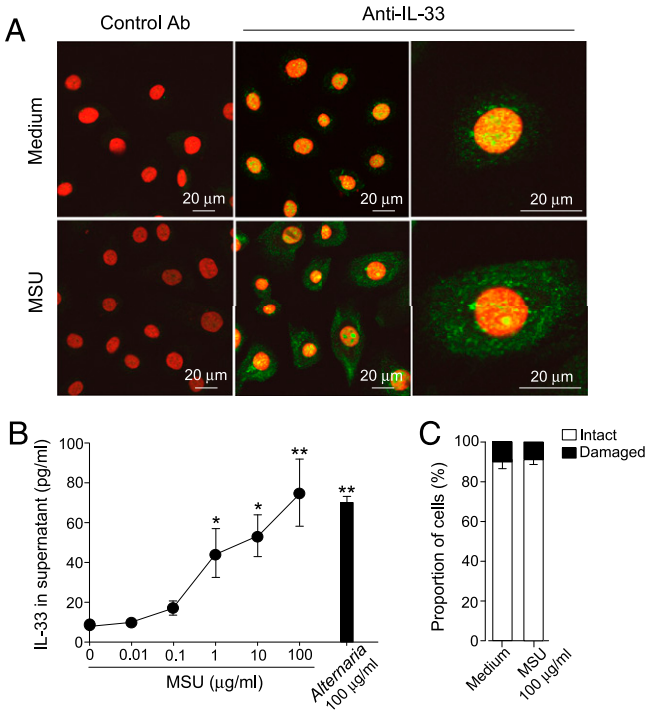

FIGURE 6. IL-33 is secreted by airway epithelial cells when exposed to MSU crystals in vitro. (**A**) NHBE cells were exposed to medium alone or MSU crystals (100 μg/ml) for 3 h, and stained with anti–IL-33 or control Ab, followed by FITC-conjugated secondary Ab. Slides were visualized using confocal microscopy. DAPI nuclear staining was pseudocolored with red. IL-33 staining is depicted as green (FITC), and colocalization of IL-33 and DAPI nuclear stains is depicted as orange (i.e., red plus green). Experiments were repeated five times; data shown are one representative experiment. Scale bars, 20 μm. (**B**) NHBE cells were exposed for 3 h to the indicated concentration of MSU crystals or *Alternaria* extract (100 μg/ml). IL-33 in cell-free supernatants was measured using ELISA. Data shown are the mean ± SEM, $*p < 0.05$, $**p < 0.01$, compared with media alone. Data shown are a pool of six experiments. (**C**) NHBE cells were exposed for 3 h to medium alone or 100 μg/ml MSU crystals. Cell membrane integrity was examined by staining cells with calcein AM and EthD-1 dyes. Data shown are the mean ± SEM. Data shown are a pool of three experiments.

allergens remains unknown. Natural allergens are a complex mixture of proteins, carbohydrates, lipid-binding molecules, and enzymes, which may cause diverse immune responses, and thus are likely difficult to regulate (40). Exposure to allergens is a risk factor for the development of asthma in humans (58), and certain allergens, such as *Alternaria*, HDM, mouse, and cockroach, are detected together at high levels in home environments (59). Therefore, to mimic natural allergen exposure in humans, we simultaneously exposed animals for 2 wk to several allergens that are relevant to human asthma (Fig. 7A).

Uricase is a potent agent that depletes UA. However, it has a short half-life in vivo and can elicit neutralizing Abs in mice (60), and thus is not suitable for multiple treatments. UA is generated from xanthine by xanthine oxidase. A pharmacologic UA synthesis inhibitor, febuxostat, inhibits xanthine oxidase activity by blocking its active site (61). Therefore, to examine the roles of endogenous UA, we treated mice with daily oral administration of febuxostat or water (as control) starting 2 d before allergen exposure and continuing throughout the experiment (Fig. 7A). When naive BALB/c mice were repeatedly exposed i.n. to a mixture of *Alternaria*, *Aspergillus*, and HDM extracts, they developed marked airway eosinophilia, as well as increased lung levels of IL-5 and IL-13 (Fig. 7B, 7C). However, no increase in IL-17 or IFN-γ was observed. Febuxostat treatment significantly reduced the numbers of eosinophils and neutrophils in BAL fluids and inhibited increased production of IL-5 and IL-13 in the lungs. In addition, exposure to these allergens significantly increased the total amount of UA in lung homogenates, and UA levels decreased upon febuxostat treatment (Fig. 7D). Mice exposed to a mixture of allergens also showed mucous hyperplasia (Fig. 7E), resembling human asthma. These airway pathologic responses were also inhibited upon febuxostat treatment. Thus, the clinically relevant xanthine oxidase inhibitor febuxostat effectively reduced lung levels of UA, as well as decreased allergen-induced airway inflammation and pathology in a mouse model of asthma.

Discussion

The molecular and cellular mechanisms that initiate type 2 immunity remain topics of considerable debate and active investigation. Evidence suggests that epithelial cells make important contributions to the development of type 2 immunity (16, 36, 62). Previous studies demonstrated that HDM extract activates TLR4 and induces type 2 immune responses (4, 16, 17). By exposing animals to HDM extract, Kool et al. (4) demonstrated that DCs activated by endogenous UA are involved in Th2-type immune responses. In the same HDM model, epithelium-derived IL-1α induced IL-33 and GM-CSF production, and initiated type 2 immunity (63); TSLP was not involved in this model. However, major questions remained whether these novel observations apply to airborne allergens in general or are unique to the TLR4 agonist HDM extract, and whether UA interacts with cells other than DCs.

In this study, we used proteases as a model "allergen." Our results show that TLR4 or IL-1α was not involved in this model. Rather, we found that UA induces both innate and adaptive type 2 immune responses by mediating production of IL-33 and TSLP. Airway epithelial cells secreted IL-33 extracellularly when they were exposed to UA. Moreover, we demonstrated that a pharmacologic inhibitor of UA synthesis effectively attenuates asthma phenotypes in mice that were exposed repeatedly to common airborne allergens. Therefore, we propose that airway UA and UA-induced IL-33 and TSLP serve an important function in the initiation of type 2 immunity in response to airborne allergens in respiratory mucosa.

Although cysteine proteases, such as papain and bromelain, have been used successfully as model allergens to investigate mechanisms of type 2 immunity (9, 22, 31, 32), molecular mechanisms that explain how these proteases are sensed by the immune system have been enigmatic. The enzymatic activities of allergens could be recognized by two mechanisms: by specific receptor(s) or by product(s) that are derived from the effects of allergens on tissues. For example, papain induces TSLP production by airway epithelial cells in vitro, and the process involves recognition of protease activity by a specific protease-sensing receptor, PAR2 (64). Alternatively, papain injected into s.c. tissues of mice induces reactive oxygen species and oxidized lipids, which, in turn, trigger TSLP production by cutaneous epithelial cells via TLR4 activation (22).

In this study, UA was rapidly released into the airway lumen upon exposure to proteases, and removal of endogenous UA attenuated innate and adaptive type 2 responses (Fig. 3). Thus, sensing of protease activity, but not the protease molecule itself, likely plays a pivotal role in the initiation of type 2 immunity to protease allergens in respiratory mucosa. However, UA depletion did not completely eliminate the protease-induced type 2 response in the airway (Fig. 3), possibly because of the presence of residual UA after depletion or involvement of other protease-induced molecules, such as fibrinogen cleavage products (65).

A major question remains as to how UA is released into airway lumen after protease exposure. High levels of UA are present in the cytosol of normal cells (49, 66, 67), and UA has been generally

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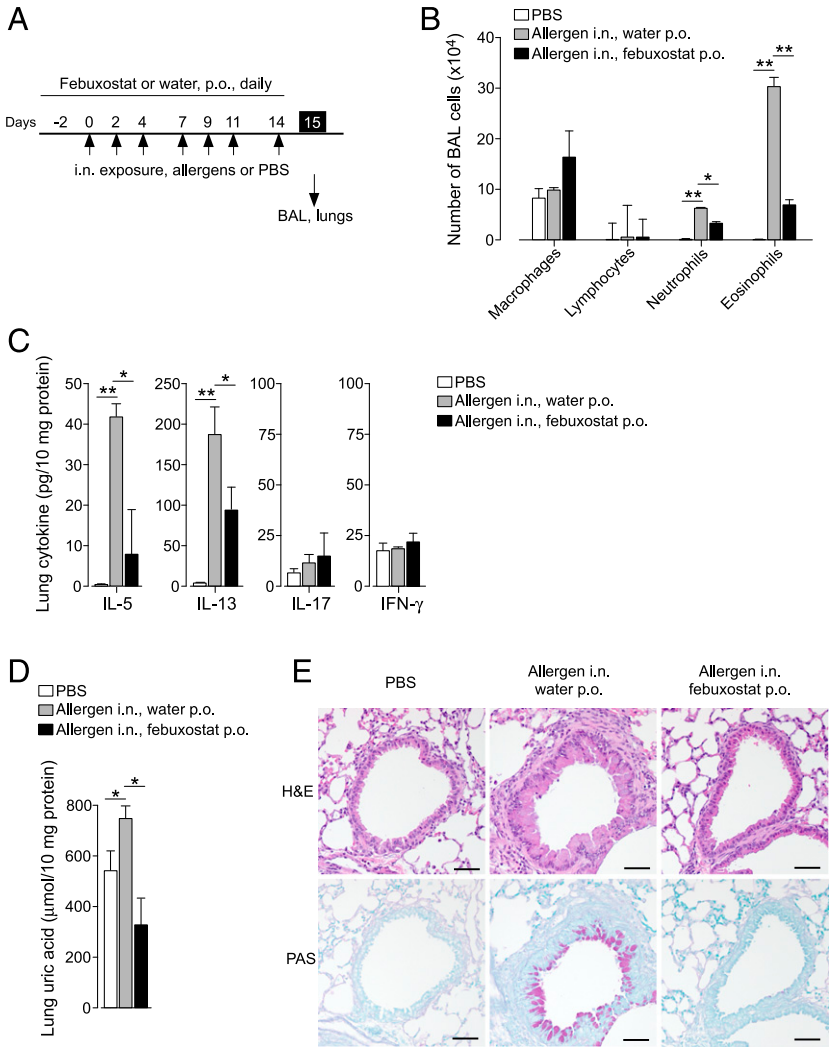

FIGURE 7. The UA synthesis inhibitor febuxostat attenuates eosinophilic airway inflammation and asthma-like pathology in mice exposed repeatedly to allergen extracts. (**A**) Experimental protocol. Naive BALB/c mice were orally administered febuxostat (5 mg/kg/dose) or distilled water daily starting on day −2 for 16 d. Mice were exposed i.n. to PBS or a mixture of allergen extracts (*Alternaria*, *Aspergillus*, and HDM, 10 μg each/dose), three times per week for 2 wk. Twenty-four hours after the last exposure, BAL and lung specimens were collected. (**B**) Total numbers of BAL cells and differentials were examined. (**C**) Lung levels of cytokines were examined using ELISA. (**D**) Lung levels of UA were examined using ELISA. (**E**) Lung sections were stained with H&E and PAS. Data shown are the mean ± SEM, $*p < 0.05$, $**p < 0.01$, $n = 6$ mice/group. Experiments were repeated twice; data shown are one representative experiment. Scale bars, 100 μm.

considered a DAMP molecule (42–44). However, in respiratory mucosa, the functions of UA may not be limited to those associated with DAMP molecules. High constitutive expression of xanthine oxidoreductase, the enzyme that generates UA, is found in many mammalian epithelial tissues (68). In healthy individuals, UA is constitutively secreted onto the surface of mucosal epithelial tissues without apparent pathologic consequences (45). Thus, theoretically, UA can be released actively, passively by cellular damage, or both by airway epithelium. Importantly, UA and its oxidation product allantoin are potent antioxidants and free radical scavengers (69, 70), suggesting that UA in the airways is beneficial for the host in resting conditions. Pathogen exposure and tissue damage rapidly increase expression of xanthine oxidoreductase (71), and UA is induced in response to various types of cellular stress, including ozone exposure and respiratory viral infection (72, 73). Furthermore, an urate transporter, the ATP-binding cassette subfamily G member 2, is expressed by epithelial cells (74). Thus, airway mucosal UA likely serves as a crucial sensor to monitor atmospheric environmental exposure and regulate respiratory mucosa behavior. At lower concentrations, UA may manage oxidative stress caused by environmental insults and maintain tissue homeostasis, whereas it may initiate immune responses at higher concentrations.

In this study, airway administration of exogenous MSU crystals was sufficient to induce IL-33 and TSLP production and innate type 2 response (Fig. 4) and recapitulate potent type 2 adjuvant activities of cysteine proteases (Fig. 5). The innate type 2 response was observed in both BALB/c and C57BL/6 mice (Supplemental Fig. 4). As a model of human gout, i.p. injection of MSU crystals induced IL-1β production by tissue resident cells, resulting in robust neutrophilic inflammation through activation of IL-1R (42, 48, 75). In contrast, our results showed that the IL-33R, but not the IL-1R, was involved in the eosinophilic responses to the proteases (Fig. 2). Thus, the different organs may preferentially use distinct IL-1 family molecules, and the route of administration (i.e., airway lumen versus peritoneal cavity) may explain the differences between the results of this study and those of previous studies.

One of the novel observations in this study is the secretion of IL-33 by airway epithelial cells exposed to MSU crystals. IL-33 is constitutively produced and stored in the nuclei of normal epithelial cells (57), and a major question remains as to how IL-33 is released to the extracellular spaces. To date, only a few physiologic agonists, other than IL-1α (63) and fungus extract (56), have been shown to induce extracellular secretion of IL-33. Our findings suggest that MSU crystals are potent agonists to induce IL-33 secretion by airway epithelial cells, and that IL-33 can be released extracellularly without apparent cellular damage. Recent studies suggest that the immunological effects of MSU crystals and other inflammation-inducing crystals are unlikely mediated by specific recognition receptors, but rather by their interaction with membrane lipids. For example, in DCs, MSU crystals interact directly with membrane cholesterol, leading to activation of Syk kinase signaling (76); Syk kinase was also involved in the type

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2 immunostimulatory functions of DCs activated by MSU crystals (4). Indeed, UA crystals directly bind to renal epithelial cells by hydrogen bonding and hydrophobic interactions (77). Interestingly, a recent study suggests that IL-1α, which is also stored in the nucleus, is released extracellularly by a calcium-dependent, inflammasome-independent pathway and an inflammasome-dependent pathway, depending on the stimuli (78). The results of our pilot study also show that IL-33 secretion induced by MSU exposure is partially dependent on the NALP3 inflammasome (Supplemental Fig. 4A). Future studies will be necessary to elucidate the molecular mechanisms involved in IL-33 secretion by airway epithelial cells in more detail and to determine whether IL-33 is secreted as a full-length form or a processed shorter form, similarly to IL-1β or IL-1α induced by inflammasome agonists (78, 79). The results of our study should provide a versatile experimental tool (i.e., MSU crystals) to facilitate this line of investigation.

In summary, despite our increasing understanding regarding the biology of pro-Th2 cytokines including IL-33, IL-25, and TSLP, the signals that control production and secretion of these cytokines from airway epithelial cells and other cells remain poorly understood. We propose a model where exposure of respiratory mucosa of naive nonsensitized animals to proteolytic enzymes or natural allergens leads to rapid luminal UA secretion, which, in turn, mediates production of IL-33 and TSLP from airway epithelial cells, ensuring innate type 2 immunity and initiation of Ag-specific Th2-type immune responses. Thus, UA in respiratory mucosa may play a central role as a sensor of allergen exposure, and may initiate and exacerbate Th2-type immunity in the airways. Indeed, oral administration of the UA synthesis inhibitor febuxostat reduced tissue UA levels and attenuated eosinophilic airway inflammation and asthma-like pathology in mice that were exposed repeatedly to several natural allergens (Fig. 7). The airway levels of UA are increased in patients with asthma after allergen exposure (4) and in patients with chronic rhinosinusitis during disease exacerbation (80). Therefore, the UA pathway may serve as a novel and realistic therapeutic target for asthma and other allergic airway diseases in humans.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Pawankar, R., G. W. Canonica, S. T. Holgate, and R. F. Lockey. 2012. Allergic diseases and asthma: a major global health concern. *Curr. Opin. Allergy Clin. Immunol.* 12: 39–41.
2. Barnes, P. J. 2008. Immunology of asthma and chronic obstructive pulmonary disease. *Nat. Rev. Immunol.* 8: 183–192.
3. Hammad, H., M. Plantinga, K. Deswarte, P. Pouliot, M. A. Willart, M. Kool, F. Muskens, and B. N. Lambrecht. 2010. Inflammatory dendritic cells—not basophils—are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J. Exp. Med.* 207: 2097–2111.
4. Kool, M., G. van Loo, W. Waelput, S. De Prijck, F. Muskens, M. Sze, J. van Praet, F. Branco-Madeira, S. Janssens, B. Reizis, et al. 2011. The ubiquitin-editing protein A20 prevents dendritic cell activation, recognition of apoptotic cells, and systemic autoimmunity. *Immunity* 35: 82–96.
5. Plantinga, M., M. Guilliams, M. Vanheerswynghels, K. Deswarte, F. Branco-Madeira, W. Toussaint, L. Vanhoutte, K. Neyt, N. Killeen, B. Malissen, et al. 2013. Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* 38: 322–335.
6. Pulendran, B., J. L. Smith, G. Caspary, K. Brasel, D. Pettit, E. Maraskovsky, and C. R. Maliszewski. 1999. Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. *Proc. Natl. Acad. Sci. USA* 96: 1036–1041.
7. van Rijt, L. S., S. Jung, A. Kleinjan, N. Vos, M. Willart, C. Duez, H. C. Hoogsteden, and B. N. Lambrecht. 2005. In vivo depletion of lung CD11c$^+$ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J. Exp. Med.* 201: 981–991.
8. Perrigoue, J. G., S. A. Saenz, M. C. Siracusa, E. J. Allenspach, B. C. Taylor, P. R. Giacomin, M. G. Nair, Y. Du, C. Zaph, N. van Rooijen, et al. 2009. MHC class II-dependent basophil-CD4+ T cell interactions promote T(H)2 cytokine-dependent immunity. *Nat. Immunol.* 10: 697–705.
9. Sokol, C. L., G. M. Barton, A. G. Farr, and R. Medzhitov. 2008. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat. Immunol.* 9: 310–318.
10. Sokol, C. L., N. Q. Chu, S. Yu, S. A. Nish, T. M. Laufer, and R. Medzhitov. 2009. Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nat. Immunol.* 10: 713–720.
11. Yoshimoto, T., K. Yasuda, H. Tanaka, M. Nakahira, Y. Imai, Y. Fujimori, and K. Nakanishi. 2009. Basophils contribute to T(H)2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. *Nat. Immunol.* 10: 706–712.
12. Allen, J. E., and R. M. Maizels. 2011. Diversity and dialogue in immunity to helminths. *Nat. Rev. Immunol.* 11: 375–388.
13. Anthony, R. M., L. I. Rutitzky, J. F. Urban, Jr., M. J. Stadecker, and W. C. Gause. 2007. Protective immune mechanisms in helminth infection. *Nat. Rev. Immunol.* 7: 975–987.
14. Spits, H., and J. P. Di Santo. 2011. The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. *Nat. Immunol.* 12: 21–27.
15. Wills-Karp, M. 2010. Allergen-specific pattern recognition receptor pathways. *Curr. Opin. Immunol.* 22: 777–782.
16. Hammad, H., M. Chieppa, F. Perros, M. A. Willart, R. N. Germain, and B. N. Lambrecht. 2009. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* 15: 410–416.
17. Trompette, A., S. Divanovic, A. Visintin, C. Blanchard, R. S. Hegde, R. Madan, P. S. Thorne, M. Wills-Karp, T. L. Gioannini, J. P. Weiss, and C. L. Karp. 2009. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 457: 585–588.
18. Phillips, C., W. R. Coward, D. I. Pritchard, and C. R. Hewitt. 2003. Basophils express a type 2 cytokine profile on exposure to proteases from helminths and house dust mites. *J. Leukoc. Biol.* 73: 165–171.
19. Porter, P. C., V. Ongeri, A. Luong, F. Kheradmand, and D. B. Corry. 2011. Seeking common pathophysiology in asthma, atopy and sinusitis. *Trends Immunol.* 32: 43–49.
20. Palm, N. W., R. K. Rosenstein, S. Yu, D. D. Schenten, E. Florsheim, and R. Medzhitov. 2013. Bee venom phospholipase A2 induces a primary type 2 response that is dependent on the receptor ST2 and confers protective immunity. *Immunity* 39: 976–985.
21. Steinfelder, S., J. F. Andersen, J. L. Cannons, C. G. Feng, M. Joshi, D. Dwyer, P. Caspar, P. L. Schwartzberg, A. Sher, and D. Jankovic. 2009. The major component in schistosome eggs responsible for conditioning dendritic cells for Th2 polarization is a T2 ribonuclease (omega-1). *J. Exp. Med.* 206: 1681–1690.
22. Tang, H., W. Cao, S. P. Kasturi, R. Ravindran, H. I. Nakaya, K. Kundu, N. Murthy, T. B. Kepler, B. Malissen, and B. Pulendran. 2010. The T helper type 2 response to cysteine proteases requires dendritic cell-basophil cooperation via ROS-mediated signaling. *Nat. Immunol.* 11: 608–617.
23. Gailhofer, G., M. Wilders-Truschnig, J. Smolle, and M. Ludvan. 1988. Asthma caused by bromelain: an occupational allergy. *Clin. Allergy* 18: 445–450.
24. Secor, E. R., Jr., A. Singh, L. A. Guernsey, J. T. McNamara, L. Zhan, N. Maulik, and R. S. Thrall. 2009. Bromelain treatment reduces CD25 expression on activated CD4$^+$ T cells in vitro. *Int. Immunopharmacol.* 9: 340–346.
25. Bartemes, K. R., K. Iijima, T. Kobayashi, G. M. Kephart, A. N. McKenzie, and H. Kita. 2012. IL-33-responsive lineage$^-$ CD25$^+$ CD44(^{hi}) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. *J. Immunol.* 188: 1503–1513.
26. Chua, K. Y., G. A. Stewart, W. R. Thomas, R. J. Simpson, R. J. Dilworth, T. M. Plozza, and K. J. Turner. 1988. Sequence analysis of cDNA coding for a major house dust mite allergen, Der p 1. Homology with cysteine proteases. *J. Exp. Med.* 167: 175–182.
27. Karp, C. L. 2010. Guilt by intimate association: what makes an allergen an allergen? *J. Allergy Clin. Immunol.* 125: 955–960, quiz 961–962.
28. Kheradmand, F., A. Kiss, J. Xu, S. H. Lee, P. E. Kolattukudy, and D. B. Corry. 2002. A protease-activated pathway underlying Th cell type 2 activation and allergic lung disease. *J. Immunol.* 169: 5904–5911.
29. Porter, P., S. C. Susarla, S. Polikepahad, Y. Qian, J. Hampton, A. Kiss, S. Vaidya, S. Sur, V. Ongeri, T. Yang, et al. 2009. Link between allergic asthma and airway mucosal infection suggested by proteinase-secreting household fungi. *Mucosal Immunol.* 2: 504–517.
30. Novey, H. S., L. E. Marchioli, W. N. Sokol, and I. D. Wells. 1979. Papain-induced asthma—physiological and immunological features. *J. Allergy Clin. Immunol.* 63: 98–103.
31. Halim, T. Y., R. H. Krauss, A. C. Sun, and F. Takei. 2012. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity* 36: 451–463.
32. Kamijo, S., H. Takeda, T. Tokura, M. Suzuki, K. Inui, M. Hara, H. Matsuda, A. Matsuda, K. Oboki, T. Ohno, et al. 2013. IL-33-mediated innate response and adaptive immune cells contribute to maximum responses of protease allergen-induced allergic airway inflammation. *J. Immunol.* 190: 4489–4499.
33. Akbari, O., R. H. DeKruyff, and D. T. Umetsu. 2001. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat. Immunol.* 2: 725–731.

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34. Brimnes, M. K., L. Bonifaz, R. M. Steinman, and T. M. Moran. 2003. Influenza virus-induced dendritic cell maturation is associated with the induction of strong T cell immunity to a coadministered, normally nonimmunogenic protein. *J. Exp. Med.* 198: 133–144.
35. Hammad, H., and B. N. Lambrecht. 2008. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat. Rev. Immunol.* 8: 193–204.
36. Paul, W. E., and J. Zhu. 2010. How are T(H)2-type immune responses initiated and amplified? *Nat. Rev. Immunol.* 10: 225–235.
37. Saenz, S. A., B. C. Taylor, and D. Artis. 2008. Welcome to the neighborhood: epithelial cell-derived cytokines license innate and adaptive immune responses at mucosal sites. *Immunol. Rev.* 226: 172–190.
38. Spits, H., and T. Cupedo. 2012. Innate lymphoid cells: emerging insights in development, lineage relationships, and function. *Annu. Rev. Immunol.* 30: 647–675.
39. Walker, J. A., J. L. Barlow, and A. N. McKenzie. 2013. Innate lymphoid cells—how did we miss them? *Nat. Rev. Immunol.* 13: 75–87.
40. Wills-Karp, M., A. Nathan, K. Page, and C. L. Karp. 2010. New insights into innate immune mechanisms underlying allergenicity. *Mucosal Immunol.* 3: 104–110.
41. Eisenbarth, S. C., D. A. Piggott, J. W. Huleatt, I. Visintin, C. A. Herrick, and K. Bottomly. 2002. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J. Exp. Med.* 196: 1645–1651.
42. Chen, G. Y., and G. Nuñez. 2010. Sterile inflammation: sensing and reacting to damage. *Nat. Rev. Immunol.* 10: 826–837.
43. Matzinger, P. 2002. The danger model: a renewed sense of self. *Science* 296: 301–305.
44. Rock, K. L., J. J. Lai, and H. Kono. 2011. Innate and adaptive immune responses to cell death. *Immunol. Rev.* 243: 191–205.
45. Peden, D. B., R. Hohman, M. E. Brown, R. T. Mason, C. Berkebile, H. M. Fales, and M. A. Kaliner. 1990. Uric acid is a major antioxidant in human nasal airway secretions. *Proc. Natl. Acad. Sci. USA* 87: 7638–7642.
46. Motojima, K., S. Kanaya, and S. Goto. 1988. Cloning and sequence analysis of cDNA for rat liver uricase. *J. Biol. Chem.* 263: 16677–16681.
47. Kobayashi, T., K. Iijima, J. L. Checkel, and H. Kita. 2013. IL-1 family cytokines drive Th2 and Th17 cells to innocuous airborne antigens. *Am. J. Respir. Cell Mol. Biol.* 49: 989–998.
48. Chen, C. J., Y. Shi, A. Hearn, K. Fitzgerald, D. Golenbock, G. Reed, S. Akira, and K. L. Rock. 2006. MyD88-dependent IL-1 receptor signaling is essential for gouty inflammation stimulated by monosodium urate crystals. *J. Clin. Invest.* 116: 2262–2271.
49. Shi, Y., J. E. Evans, and K. L. Rock. 2003. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* 425: 516–521.
50. Terkeltaub, R. 2010. Update on gout: new therapeutic strategies and options. *Nat. Rev. Rheumatol.* 6: 30–38.
51. Ikutani, M., T. Yanagibashi, M. Ogasawara, K. Tsuneyama, S. Yamamoto, Y. Hattori, T. Kouro, A. Itakura, Y. Nagai, S. Takaki, and K. Takatsu. 2012. Identification of innate IL-5-producing cells and their role in lung eosinophil regulation and antitumor immunity. *J. Immunol.* 188: 703–713.
52. Neill, D. R., S. H. Wong, A. Bellosi, R. J. Flynn, M. Daly, T. K. Langford, C. Bucks, C. M. Kane, P. G. Fallon, R. Pannell, et al. 2010. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 464: 1367–1370.
53. Bartemes, K. R., and H. Kita. 2012. Dynamic role of epithelium-derived cytokines in asthma. *Clin. Immunol.* 143: 222–235.
54. Lamkanfi, M., and V. M. Dixit. 2009. IL-33 raises alarm. *Immunity* 31: 5–7.
55. Lüthi, A. U., S. P. Cullen, E. A. McNeela, P. J. Duriez, I. S. Afonina, C. Sheridan, G. Brumatti, R. C. Taylor, K. Kersse, P. Vandenabeele, et al. 2009. Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. *Immunity* 31: 84–98.
56. Kouzaki, H., K. Iijima, T. Kobayashi, S. M. O'Grady, and H. Kita. 2011. The danger signal, extracellular ATP, is a sensor for an airborne allergen and triggers IL-33 release and innate Th2-type responses. *J. Immunol.* 186: 4375–4387.
57. Moussion, C., N. Ortega, and J. P. Girard. 2008. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? *PLoS ONE* 3: e3331.
58. Platts-Mills, T. A., D. Vervloet, W. R. Thomas, R. C. Aalberse, and M. D. Chapman. 1997. Indoor allergens and asthma: report of the Third International Workshop. *J. Allergy Clin. Immunol.* 100: S2–S24.
59. Salo, P. M., S. J. Arbes, Jr., P. W. Crockett, P. S. Thorne, R. D. Cohn, and D. C. Zeldin. 2008. Exposure to multiple indoor allergens in US homes and its relationship to asthma. *J. Allergy Clin. Immunol.* 121: 678–684.e2.
60. Cammalleri, L., and M. Malaguarnera. 2007. Rasburicase represents a new tool for hyperuricemia in tumor lysis syndrome and in gout. *Int. J. Med. Sci.* 4: 83–93.
61. Stamp, L. K., J. L. O'Donnell, and P. T. Chapman. 2007. Emerging therapies in the long-term management of hyperuricaemia and gout. *Intern. Med. J.* 37: 258–266.
62. Artis, D., and R. K. Grencis. 2008. The intestinal epithelium: sensors to effectors in nematode infection. *Mucosal Immunol.* 1: 252–264.
63. Willart, M. A., K. Deswarte, P. Pouliot, H. Braun, R. Beyaert, B. N. Lambrecht, and H. Hammad. 2012. Interleukin-1α controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. *J. Exp. Med.* 209: 1505–1517.
64. Kouzaki, H., S. M. O'Grady, C. B. Lawrence, and H. Kita. 2009. Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. *J. Immunol.* 183: 1427–1434.
65. Millien, V. O., W. Lu, J. Shaw, X. Yuan, G. Mak, L. Roberts, L. Z. Song, J. M. Knight, C. J. Creighton, A. Luong, et al. 2013. Cleavage of fibrinogen by proteinases elicits allergic responses through Toll-like receptor 4. *Science* 341: 792–796.
66. Becker, B. F. 1993. Towards the physiological function of uric acid. *Free Radic. Biol. Med.* 14: 615–631.
67. Vorbach, C., R. Harrison, and M. R. Capecchi. 2003. Xanthine oxidoreductase is central to the evolution and function of the innate immune system. *Trends Immunol.* 24: 512–517.
68. Linder, N., J. Rapola, and K. O. Raivio. 1999. Cellular expression of xanthine oxidoreductase protein in normal human tissues. *Lab. Invest.* 79: 967–974.
69. Dröge, W. 2002. Free radicals in the physiological control of cell function. *Physiol. Rev.* 82: 47–95.
70. Sheehan, D., G. Meade, V. M. Foley, and C. A. Dowd. 2001. Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem. J.* 360: 1–16.
71. Harrison, R. 2002. Structure and function of xanthine oxidoreductase: where are we now? *Free Radic. Biol. Med.* 33: 774–797.
72. Akaike, T., M. Ando, T. Oda, T. Doi, S. Ijiri, S. Araki, and H. Maeda. 1990. Dependence on O2- generation by xanthine oxidase of pathogenesis of influenza virus infection in mice. *J. Clin. Invest.* 85: 739–745.
73. Papi, A., M. Contoli, P. Gasparini, L. Bristot, M. R. Edwards, M. Chicca, M. Leis, A. Ciaccia, G. Caramori, S. L. Johnston, and S. Pinamonti. 2008. Role of xanthine oxidase activation and reduced glutathione depletion in rhinovirus induction of inflammation in respiratory epithelial cells. *J. Biol. Chem.* 283: 28595–28606.
74. Woodward, O. M., A. Köttgen, J. Coresh, E. Boerwinkle, W. B. Guggino, and M. Köttgen. 2009. Identification of a urate transporter, ABCG2, with a common functional polymorphism causing gout. *Proc. Natl. Acad. Sci. USA* 106: 10338–10342.
75. Chan, J. K., J. Roth, J. J. Oppenheim, K. J. Tracey, T. Vogl, M. Feldmann, N. Horwood, and J. Nanchahal. 2012. Alarmins: awaiting a clinical response. *J. Clin. Invest.* 122: 2711–2719.
76. Ng, G., K. Sharma, S. M. Ward, M. D. Desrosiers, L. A. Stephens, W. M. Schoel, T. Li, C. A. Lowell, C. C. Ling, M. W. Amrein, and Y. Shi. 2008. Receptor-independent, direct membrane binding leads to cell-surface lipid sorting and Syk kinase activation in dendritic cells. *Immunity* 29: 807–818.
77. Koka, R. M., E. Huang, and J. C. Lieske. 2000. Adhesion of uric acid crystals to the surface of renal epithelial cells. *Am. J. Physiol. Renal Physiol.* 278: F989–F998.
78. Gross, O., A. S. Yazdi, C. J. Thomas, M. Masin, L. X. Heinz, G. Guarda, M. Quadroni, S. K. Drexler, and J. Tschopp. 2012. Inflammasome activators induce interleukin-1α secretion via distinct pathways with differential requirement for the protease function of caspase-1. *Immunity* 36: 388–400.
79. Lefrançais, E., and C. Cayrol. 2012. Mechanisms of IL-33 processing and secretion: differences and similarities between IL-1 family members. *Eur. Cytokine Netw.* 23: 120–127.
80. Rank, M. A., J. B. Hagan, S. A. Samant, and H. Kita. 2013. A proposed model to study immunologic changes during chronic rhinosinusitis exacerbations: data from a pilot study. *Am. J. Rhinol. Allergy* 27: 98–101.

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Supplemental Data

Airway uric acid is a sensor of inhaled protease allergens and initiates type 2 immune responses in respiratory mucosa

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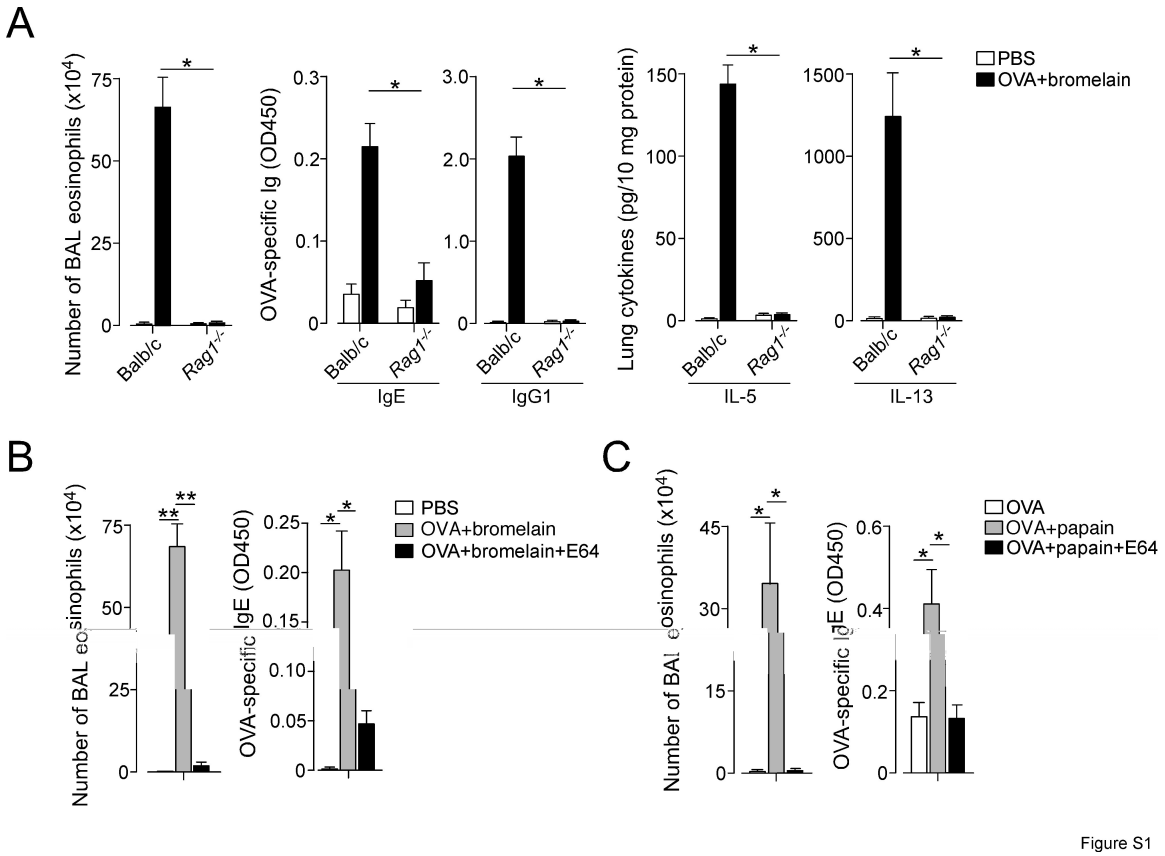


Supplemental Figure S1. Type 2 immune responses in mice that are previously exposed to proteases plus OVA are dependent on adaptive immunity and protease-activity. (A) As described in Figure 1B, naïve WT BALB/c mice or *Rag1*$^{-/-}$ mice were exposed i.n. to PBS or OVA (100 µg/dose) plus bromelain (10 µg/dose) on days 0 and 7. All mice were challenged i.n. with OVA alone on days 21, 22, and 23. On day 14, plasma concentrations of OVA-specific IgE and IgG1 were determined. The number of eosinophils in BAL fluids and cytokine levels in lung homogenates were examined on day 24. Data shown are the mean±SEM, * $p<0.05$, n=6 mice/group. Experiments were repeated twice; data shown are one representative experiment. (B) Naïve WT BALB/c mice were exposed i.n. to PBS, OVA (100 µg/dose) plus bromelain (10 µg/dose), or OVA plus bromelain that was pretreated with E64 (10 µM) on days 0 and 7. All mice

were challenged i.n. with OVA on days 21, 22, and 23. Mice were analyzed similarly to Panel A. Data shown are the mean±SEM, * $p<0.05$, ** $p<0.01$, n=6 mice/group. Experiments were repeated twice; data shown are one representative experiment. (C) Naïve WT BALB/c mice were exposed i.n. to endotoxin-free OVA (100 μg/dose), OVA plus papain (50 μg/dose), or OVA plus E64 (10 μM)-treated papain on days 0 and 7. All mice were challenged i.n. with OVA alone on days 21, 22, and 23. Mice were analyzed similarly to Panel A. Data shown are the mean±SEM, * $p<0.05$, compared to OVA group, n=5 mice/group. Experiments were repeated twice; data shown are one representative experiment.

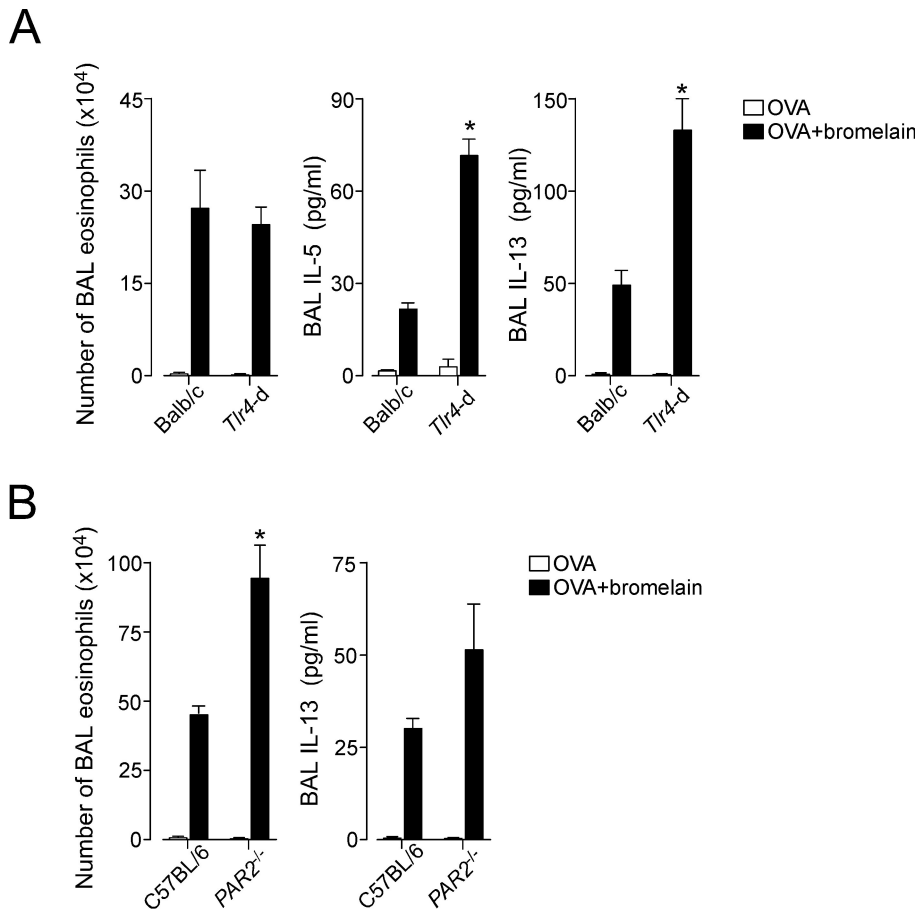
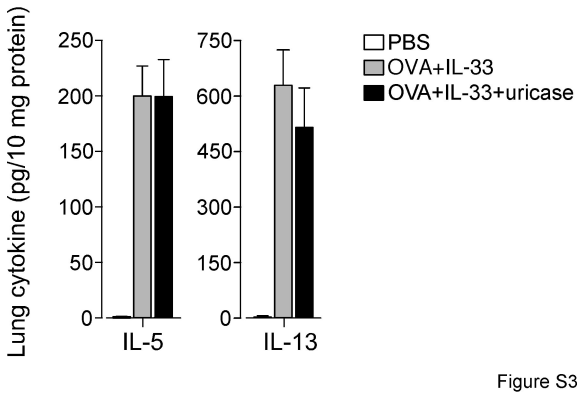
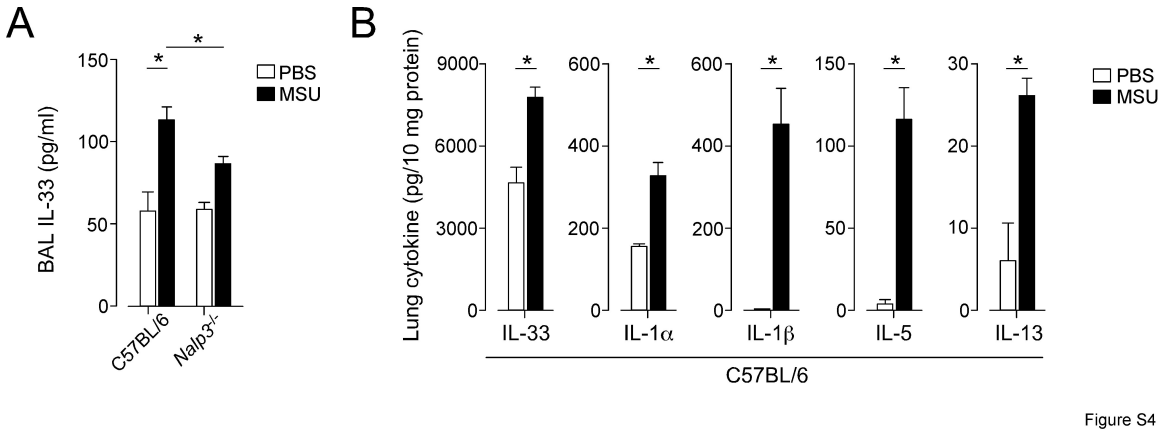


Figure S2

Supplemental Figure S2. TLR4 and PAR2 are not required for protease-induced type 2 immune responses. (A and B) As described in Figure 1B, naïve WT BALB/c mice or *Tlr4*-d mice (A) or naïve WT C57BL/6 mice or *PAR2*$^{-/-}$ mice (B) were exposed i.n. to endotoxin-free OVA alone (100 µg/dose) or OVA plus bromelain (10 µg/dose) on days 0 and 7. All mice were challenged i.n. with OVA alone on days 21, 22, and 23. Eosinophil numbers and cytokine levels in BAL fluids were examined on day 24. Data shown are the mean±SEM, * $p<0.05$ compared to WT mice, n=5 mice/group. Experiments were repeated twice; data shown are one representative experiment.



Supplemental Figure S3. Uricase does not affect development of type 2 immunity induced by IL-33. Naïve BALB/c mice were exposed i.n. to PBS or endotoxin-free OVA (100 µg/dose) plus IL-33 (1 µg/dose) with or without uricase (1 U/ml) on days 0 and 7. All mice were challenged i.n. with OVA on days 21, 22, and 23. Cytokine levels in lung homogenates were examined on day 24. Data shown are the mean±SEM, n=5 mice/group. The experiment was performed once.



Supplemental Figure S4. Innate type 2 response in C57BL/6 mice. Naïve WT C57BL/6 mice or *Nlrp3*$^{-/-}$ mice were administered once i.n. with MSU crystals. After 3 hr, cytokine levels in BAL fluid supernatants (Panel A) and lung homogenates (Panel B) were analyzed using ELISA. Data shown are the mean±SEM, *$p<0.05$, n=5 mice/group. Experiments were repeated twice; data shown are one representative experiment.